The extraction and fractionation of waxes from biomass

Emily H. K. Sin

PhD

University of York

Department of Chemistry

June 2012

Abstract

The aim of this project was to extract and fractionate waxes from abundant and low-cost under-utilised renewable resources using a green alternative technology. Through a review of the literature, the waxes covering agricultural by-products such as straw were identified as a potential source of high value chemicals for a wide range of applications.

Wheat straw waxes were extracted using organic solvents to demonstrate that straw contained high value wax compounds including free fatty acids, fatty alcohols, alkanes, wax esters, sterols, aldehydes and β-diketones. The solvent properties did not affect the composition of the extracts but changed the relative abundance of the different compounds. Linear solvation energy relationship (LSER) was used to model the extraction selectivity relating to total extraction yield and the various wax compounds. Lipophilic and aqueous fractions were separated and LSER results identified that the solvent properties affect only on the quantity of aqueous fraction recovered indicating the selectivity of the solvent.

Extraction of wheat straw wax was carried out using a more environmentally friendly supercritical CO_2 extraction. The compositional profiles can be tuned by the manipulation of temperature and pressure and compared with the organic solvent extractions. Optimisation of temperature and pressure was carried out and the total crude yields and wax chemical group yields were modelled using the Chrastil equation to gain a better understanding of conditions required to achieve optimum extraction. The optimisation was used as part of the industrial collaboration scale up with Sundown Products Limited and Evonik Industries where a total of three tonnes of wheat, barley and oat straws were extracted using supercritical CO_2 which yielded approximately 60 kg of wax. The three cereal straws were selected based on yield and composition as raw materials for the scale up from the biomass screen of seven different straws using hexane and ethanol extractions.

Economical assessment was carried out based on the scale up trial and it was concluded that currently the cereal straw wax would cost £12 per kg which is about 2 – 3 times higher than commercial waxes. The straw waxes were characterised and physical properties such as melting point were determined and found to be similar to commercial waxes such as beeswax. Fractionation by $scCO_2$, GPC and saponification were used to further separate the

wax products for formulation trials and product tests with the project sponsor, Croda. The crude wax products were deeply coloured and highly hydrophobic with no emulsification properties therefore applications such as coatings and polishes were suggested.

Table of Contents

Abtract 3

List of tables, figure and equations 9

List of abbreviations 17

Acknowledgements 21

Declaration 23

1. INTRODUCTION 27

1.1 Scope of the project 27
1.2 Sustainable development 28
1.2.1 Bio-refinery Concept 29
1.2.2 The twelve principles of Green Chemistry 31
1.2.3 Biorefinery and Green Chemistry as sustainable future 33
1.3 Agricultural waste 33
1.3.1 Availability 33
1.3.2 Straw and husk 36
1.3.3 The plant cuticle 37
1.4 Plant waxes 39
1.4.1 Classes of plant epicuticular waxes 39
1.5 Biosynthetic pathway of plant cuticular wax 42
1.5.1 Biosynthetic pathway 42
1.5.2 Cereal waxes 49
1.6 Commercial waxes 52
1.6.1 Manufacturing of commercial waxes 52
1.6.2 Chemical composition and physical properties 56
1.6.3 Wax market and applications 57
1.6.4 Wax market outlook and opportunities 58
1.7 Introduction to work in thesis 60

2. SOLVENT EXTRACTION OF WHEAT STRAW WAX 65

2.1 Introduction 65
2.2 Solvent selection and extraction 65
2.2.1 Solvent parameters and linear solvation energy relationships 65
2.2.2 Solvent green guides 67
2.2.3 Biomass pre-treatment techniques 69
2.2.4 Solvent extraction techniques 70
2.3 Optimisation of solvent extraction using LSER 72
2.4 Chemical composition identification using GC and GC-MS 84

2.4.1 Free fatty acids 87
2.4.2 Hydrocarbons 91
2.4.3 Free fatty alcohols 94
2.4.4 Aldehydes 96
2.4.5 Wax Esters 99
2.4.6 Sterols 104
2.4.7 Beta-diketones 113
2.4.8 Chemical composition of wheat straw wax summary 117
2.5 Quantification of key wax components in wheat straw wax 117
2.5.1 Quantification of key wax components by calibration 119
2.5.2 Free fatty acids 121
2.5.3 Hydrocarbons 123
2.5.4 Free fatty alcohols 125
2.5.5 Aldehydes 126
2.5.6 Wax esters 127
2.5.7 Sterols 130
2.5.8 β-Diketones 132
2.5.9 Summary of wax components quantified in organic solvent extracts
2.6 Conclusion and future work 141

3. SUPERCRITICAL CARBON DIOXIDE EXTRACTION OF WHEAT STRAW WAX 141

3.1 Introduction 141
3.2 Carbon dioxide extraction of wheat straw 151
3.2.1 Raw materials, pre-treatment and moisture content 151
3.2.2 Comparison of organic solvents and carbon dioxide extraction yields 152
3.2.3 Effect of temperature and pressure in supercritical carbon dioxide crude extraction yields by first order polynomial modelling 154
3.2.4 Effect of temperature and pressure in supercritical carbon dioxide extraction yields of different wax groups 159
3.2.5 Optimisation of wheat straw wax extraction using supercritical carbon dioxide by Chrastil modelling 164
3.3 Identification and quantification of key wax components in wheat straw wax 171
3.3.1 Free fatty acids 172
3.3.2 Hydrocarbons 175
3.3.3 Fatty alcohols 177
3.3.4 Aldehydes 178
3.3.5 Wax esters 179
3.3.6 Sterols 180
3.3.7 β-Diketones 182
3.3.8 Summary of wax components quantified in CO_2 extracts 183
3.4 Conclusion and future work 189

4. STRAW WAX SCREEN AND PRODUCTION SCALE SUPERCRITICAL CARBON DIOXIDE EXTRACTION OF CEREAL STRAW WAXES 196

4.1 Introduction 196
4.2 Raw materials selection 196
4.2.1 Raw materials 196
4.2.2 Identification of hexane and ethanol extraction of raw materials 197
4.2.3 Quantification of hexane and ethanol extraction of raw materials 213
4.3 Production scale extraction of cereal straw wax 226
4.3.1 Raw materials 226
4.3.2 Laboratory extraction trials at York Green Chemistry Centre 227
4.3.3 Production scale extraction trials at Evonik Industries 229
4.4 Conclusion and future work 241

5. STRAW WAX PROCESSING, PHYSICAL PROPERTIES AND ECONOMIC CONSIDERATIONS 245

5.1 Introduction 245
5.2 Straw wax processing 245
5.2.1 Co-extraction of water 245
5.2.2 Fractionation by supercritical carbon dioxide 248
5.2.3 Fractionation by gel permeation chromatography (GPC) 253
5.2.4 Saponification 263

5.3 Physical properties 271
5.3.1 Melting point 271
5.3.2 Acid value, saponification value and drop point 281
5.3.3 Appearance and aroma 284
5.3.4 Water absorption 286
5.4 Applications and costs 287
5.4.1 Potential applications 287
5.4.2 Economic considerations 288
5.5 Conclusion and future work 293

6. EXPERIMENTAL PROCEDURES 299

6.1 Materials and reagents 299
6.2 Pre-treatment, extraction and fractionation procedures 300
6.2.1 Moisture content of raw materials 300
6.2.2 Pre-treatment of raw materials 300
6.2.3 Soxhlet extraction 301
6.2.4 FexIKA® extraction 301
6.2.5 Supercritical fluid extraction 302
6.2.6 Purification and fractionation of wax extracts 304
6.2.7 Saponification 305

6.3 Chemical compositions analysis procedures 306
6.3.1 GC analysis 306
6.3.2 GC-MS analysis 307
6.3.3 Derivatisation for GC analysis 307
6.3.4 FAME analysis 307
6.3.5 IR analysis 308
6.3.6 GPC analysis 308
6.3.7 MALDI TOF MS analysis and synthesis of LiDHB matrix 309
6.4 Physical properties of wax extracts 310
6.4.1 Decomposition by STA analysis 310
6.4.2 Thermal transition temperatures by DSC analysis 310
6.4.3 Acid value 310
6.4.4 Saponification value 311
6.4.5 Drop point 312
6.4.6 Slip point 312
6.4.7 Gardner colour 312
6.4.8 Water absorption 313
6.4.9 Base value 313

7. CONCLUSION AND FUTURE WORK 317

8. REFERENCES 325

List of tables, figures and equations

1. INTRODUCTION

Figure 1.1: Comparison of oil-refinery vs. bio-refinery (originally in colour) 30
Figure 1.2: Straw bio-refinery (originally in colour) 31
Figure 1.3: The "Twelve Principles of Green Chemistry" (originally in colour) 32
Figure 1.4: Total breakdown of cereal and oilseed crops in UK in 2007 (originally in colour) 34
Figure 1.5: Breakdown of wheat, barley, oat and oilseed rape straw in the UK in 2007 (originally in colour) 35
Figure 1.6: Cereal straw and its botanical components (originally in colour) 36
Figure 1.7: The structure of the plant cuticle (originally in colour) 38
Figure 1.8: Molecular structure of cuticular waxes 39
Table 1.1: Common wax classes 41
Figure 1.9: Fatty acids *de novo* synthetic pathway 44
Figure 1.10: Wax biosynthesis including acyl reduction, fatty acids elongation and alkane pathways 46
Figure 1.11: Beta-diketone biosynthetic pathway 47
Figure 1.12: A simplified sterol biosynthetic pathway from the isoprenoid pathway 48
Table 1.2: Epicuticular wax of cereal plants 49
Table 1.3: Epicuticular wax from cereal waste 51
Figure 1.13: Wool grease retrieval from sheep wool 54
Figure 1.14: Wool grease processing at Croda 55
Table 1.4: Chemical composition and physical properties of four commercial waxes 57
Figure 1.15: Global wax applications 58
Figure 1.16: Wax market outlook in 2020 59
Figure 1.17: A poster by B&T Company at In-Cosmetics® 2010 (Picture taken at the exhibition) (originally in colour) 60

2. SOLVENT EXTRACTION OF WHEAT STRAW WAX

Table 2.1: List of selected solvents and their Reichardt's number (E_T^N) and Kamlet-Taft polarity parameters (α, β and π^*) 67
Equation 2.1: Kamlet-Taft linear solvation energy relationships 67
Table 2.2: List of selected solvents and their environmental, health and safety issues[1] (originally in colour) 69
Figure 2.1: Soxhlet extractor (originally in colour) 70
Figure 2.2: FexIKA® Vario Control Series Extractor (originally in colour) 71
Equation 2.2: Calculation of percentage crude yield 72
Table 2.3: Percentage crude yield with percentage of DCM and water soluble fractions 73
Figure 2.3: Percentage crude yield with percentage of DCM and water soluble fractions (originally in colour) 73
Equation 2.3: Calculation of percentage selectivity 74
Figure 2.4: The selectivity of different organic solvent extractions (originally in colour) 74
Table 2.4: Comparison of data with literature values 75
Equation 2.4: New Gibbs free energy equation 76
Equation 2.5: New normalised temperature LSER equation with percentage crude yield 76

Table 2.5: Solvent parameters in LSER equation 2.5 77
Figure 2.5: A 3D plot of the percentage crude yield of the solvents (originally in colour) 78
Figure 2.6: A comparison of experimental and predicted percentage crude yield (originally in colour) 79
Equation 2.6: New normalised temperature LSER equation with percentage aqueous yield 80
Equation 2.7: Calculation for residual error 80
Figure 2.7: Residual error plot for experimental and predicted percentage crude yield (originally in colour) 81
Figure 2.8: A comparison of experimental and predicted percentage aqueous soluble yield 81
Figure 2.9: Residual error plot for experimental and predicted percentage aqueous soluble yield (originally in colour) 82
Figure 2.10: A comparison of DCM soluble, aqueous soluble and crude yield (originally in colour) 83
Equation 2.8: LSER equation for percentage crude yield 83
Equation 2.9: LSER equation for percentage DCM soluble yield 83
Equation 2.10: LSER equation for percentage aqueous yield 83
Figure 2.11: GC chromatogram of whole heptane wheat straw extract (originally in colour) 84
Figure 2.12: GC chromatogram of ethanol wheat straw extract (originally in colour) 85
Equation 2.11: Kovats Index (KI) calculation as modified by Van Den Dool and Dec Kratz 85
Figure 2.13: Reference alkane mix analysed by two different capillary columns (originally in colour) 87
Figure 2.14: Formation of EI fragment ion m/z = 60 88
Figure 2.15a: EI mass spectrum of hexadecanoic acid ($C_{16:0}$) (magnified three times from m/z 213 - 260) (originally in colour) 88
Figure 2.15b: EI mass spectrum of hexadecanoic acid ($C_{16:0}$) from the NIST library
Figure 2.16: Formation of EI fragment ion m/z = 73 88
Table 2.6: Identification of free fatty acids presented in solvent extracts including the EI fragmentation pattern with percentage base peak intensity and KI values 90
Table 2.7: Aliphatic alkyl chain fragmentation peaks in mass spectra 90
Figure 2.17a: EI mass spectrum of nonacosane (C_{29}) (magnified two times from m/z 100 – 400) (originally in colour) 92
Figure 2.17b: EI mass spectrum of nonacosane (C_{29}) from the NIST library (originally in colour) 93
Table 2.8: Identification of *n*-alkanes presented in solvent extracts including the EI fragmentation pattern with percentage base peak intensity and KI values 93
Figure 2.18a: EI mass spectrum of octacosanol (C_{28}) (magnified five times from m/z 126 – 410) (originally in colour) 95
Figure 2.18b: EI mass spectrum of octacosanol (C_{28}) from the NIST library 95
Table 2.9: Identification of octacosanol in solvent extracts including the EI fragmentation pattern with percentage base peak intensity and KI values 95
Figure 2.20: Formation of EI fragment ion m/z = 393 and m/z = 365for octacosanol (C_{28}) 96
Figure 2.21: EI mass spectrum of octacosanal (C_{28}) (magnified five times from m/z 125 – 410) (originally in colour) 97
Figure 2.22: Mass fragment ion of aliphatic aldehyde (originally in colour) 97
Figure 2.23: Formation of mass fragment ion m/z = 390 98
Figure 2.24: FI mass spectrum of octacosanal (C_{28}) 98

Table 2.10: Identification of octacosanal in solvent extracts including the EI fragmentation pattern with percentage base peak intensity and calculated KI 99
Figure 2.25: Formation of base peak ion m/z = 57 for aliphatic aldehydes 99
Figure 2.26: GC chromatogram of wheat straw wax esters (originally in colour) 100
Figure 2.27: Formation of EI fragment ions m/z = 393, 257 and 256 for octacosanyl hexadecanoate (C_{28}:C_{16}) 101
Figure 2.28: EI mass spectrum of octacosanyl hexdecanoate (C_{28}:C_{16}) (originally in colour) 101
Table 2.11: Identification of wax esters in solvent extracts including EI fragmentation pattern with percentage base peak intensity and calculated KI 103
Table 2.12: List of characteristic fragment ions for even-numbered wax 104
Figure 2.29: Four cycloalkane carbon skeleton rings found in sterols and 3'-ketosteroid 105
Figure 2.30: Structure of sterols present in wheat straw wax 106
Figure 2.31a: EI mass spectrum of stigmasterol (magnified four times from m/z 400 – 415) (originally in colour) 107
Figure 2.31b: EI mass spectrum of stigmasterol from the NIST library 107
Figure 2.32: Fragmentation of a sterol to give characteristic mass ions 108
Figure 2.33: Dehydration step of a sterol to give characteristic ion 108
Table 2.13: List of characteristic fragment ions for sterols 109
Figure 2.35: EI mass spectrum of Δ^4-Sitosten-3-one (originally in colour) 110
Figure 2.36: FI mass spectrum of Δ^4-Sitosten-3-one 110
Figure 2.37: Formation of base peak ion m/z = 124 111
Figure 2.38: Formation of fragment ions m/z = 230 and m/z = 149 112
Table 2.14: Identification of sterols and sterol derivatives in solvent extracts including EI fragmentation pattern with percentage base peak intensity and calculated KI 113
Figure 2.39: Keto-enol tautomerism of β-diketones 114
Figure 2.40a: EI spectrum of hentriacontane-14,16-dione (magnified two times from m/z 212 – 500) (originally in colour) 115
Figure 2.40b: EI spectrum of hentriacontane-14,16-dione from the NIST library 115
Figure 2.41: Characteristic fragment ions for β-diketones 116
Table 2.15: Identification of β-diketones in solvent extracts including EI fragmentation pattern with percentage base peak intensity and calculated KI 116
Figure 2.42: Mass fragmentation of the 14,16 hentriacontanedione (C_{31}) and 16,18 triatriacontanedione (C_{33}) (originally in colour) 117
Figure 2.43: Typical GC chromatogram of solvent wheat straw wax (Viscount 09) extract (originally in colour) 118
Figure 2.44: Calibration graph for octadecanoic acid (free fatty acid representative) (originally in colour) 119
Equation 2.12: Calculation of area ratio 120
Equation 2.13: Calculation of mass ratio 120
Table 2.16: List of standards and its calibration range with r^2 and calculated response factor value 120
Equation 2.14: Calculation of response factor value using ECN method 121
Table 2.17: List standards with the calculated response factor value from ECN method 121
Figure 2.45: Percentage of total fatty acids in solvent crude extracts (originally in colour) 122
Figure 2.46: Percentage of individual free fatty acids in solvent crude extracts (originally in colour) 123
Figure 2.47: Percentage of total *n*-alkanes in solvent crude extracts (originally in colour) 124

Figure 2.48: Percentage of individual *n*-alkanes in solvent crude extracts (originally in colour) 125
Figure 2.49: Percentage of octacosanol (C_{28}) in solvent crude extracts (originally in colour) 126
Figure 2.50: Percentage of octacosanal (C_{28}) in solvent crude extracts (originally in colour) 127
Figure 2.51: Percentage of wax esters in solvent crude extracts (originally in colour) 128
Equation 2.15: New normalised LSER equation with percentage wax ester 129
Figure 2.52: Percentage of wax esters in solvent crude extracts (originally in colour) 130
Figure 2.53: A comparison of experimental and predicted percentage wax esters in crude extracts (originally in colour) 130
Figure 2.54: Residual error plot for experimental and predicted percentage wax esters in crude extracts (originally in colour) 131
Figure 2.55: Percentage of sterols in solvent crude extracts (originally in colour) 132
Figure 2.56: Percentage of individual sterols and its derivatives in solvent crude extracts (originally in colour) 132
Figure 2.57: Percentage of β-diketones in solvent crude extracts (originally in colour) 132
Figure 2.58: Percentage of individual β-diketones in solvent crude extracts (originally in colour) 133
Figure 2.59: The lipophilic fraction of the wheat straw wax in various crude solvent extracts (originally in colour) 134
Figure 2.60: FAME analysis of wheat straw wax (originally in colour) 135
Figure 2.61a: EI spectrum of methyl octadecanoate (magnified two times from m/z 260 – 300) (originally in colour) 136
Figure 2.61b: EI spectrum of methyl octdecanoate from the NIST library 136
Equation 2.16: Calculation for percentage solvent efficiency 137
Table 2.18: Quantification of wheat straw wax components in various solvent extracts 139
Table 2.19: Normalised percentage solvent efficiency for different wax grou 140

3. SUPERCRITICAL CARBON DIOXIDE EXTRACTION OF WHEAT STRAW WAX

Figure 3.1: Phase Diagram for supercritical fluid 148
Table 3.1: Physical properties of gaseous, supercritical and liquid CO_2 149
Table 3.2: Physical properties of CO_2 compared with organic solvents 149
Figure 3.2: Density of CO_2 with change in temperature and pressure 150
Figure 3.3: Comparison of percentage crude yields on organic solvents and carbon dioxide 152
Table 3.3: CO_2 extraction conditions outlined in figure 3.3 153
Table 3.4: Comparison of supercritical CO_2 and hexane lipids extraction yields 154
Figure 3.4: Experimental design for extraction conditions optimisation 155
Table 3.5: Experimental design with the coded coordinate values 156
Table 3.6: Experimental design for optimisation 156
Equation 3.1: First order polynomial function 156
Figure 3.5: Percentage crude yields for $scCO_2$ extraction at different pressure and temperature (originally in colour) 157
Equations 3.2, 3.3, 3.4 and 3.5: Calculation for coefficients of the first order polynomial function 158

Equation 3.6: First order function modelling the $scCO_2$ extraction of wheat straw waxes 158
Table 3.7: Percentage of different wax groups extracted from wheat straw 160
Table 3.8: Coefficients for different wax groups 160
Table 3.9: Prediction of percentage yield for different wax groups 161
Equation 3.7 and 3.8: First order function modelling the wax esters and β-diketones extraction in wheat straw waxes 161
Figure 3.6: Coefficients in first order modelling for wax ester and β-diketone 163
Equation 3.9: Chrastil equation 165
Table 3.10: Extraction conditions and percentage crude yield for Chrastil modelling 166
Figure 3.7: Percentage crude yield of wheat straw waxes from $scCO_2$ extraction 166
Equation 3.10: Chrastil equation modelling the $scCO_2$ extraction of wheat straw waxes 167
Figure 3.8: Experimental ln(*c*) against predicted ln(*c*) for percentage crude yield 168
Table 3.11: Chrastil modelling for different wax groups 169
Equation 3.11: Chrastil equation modelling the $scCO_2$ extraction of wax esters in the wheat straw waxes 170
Figure 3.9: Experimental ln(*c*) against predicted ln(*c*) for percentage wax esters 170
Figure 3.10: Percentage of total free fatty acids in CO_2 crude extracts 172
Figure 3.11: Percentage of individual free fatty acids in CO_2 extracts 174
Table 3.12: Published solubility data for fatty acids in CO_2 175
Figure 3.12: Percentage of total *n*-alkanes in CO_2 crude extracts 176
Figure 3.13: Percentage of individual *n*-alkanes in CO_2 extracts 176
Table 3.13: Published solubility data for *n*-alkanes in CO_2 177
Figure 3.14: Percentage of total octacosanol (C_{28}) in CO_2 crude extracts 178
Figure 3.15: Percentage of total octacosanal (C_{28}) in CO_2 crude extracts 178
Figure 3.16: Percentage of wax esters in CO_2 crude extracts 179
Figure 3.17: Percentage of individual wax esters in CO_2 extracts 180
Figure 3.18: Percentage of sterols and its derivatives in CO_2 crude extracts 181
Figure 3.19: Percentage of individual sterols and its derivatives in CO_2 extracts 181
Figure 3.20: Percentage of β-diketones in CO_2 crude extracts 182
Figure 3.21: Percentage of individual β-diketones in CO_2 extracts 183
Figure 3.22: The lipophilic fraction of the wheat straw wax in various crude CO_2 extracts 186
Table 3.14: Quantification of wheat straw wax components in various solvent extracts 187

4. STRAW WAX SCREEN AND PRODUCTION SCALE SUPERCRITICAL CARBON DIOXIDE EXTRACTION OF CEREAL STRAW WAXES

Figure 4.1: Straw samples (wheat, oilseed rape, miscanthus, bagasse and sunflower) used for wax screening (originally in colour) 197
Figure 4.2: Hexane and ethanol extracts of the seven different straws (originally in colour) 198
Table 4.1: Description of straw 199
Figure 4.3: Percentage crude yield of hexane and ethanol extracts of different straws (originally in colour) 200
Figure 4.4: GC chromatogram of hexane extract for wheat straw (Oakley 08) (WS4) (originally in colour) 201
Figure 4.5: GC chromatogram of hexane extract for barley straw (Carat 08) (BS1) (originally in colour) 202
Figure 4.6: GC chromatogram of hexane extract for oat straw (OS) (originally in colour) 203

Figure 4.7: GC chromatogram of hexane extract for oilseed rape straw (RS1) (originally in colour) 204
Figure 4.8: GC chromatogram of hexane extract for sunflower straw (SS) (originally in colour) 205
Figure 4.9: GC chromatogram of hexane extract for miscanthus straw (MS) (originally in colour) 206
Figure 4.10: GC chromatogram of hexane extract for bagasse (Bag) (originally in colour) 207
Table 4.2: List of wax compounds identified in straw waxes 208
Figure 4.11: Structures of sterols presented in sunflower oil 210
Figure 4.12: EI spectrum of nonacosan-15-one (magnified eight times from m/z 400 – 425) (originally in colour) 212
Figure 4.13: FI mass spectrum of nonacosan-15-one 212
Figure 4.14: Characteristic fragment ions m/z = 225 for nonacosan-15-one (originally in colour) 212
Figure 4.16: Characteristic fragment ions m/z = 240 and m/z = 241 for nonacosan-15-one 207
Table 4.3: Identification of noncosan-15-one presented in oilseed rape straw including the EI fragmentation pattern with percentage base peak intensity and KI value 207
Table 4.4: Quantification of hexane Soxhlet straw waxes (% in extracts) 211
Table 4.5: Quantification of hexane Soxhlet straw waxes (μgg^{-1} of biomass) 213
Table 4.6: Quantification of ethanol Soxhlet straw waxes (% in extract) 215
Table 4.7: Quantification of ethanol Soxhlet straw waxes (μgg^{-1} of biomass) 217
Table 4.8: Quantification of laboratory scale heptane Soxhlet extraction wheat, barley and oat straw wax components 222
Table 4.9: Summary of production scale scCO_2 extraction conditions 224
Table 4.10: Summary of carrier oil-containing wheat straw waxes 224
Figure 4.17: Laboratory heptane/ hexane Soxhlet and production scale supercritical CO_2 percentage crude yields 226
Figure 4.18: Comparison of supercritical CO_2 and heptane re-extracted yields against heptane Soxhlet yields 226
Table 4.11: Quantification of industrial scale scCO_2 extraction of cereal straw waxes 229
Table 4.12: Quantification of laboratory scale re-heptane Soxhlet extraction of cereal straw waxes 230
Table 4.14: Comparison of the chemical compositions from heptane Soxhlet, supercritical CO_2 and heptane re-extracted wax extracts 212

5. STRAW WAX PROCESSING, PHYSICAL PROPERTIES AND ECONOMIC CONSIDERATIONS

Table 5.1: Percentage moisture level of biomass before and after extraction 246
Figure 5.1: Co-extraction of water in industrial scale supercritical CO_2 extraction (originally in colour) 246
Figure 5.2: Co-extracted water separated from molten wheat straw wax (originally in colour) 247
Figure 5.3: Coloured co-extracted water from cereal straw waxes (originally in colour) 248
Figure 5.4: Percentage of co-extracted water within the cereal straw waxes (originally in colour) 248
Figure 5.5: Wheat and oat wax fractions separated from scCO_2 fractionation 249
Figure 5.6: Percentage of wax present in each separator after scCO_2 fractionation 251

Table 5.2: Identification and quantification of wheat and oat straw fractions from $scCO_2$ fractionation 252
Figure 5.7: A plot of retention time against Ln (molecular weight) 253
Figure 5.8: GPC trace showing DAD signal of wool grease (originally in colour) 254
Figure 5.9: GPC separation of wool grease and wheat straw wax by size (originally in colour) 255
Figure 5.10: Mass balance for preparative GPC fractions (originally in colour) 255
Figure 5.11: IR spectra (full) of selected wheat straw wax fractions (originally in colour) 257
Figure 5.12: IR spectra (1800 – 1550 cm^{-1}) of selected wheat straw wax fractions (originally in colour) 259
Figure 5.13: MALDI-TOF mass spectrum for wool grease fraction 5 (originally in colour) 262
Figure 5.14: MALDI-TOF mass spectrum for wheat straw wax fraction 5 (originally in colour) 262
Figure 5.15: MALDI-TOF mass spectrum for oat straw wax fraction 5 (originally in colour) 263
Figure 5.16: Saponification reaction scheme for triglycerides and wax esters 264
Figure 5.17: Wheat alcohol and acid fractions of wheat straw wax (originally in colour) 264
Figure 5.18: GC chromatograms of saponified wheat alcohol and acid fractions (originally in colour) 266
Figure 5.19: EI mass spectrum of 2-heptadecanone (originally in colour) 267
Figure 5.20: Formation of EI fragment ion of m/z = 58 267
Table 5.4: Identification of ketones presented in wheat alcohol fraction 267
Figure 5.21: Proposed structures for odd-numbered C_{41} wax ester in wheat straw wax 269
Table 5.5: Wax compounds presented in crude wheat straw wax, wheat alcohol and acid fraction 269
Figure 5.22: An example of the Retro-Claisen reaction on β-diketone 270
Figure 5.23: STA thermogram of wheat straw wax (originally in colour) 271
Figure 5.24: DSC thermograms of four commercial waxes (originally in colour) 273
Table 5.6: The melting points of wheat straw wax compounds 274
Figure 5.25: DSC thermograms of $scCO_2$ extracts compared with organic solvent extracts (originally in colour) 275
Figure 5.26: DSC thermograms of $scCO_2$ extracts under different temperature and pressure (originally in colour) 276
Table 5.7: Percentage of wax groups in crude extracts from CO_2 and organic solvent extractions 277
Table 5.8: Percentage of wax group in the three cereal straw waxes 277
Figure 5.27: DSC thermograms of wheat, barley and oat straw waxes from scale up trials (originally in colour) 280
Figure 5.28: DSC thermograms of wheat alcohols and acids fractions compared to wheat straw wax (originally in colour) 281
Table 5.9: Acid value and saponification value for commercial and cereal straw waxes 283
Table 5.10: Drop point for commercial and cereal straw waxes 284
Figure 5.29: Production scale cereal straw waxes 285
Table 5.11: Gardner colour for commercial and cereal straw waxes 285
Figure 5.30: Barley straw wax and super-refined barley straw wax 286

Table 5.12: Potential applications for different wax groups identified in cereal straw waxes 288
Figure 5.31: The breakdown of operation costs for CO_2 extraction 290
Table 5.13: Costs of raw materials and palletisation 290
Equation 5.1: Cost of pelletised materials 291
Equation 5.2: Calculation of net cost of pelletised straw 291
Table 5.14: Estimated net costs for pelletised raw materials 292
Table 5.15: Costs for CO_2 extraction 292

6. EXPERIMENTAL PROCEDURES

Equation 6.1: Calculation of moisture level 300
Figure 6.1: Alternative pre-treatment processes: milled, chopped, pellets and briquettes (originally in colour) 301
Table 6.1: Extraction information for ethanol and hexane 302
Figure 6.2: Thar SFE 500 system used for carbon dioxide extractions (originally in colour) 303
Equation 6.2: Calculation for quantity of sodium hydroxide 305
Equation 6.3: Calculation of extra sodium hydroxide for low base value 305
Equation 6.4: Calculation of acid value 311
Equation 6.5: Calculation of saponification number 311
Equation 6.6: Calculation for water absorption 313
Equation 6.7: Calculation for base value 314

List of abbreviations

The following abbreviations are included in the thesis in addition to the Mendeleev periodic table symbols:

ACP	-	Acyl Carrier Protein
AMP	-	Adenosine Mono-Phosphate
ASTM	-	American Society for Testing and Materials
ASE	-	Accelerated Solvent Extraction
ATR	-	Attenuated Total Reflectance
bp	-	Boiling point
CI	-	Chemical Ionisation
DAD	-	Diode Array Detector
Dardni	-	Department of Agriculture and Rural Development (Northern Ireland)
DCM	-	Dichloromethane
Defra	-	Department of Environment, Food and Rural Affairs
DHB	-	Dihydroxybenzoic acid
DMAPP	-	Dimethylalkyl diphosphate
DMC	-	Dimethyl Carbonate
DMSO	-	Dimethyl Sulphoxide
DSC	-	Differential Scanning Calorimetry
ECN	-	Effective Carbon Number
EHS	-	Environment, Health and Safety
EI	-	Electron Impact
ER	-	Endoplasmic Reticulum

FAE	-	Fatty Acid Elongases
FAME	-	Fatty Acid Methyl Ester
FAO	-	Food and Agriculture Organization of the United Nations
FAS	-	Fatty Acid Synthase
FI	-	Field Ionisation
FID	-	Flame ionisation detector
FPP	-	Farnesyl pyrophosphate
FT-IR	-	Fourier Transform Infra-Red
GC	-	Gas Chromatography
GC-MS	-	Gas Chromatography coupled with Mass Spectroscopy
gL	-	Gigalitres
GPC	-	Gel Permeation Chromatography
HβD	-	Hydroxy β-diketone
HDL	-	High Density Lipoprotein
HTGC	-	High Temperature Gas Chromatography
IEA	-	International Energy Agency
IMS	-	Industrial Methylated Spirits
IPP	-	Isopentenyl pyrophosphate
IR	-	Infra Red
IUCN	-	International Union for Conservation of Nature and Natural Resources
KAS	-	β-Ketoacyl Synthase
KI	-	Kovats Index
LCA	-	Life Cycle Assessment
lCO_2	-	Liquid Carbon Dioxide
LDL	-	Low Density Lipoprotein
LOQ	-	Limit of quantification

LSER	-	Linear Solvation Energy Relationship
MALDI	-	Matrix-Assisted Laser Desorption/Ionisation
MeTHF	-	2-Methyl Tetrahydrofuran
$M^{\cdot+}$	-	Molecular Ion
mp	-	Melting Point
MS	-	Mass Spectroscopy
MTBE	-	Methyl *tert*-butyl ether
MW	-	Molecular weight
m/z	-	Mass-to-charge ratio
ppm	-	Parts Per Million
PTFE	-	PolytetraFluoroethylene
Rf	-	Response factor
RT	-	Room Temperature
R^2	-	Correlation Coefficient
$scCO_2$	-	Supercritical carbon dioxide
SFE	-	Supercritical fluid extraction
STA	-	Simultaneous Thermal Analysis
ster	-	Sterol and Sterol Derivatives
TFA	-	Trifluoroacetic Acid
TGA	-	Thermal Gravimetric Analysis
THF	-	Tetrahydrofuran
TMS	-	Trimethylsilyl
TOF	-	Time-Of-Flight
Tr	-	Trace level
Unesco	-	United Nations Educational Scientific and Cultural Organization
UNEP	-	United Nations Environment Programme
UK	-	United Kingdom

US	-	United States
V_m	-	Molar Volume
VOC	-	Volatile Organic Carbon
v/v	-	Volume/volume
v/v/v	-	Volume/volume/volume
WCED	-	World Commission on Environment and Development
WWF	-	World Wildlife Fund
w/v	-	Weight/volume

Acknowledgements

Firstly, I would like to make a special thank you to Professor Ray Marriott for the continuous help and guidance through my PhD. He was a true inspiration to me and I feel very lucky to have him as my supervisor for 2 years. I would to thank my supervisor Professor James Clark for giving me the opportunity to do such a great PhD in his group. His advices and support had been invaluable to me. I am very grateful for all the chances I was provided with to attend and present in conferences. I would also like to thank my other supervisor Dr. Andy Hunt for his help towards the end of my PhD and being understanding and patient with me.

For financial assistance, I would like to thank Croda Enterprises Limited and BBSRC. Thank you to everyone especially Graham Atkinson, Julie Humphrey and Richard Cawthorne in Croda Rawcliffe Bridge for the assistance whilst working there. Thank you to Dass Chalal, Kim Carmichael, Helen Sarginson, Will Cannon and Damian Kelly from Croda for being part of my project.

I want to thank Trevor Dransfield, Karl Heaton and Adam Dowle for all the mass spectroscopy help. I would also like to thank David Cubitt from Sundown Products Limited for giving opportunity to work on the scale up trials. I would also like thank Grant Coupland and his team in Colep UK Limited for the wax formulations experience.

For proof-reading this thesis, I would like to thank Professor Ray Marriott, Dr. Duncan Macquarrie, Dr. Andy Hunt, Professor James Clark and Paul Brown. I would like to thank Dr. Simon Breeden, Paul Elliott and Owain Samuels for general laboratory help. Another thank you must to go to Dr. Peter Shuttleworth and James Sherwood for the data analysis help and Dr. Colin Ellis for all the computer help he has given me.

Huge thanks must go to the past and present members of the group for the ideas, support, laboratory help and general office banter in particular Leona and Alice. I would also like to send special thanks all my amazing friends (you know who you are!) pushing me to write during the stressful period. A special thanks to my housemate James for reminding me that I have to write and listen to me complaining every day.

Last but most certainly not least, massive thanks go to my family especially my parents, grandma and brother for their unconditional support and believing in me.

Declaration

Some of the results presented in this thesis were obtained by, in collaboration with other workers, who are fully acknowledged in the text. All other results are the work of the author.

Miss Emily H. K. Sin

2012

Chapter 1

Introduction

1. INTRODUCTION

1.1 Scope of the project

The scope of this study is the extraction of waxes from biomass in a sustainable manner to meet tighter legislation and higher demand for natural waxes to replace some of the existing petroleum and synthetic waxes that are currently in the market. Through literature review, agricultural waste was found to be an abundant and low cost renewable resource that contains valuable waxes which can be a new potential high value source of natural wax for many industrial products. Six distinct areas of research were identified:

Extraction of wheat straw waxes using organic solvents – Wheat straw waxes were extracted using a traditional extraction method (Soxhlet apparatus). Linear solvation energy relationship (LSER) was applied to correlate yields of extracts from wheat straw with solvent parameters. The effect and significance of solvent parameters and their selectivity were determined and discussed.

Raw materials screening – Seven different crops were extracted using ethanol and hexane with an automated FexIKA® extractor to compare the yield and chemical composition. New compounds not found in wheat straw waxes were highlighted. The potential for the raw materials extracted as renewable resources for waxes is discussed.

Characterisation and quantification of straw waxes - Qualitative characterisation and quantification of the key wax components of the straw extracts was carried out using GC-FID and GC-MS. The wax classes of commercial interest were prioritised in order to identify potential applications for this new novel wax product.

Alternative green extraction of wheat straw waxes using $scCO_2$ – For a sustainable extraction of waxes from biomass, an environmentally-benign technology, supercritical CO_2 extraction was employed. The Chrastil model was applied to the extraction of wheat straw waxes to determine the relationship between solvent density and solvating power on extraction yields to obtain an optimised extraction temperature and pressure.

Production scale $scCO_2$ extraction of cereal straw waxes – Following successful laboratory $scCO_2$ trials, production scale on a total of three tonnes of wheat, barley and oat straw were successfully extracted using $scCO_2$ and the effect of scale up is discussed.

Wax processing and physical properties – Wax from the production scale extraction was processed using similar methods to current commercial wax lanolin e.g.: saponification. Fractionation using GPC and $scCO_2$ were explored as potential new methods to separate the complex mixtures. Physical properties such as melting point were determined and compared with existing commercial waxes. The potential for straw wax to compete in the current wax market is considered.

1.2 Sustainable development

From the growing environmental concerns in the 1960s and 1970s, debates across the world on sustainability arose.[2] The term "sustainable development" was first used by the World Conservation Strategy in 1980.[3] However, this term was firstly expressed and defined in the Brundtland Report in 1987. The definition of sustainable development is "development that meets the needs of the present without compromising the ability of future generations to meet their needs".[4] The concept of sustainable development is an attempt to combine growing concerns about a range of environmental issues with socio-economic issues.[2] Current industrial economies are heavily dependent on crude oil for both energy and chemical products. With the depletion of crude oil at an increasing rate and a higher world demand, sustainable development by government, industries and the public is necessary.[5] Tighter legislation means industries are forced to switch to alternative processes and minimise waste in order to battle climate change.[6] To supply the changing world demands without further damage to the environment, the current oil-based economy must be switched to a bio-based economy.[7] Bio-based industrial products can be developed to replace existing petroleum-based products.[7] Waxes extracted from renewable resources using an environmental benign technology to replace petroleum waxes are recognised to be sustainable. Due to the decrease in petroleum waxes, the production of synthetic waxes must be increased in order to maintain a stable wax market. To achieve a sustainable development, it is important to assess and identify the natural resources that are in sufficient quantity and readily available.[8]

1.2.1 Bio-refinery Concept

The bio-refinery concept is the development of bio-based industrial products from waste biomass. Comparable to the well-established oil-refinery, the bio-refinery will be an integrated system for securing renewable materials to convert into bio-chemicals, bio-energy and bio-materials in order to maximise the valuable products and minimise the waste as shown in Figure 1.1.[8] The challenge is to minimise the strong dependence on fossil fuels and migrate to renewable natural resources globally. There is a prediction of an average of 84% global increase in fossil fuels usage between 2005 and 2030.[9] The world's primary source for chemicals and energy is oil, with a demand of approximately 84 million barrels per day in 2007.[9] With efforts across the globe, the level of oil consumption was similar in 2009 but despite the rate of increase of oil usage slowing; it is still predicted to increase to 99 million barrels per day by 2035.[10] Targets have been made to cut oil usage by 4.7 million barrels per day by 2020 globally and in 2009 help, government support of $20 billion per year was made and this is set to rise up to $45 billion per year by 2020.[10] Governments across different countries are attempting to push forward the idea of bio-refinery and setting goals for bio-based products industries.[11] An agreement by G8 leaders to cut global emissions by 50% by 2050 was taken at their summit in July 2009.[10] With government support, targets set and the rising oil price, bio-based products such as biofuels are set to be in competition with petroleum-derived products. Biofuel production of 1 million barrels per day in 2010 is projected to grow to 4.4 million barrels per day in 2035.[10]

The bio-feedstocks must be extensively and optimally exploited using sustainable processes to be in competition with petroleum-derived products. There are four types of bio-feedstocks from distinct sectors: agriculture, forestry, industries or household and aquaculture.[12] The raw materials are very different to crude oil which is a mixture of hydrocarbons and small amounts of impurities. These can be removed and fractional distillation can be carried out to manufacture a full range of products from gasoline to lubricating oils.[12] In contrast, biomass is not homogeneous and mainly consists of C, H and O. [12] With the vast range of biomass waste, that differs seasonally, there is need for a large range of refining and processing is needed by comparison. Based on the oil refinery concept, a list of target bio-platform molecules

must be identified for the development of a series of chemicals.[12] The idea of bio-platform molecules is to replace the existing petroleum-derived ethylene, propylene, C_4-olefines and aromatics.[12] A list of twelve bio-platform molecules was identified by the US Department of Energy in 2004 which can be used for further reactions to create new and existing key molecules.[13]

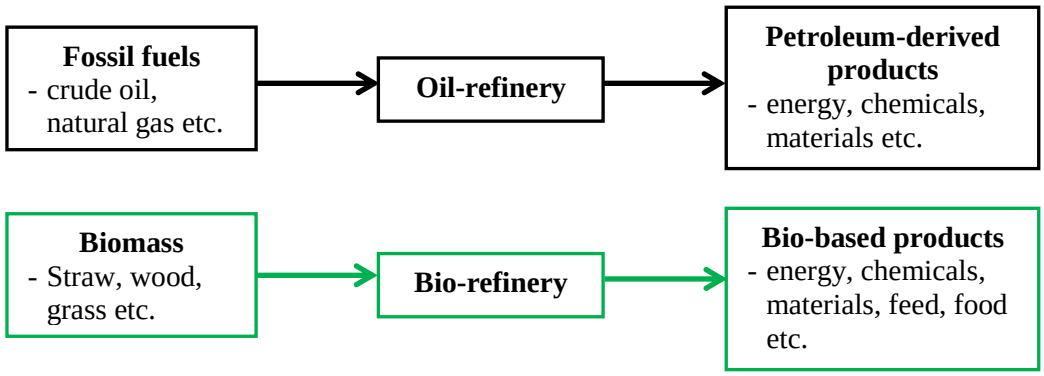


Figure 1.1: Comparison of oil-refinery vs. bio-refinery (originally in colour)

The main drawbacks for using biomass as bio-feedstock are the wide distribution geographically with very low bulk density and relatively high water contents making transportation costly and complicated.[14] The bulk density is intensively increased by pre-treatments of biomass such as pelletisation prior to distribution and this is necessary for both economic and conversion processing reasons.[15] Alternatively, localised bio-refineries beside farmlands can be established to stimulate local industries, create new markets and generate job opportunities.[16, 17] Other challenges include the developing conversion processes and acceptance of new products.[8]

Using wheat straw as bio-feedstock for whole crop bio-refinery has been demonstrated by Deswarte *et al*.[15] The study showed the agricultural waste being fully exploited by extracting the high value wax products using $scCO_2$ in the initial stage of bio-refinery prior to converting the lignocellulosic fraction into paper, strawboard, mulch or energy as illustrated in fig 1.2.[15, 18, 19, 20] The University of York and a number of collaborators (Velcourt Group Plc and Botanix Limited) have demonstrated the success of a pilot scale $scCO_2$ extraction of wheat straw in 2005.[16] Calorimetric studies showed that dewaxing the straw does not affect its calorific value as the $scCO_2$ processing step also removes most of the water that is present in the straw.[15, 19] The negative effect of wax removal is compensated by the positive effect of water removal

in the process giving the same calorific value.[15, 19] This new valuable wax product has shown high potential to replace some commercial waxes. Bio-materials such as strawboards have also shown added strength when waxes were removed.[15]

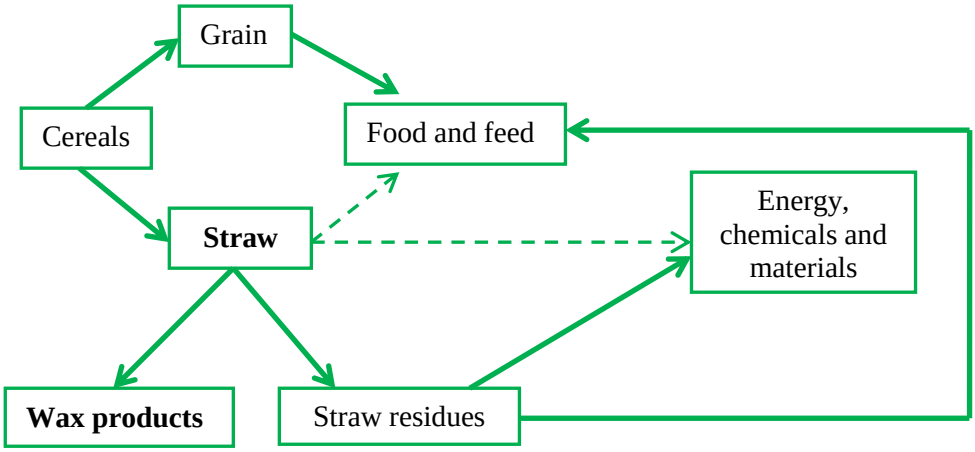


Figure 1.2: Straw bio-refinery (originally in colour)

1.2.2 The twelve principles of Green Chemistry

Green Chemistry is a term which describes environmentally benign chemical synthesis and processes.[21] This is a field of chemistry based on the utilisation of a set of principles that reduces or eliminates hazardous waste by design, manufacture and application of chemical products.[22] The "Twelve Principles of Green Chemistry" (Figure 1.3) was established by Paul Anastas and John Warner in 1998 to help chemists to achieve sustainable chemical designs.[22, 23]

1) **Prevention**

It is better to prevent waste than to treat or clean up waste after it is formed.

2) **Atom economy**

Synthetic methods should be designed to maximise the incorporation of all materials used in the process into the final product.

3) **Less hazardous chemical synthesis**

Wherever practicable, synthetic methodologies should be designed to use and generate substances that possess little or no toxicity to human health and the environment.

4) **Designing safer chemicals**

Chemical products should be designed to preserve efficacy of function whilst reducing toxicity.

5) **Safer solvents and auxiliaries**

The use of auxiliary substances (e.g.: solvents, separation agents etc.) should be made unnecessary wherever possible and, innocuous when used.

6) **Design for energy efficiency**

Energy requirements should be recognised for their environmental and economic impacts and should be minimised. Synthetic methods should be conducted at ambient temperature and pressure.

7) **Use of renewable feedstocks**

A raw material of feedstock should be renewable rather than depleting wherever technically and economically practicable.

8) **Reduce derivatives**

Unnecessary derivatisation (blocking group, protection/deprotection and temporary modification of physical/chemical processes) should be avoided whenever possible.

9) **Catalysis**

Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.

10) **Design for degradation**

Chemical products should be designed so that at the end of their function they do not persist in the environment and break down into innocuous degradation products.

11) **Real-time analysis for pollution prevention**

Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.

12) **Inherently safer chemistry for accident prevention**

Substances and the form of a substance used in a chemical process should be chosen so as to minimize the potential for chemical accidents, including releases, explosions and fires.

Figure 1.3: The "Twelve Principles of Green Chemistry"[22] (originally in colour)

1.2.3 Biorefinery and Green Chemistry as sustainable future

The University of York have previously demonstrated that the incorporation of green chemistry into the bio-refinery concept could help towards a sustainable future for the 21st century.[24] The group showed a number of green technologies such as $scCO_2$ extraction being implemented into conversion processes, use of agricultural waste such as wheat straw and incorporation of renewable starch as chromatography stationary phase into biorefineries.[24] For a sustainable future, it is therefore the aim of this thesis to incorporate an intermediate step in existing uses of agricultural waste with a green technology, $scCO_2$ extraction, in the extraction of valuable waxes. This concept will prevent waste by utilisation of renewable agricultural wastes and CO_2, use safer and greener solvents to minimise pollution and reduce and replace existing petroleum-derived wax products.

1.3 Agricultural waste

1.3.1 Availability

For every tonne of cereal produced worldwide, approximately 1.5 tonnes of straw is produced as an agricultural by-product. This give a world production of 1000 million tonnes of cereals per annum and about 1500 million tonnes of cereal straw is obtained. China is the main contributor of this enormous amount of straw as this single country can produce more than 700 million tonnes of cereal straw per year.[25]

Reported by the NNFCC, there were 11.8 million tonnes of cereal straws in the UK in 2007 and of which 8.3 million tonnes are from cereals and 2.5 million tonnes are from oilseeds. Wheat, the most abundant cereal crop in the UK, covers 2 million hectares which can produce about 3.5 tonnes per hectare of straw giving approximately 7.5 million tonnes of wheat straw alone.[26] Other cereal crops such as rye and triticale are also grown. Figure 1.4 shows the individual breakdown of each crop grown in UK in 2007.

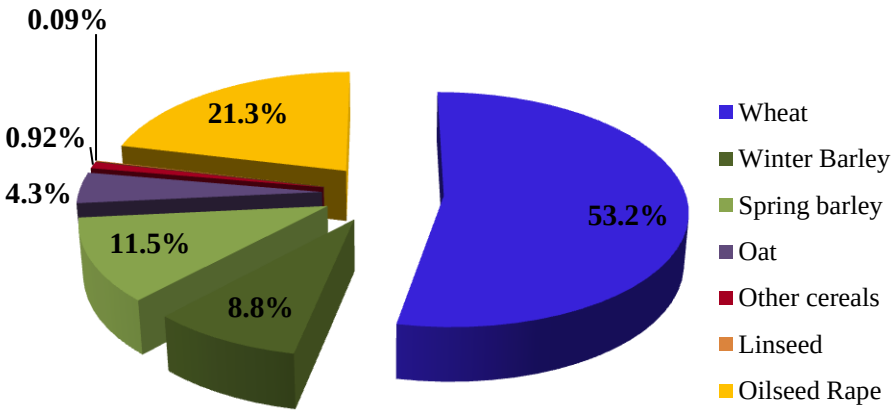


Figure 1.4: Total breakdown of cereal and oilseed crops in UK in 2007[26] (originally in colour)

These four crops are grown in all parts of the UK as shown in Figure 1.5. Approximately 1.6 million tonnes of wheat straw is produced in eastern England which makes it the greatest amount of wheat straw yielded in a single region. Barley is also predominately grown in eastern England giving 3.6 million tonnes of barley straw making this region the highest straw producing region. Oat tends to be grown in south England and can produce about 1700 tonnes in total. Oilseed rape is the major oilseed crops grown and is located mainly in the midlands which can produce about 1.1 million tonnes of straw just in this region.[26]

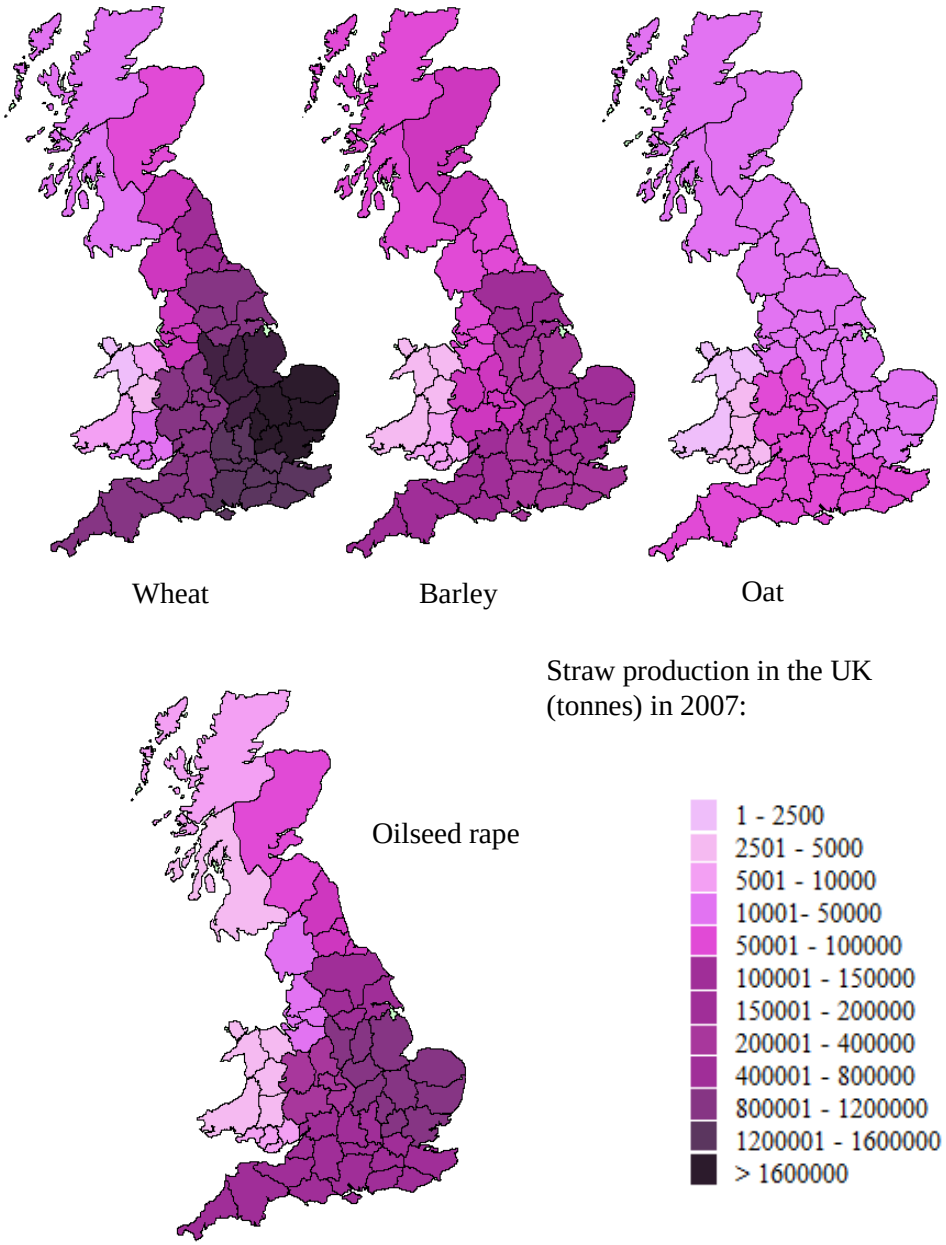


Figure 1.5: Breakdown of wheat, barley, oat and oilseed rape straw in the UK in 2007[26]

(originally in colour)

1.3.2 Straw and husk

The main agricultural waste in cereal crops are its straw and husk. Straw is the stalk of the cereal plant that is left after the grains or seeds are removed and husk is the protective outer layer of the grains or seeds and is left as a by-product after dehulling as shown in Figure 1.6. The straw alone can make up more than 50% of the dry weight of the crop.[27]

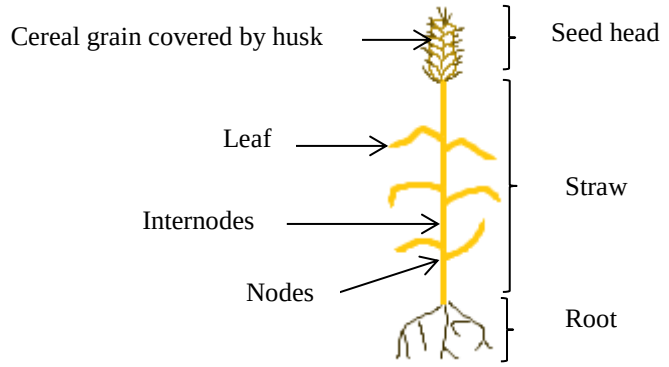


Figure 1.6: Cereal straw and its botanical components (originally in colour)

Straw consists of mainly three groups of organic compounds which are cellulose, hemicellulose and lignin and together these three components add up to more than 80% of the dry weight.[28] The minor constituents include waxes, protein, sugars, salts and ash. As discussed in Section 1.3.1, there is an enormous quantity of straw available worldwide; utilisation of this major agricultural by-product is paramount. The price of straw fluctuates on a yearly basis due to demand, weather and harvesting results. Straw prices were higher in 2010 compared to 2009 and the value of straw production rose by 15% to £346 million in the UK.[29]

Traditionally, straw is used as an animal feedstock due to its high cellulose content. About 40% of straw is cellulose which makes it a great energy source but the digestibility is limited to about 30% due to the chemical structure of straw.[30, 31] In 1800, straw was being used as source of non-wood fibre for paper for the first time. With the shrinking forests and dramatic increase in demands for paper, there was a rise in the use of non-wood fibres derived pulp from 6.7% to 10.6% from 1970 to 1993.[27] With the growing demand for energy and a declining supply of petroleum, straw is becoming important as an alternative and renewable energy source. Straw can

be converted to a useful energy source via thermochemical conversion (combustion, pyrolysis, gasification and liquefaction) or biochemical processing (digestion and fermentation). A potential total of 442 gL of bio-ethanol can be produced with agricultural waste (corn stover, wheat straw, barley straw, oat straw, rice straw, sorghum straw and bagasse) worldwide, as reported in 2004.[32] Other applications of straw include bio-based materials such as particleboards, bio-degradable plastics and adsorbents. Successful straw boards have been manufactured as a replacement in the wood-based panel industries.[33] Straw can be decomposed to soluble sugars and chemicals such as lactic acid which is a critical chemical for producing bio-degradable plastics.[34] The market for bio-degradable plastics grew five-fold between 1996 and 2001.[35] As well as using straw as animal feed, paper, energy, particle boards and biodegradable plastics, it is also used as an absorbent to battle water pollution problems. Traditionally, activated carbons are used for the absorption of dyes and heavy metals in waste water but bio-derived activated carbons have been synthesised from different straw and husk.[36, 37] The excess straw is also being incorporated into the soil and research shows that incorporation can change the soil properties both positively e.g.: nutrient gain and negatively e.g.: in clay-based soil.[38, 39] Other current uses include animal bedding, garden mulch, bio-composites, oil spillage clean-up, mushroom compost control of the algae and cyanobacteria growth in aquatic reservoir.[40, 41, 42, 43, 44, 45] Although there are many uses of straw, it has been mainly used as a whole form and not as individual components that can be utilised separately. Waxes can be extracted to create another bio-based product prior to its current uses e.g.: energy, which can add value to this low cost, high volume biomass.[19]

1.3.3 The plant cuticle

The plant cuticle is a multi-layered structure as shown in Figure 1.7. The epidermal cells are highly protected by the cell wall and a pectin cuticular layer. This pectin layer provides the surface for a layer of semi-crystalline epicuticular wax, the main function of which is to minimise water loss. Its reflective property also helps to protect the plant from ultra-violet radiation.[46, 47]. This protective waxy layer also helps in plant defence against bacterial and fungal pathogens.[46] Its hydrophobicity can help with the reduction of water retention on the surfaces and therefore minimise a build-up of air pollutants.[46, 47] It was also shown that there are small amounts of embedded

wax particles in the cutin making it a support for both intracuticular and epicuticular wax.[48] The content of the wax in the straw is low and only comprised of 0.5 – 1.0% of the dry weight but with the huge volume of this agricultural by-product worldwide, a potential of 7.5 – 25 million tonnes of valuable waxes can be obtained each year. [49] Epicuticular wax on straw consists of three structurally distinctive fractions with varied degrees of order and composition: crystalline, solid amorphous and liquid amorphous.[48] The crystalline fraction consists of regularly aligned long aliphatic chains of the wax which are packed in an orderly fashion. As the chain length of the different wax constituent compounds vary so some of the crystalline regions would result in a less ordered fraction which is the solid amorphous region within the layers as shown in Figure 1.8. [48] The cuticular wax is a dynamic structure therefore the crystalline and solid amorphous can break and undergo rearrangement. The packed array of aliphatic chains are surrounded by the solid amorphous region and with elevated temperature, this would convert into liquid amorphous.[50] The crystalline regions are assembled as orthorhombic crystal lattice at a lower temperature and is rearranged to an hexagonal structure with increased temperature but prior to melting point.[50, 51]

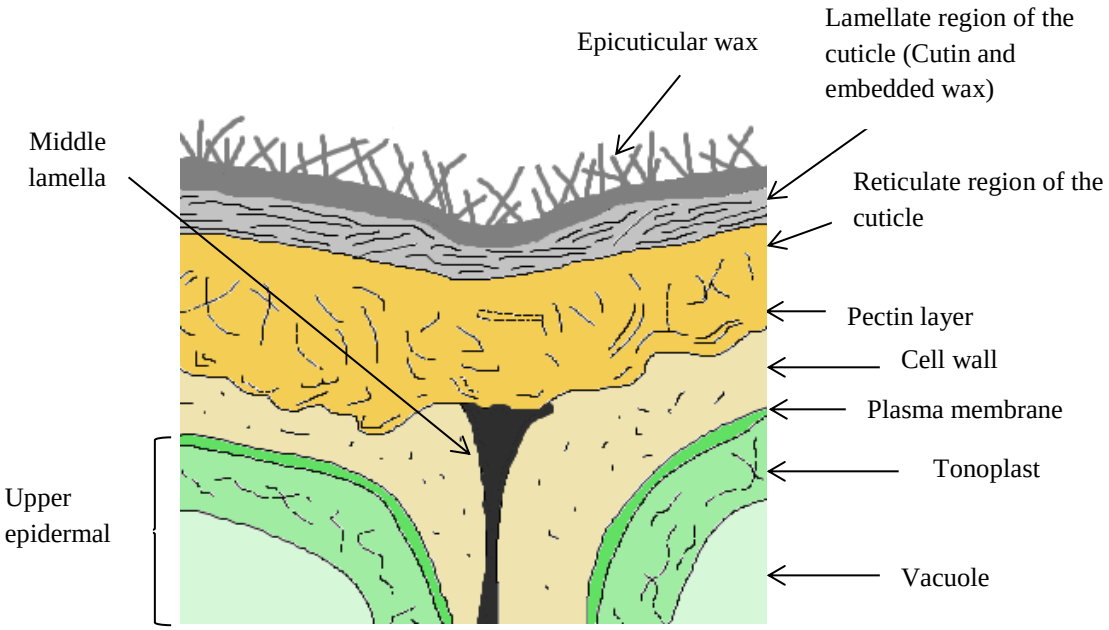


Figure 1.7: The structure of the plant cuticle (originally in colour)

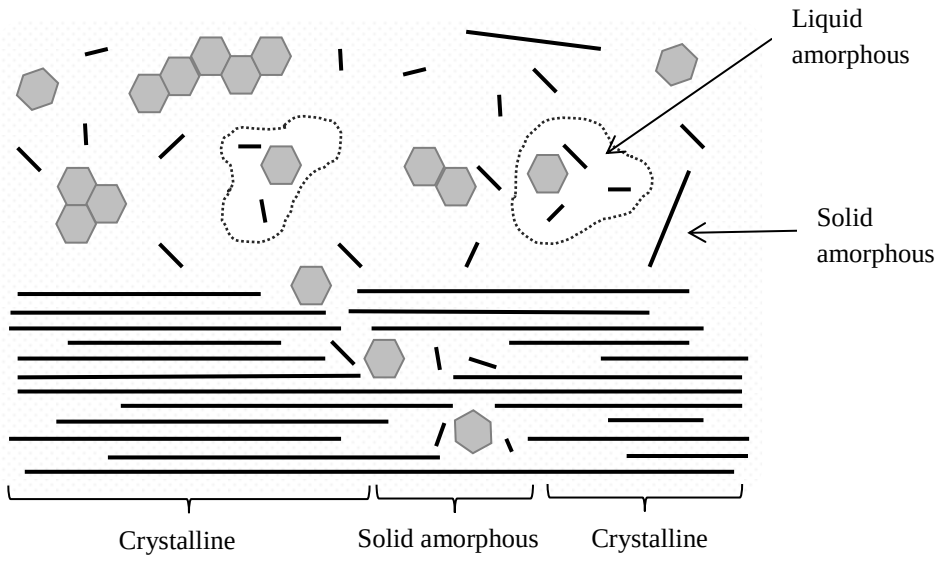


Figure 1.8: Molecular structure of cuticular waxes

1.4 Plant waxes

1.4.1 Classes of plant epicuticular waxes

Plant epicuticular waxes are complex mixtures of both aliphatic and cyclic compounds with various functional groups, chain lengths and abundance in different species. Table 1.1 shows the common wax classes with emphasis on the major components.

Hydrocarbons in plant waxes can exist as *n*-alkanes, branched- chain alkanes and alkenes. *n*-Alkanes are a universal wax class and account for more than 50% of the wax.[52] They predominately exist as odd-chain numbers and include chains of C_{27}, C_{29}, C_{31} and C_{33}.[53] Even-chain *n*-alkanes are also present in some species but it is at much lower proportions in comparison.[53] Branched-chain alkanes found are usually even-numbered but it has been reported that both odd and even-numbered are present.[53] Alkenes in plant lipids tend to be mono-unsaturated with the double bond in position 1.[54]

Wax esters are another ubiquitous class of compounds which can exist up to 80%. Mono-esters are the most common and are typically made up of saturated long even-chain acids (C_{12} - C_{30}) and alcohols (C_{20} – C_{32}).[55] Unsaturation of wax esters has been reported and the double bond(s) are usually found in the acid moiety.[56] The wax ester fractions are found to be constituted by the esterification of the homologous acids and alcohols of several isomers and/or a single homologue acid or alcohol.[55] Free fatty

acids in plants can be found unbranched, branched, saturated and unsaturated but usually exist as even-chain saturated forms and have carbon numbers of about C_{16} to C_{34}.[57] These are quite dominant in some plants and up to 40% of free fatty acids can be found [58]

Primary alcohols are commonly found and can be up to 60% of the total wax. These molecules are usually straight chain and comprise of chain lengths of C_{20} to C_{34}. In cereal crops, a single primary alcohol tends to be predominant and in wheat, hexacosanol (C_{26}) or octacosanol (C_{28}). [59, 60, 61] Secondary alcohols are also identified in plants, however they are predominately odd-chain numbers and the hydroxyl group is commonly found in the 10^{th} or 15^{th} position in a C_{29} alkyl chain and 9^{th} or 16^{th} position in a C_{31} chain.[62, 63, 64, 65] Ketones are also found in a similar pattern as these are formed from the oxidation of the corresponding alcohol.[65] Aldehydes are intermediates formed from oxidation of alcohols to fatty acids and are usually found in small quantities. If aldehydes are observed, its compositional pattern tends to be similar to the fatty alcohols.[66]

β-Diketones can be present as a major wax constituent of up to 70% in some plant species. About 10-50% of β-diketones have been reported in cereals such as wheat, barley, oats and rye.[67] The 1,3-dicarbonyl group is usually situated on positions 12,14-, 14,16- and 16,18- in C_{31} and C_{33} alkyl chains.[68] Hydroxy-β-diketones are also mainly found in cereals as the two groups are closely related and are mostly derived from the most abundant hentriacontane-14,16-dione.[67]

The cyclic components consist mainly of sterols and triterpenoids. Plant sterols, also called phytosterols, are tetracyclic groups of molecules found in plants and the most common are stigmasterol, β-sitosterol and campesterol.[69] Plants can contain up to 3 mg of sterols per gram dry weight.[70] Sterols in plants tend to be membrane constituents with main functions in regulation of its fluidity and water permeability.[71] These sterols can react with free fatty acids present on the plant surfaces and form an ester bond to make steryl esters.[70] It has been reported that the structures of steryl esters are formed from the three major free sterols identified.[72] The fatty acid moieties have been reported to be in the range of C_{12} to C_{22}.[72]. Hexadecanoic, octadecanoic and octadecenoic acids are predominately found and poly-unsaturated fatty acids have also

previously been reported as a moiety of steryl esters.[73] Esterification of free sterols and free fatty acids to form steryl esters is a way to regulate the levels these wax constituents in plants and assist the intracellular and extracellular movement of free sterols.[72, 74] Triterpenoids are pentacyclic compounds and often exist as triterpene alcohols, ketones and acids and the most common are amyrins, ursanes, lupanes and oleane.[75, 76, 77, 78] The pattern and abundance of the different wax classes in various species presented in the plant cuticle are dependent on the genetic expression via the biosynthetic pathway and environmental influences.[59, 79]

Table 1.1: Common wax classes[80]

Compounds	Carbon number and range of major components	Major components
Aliphatic wax classes		
n-Alkanes	Odd C_{21} - C_{35}	C_{27}, C_{29}, C_{31}
Wax esters	Even C_{34} - C_{62}	C_{40}, C_{50}
Free fatty acids	Even C_{16} - C_{32}	C_{22}, C_{24}, C_{26}, C_{28}
Primary fatty alcohols	Even C_{22} - C_{32}	C_{26}, C_{28}, C_{30}, C_{32}
Aldehydes	Even C_{22} - C_{32}	C_{26}, C_{28}, C_{30}, C_{32}
Ketones	Odd C_{23} - C_{33}	C_{29}, C_{31}
Secondary fatty alcohols	Odd C_{23} - C_{33}	C_{29}, C_{31}
β-Diketones	Odd C_{27} - C_{33}	C_{31}, C_{33}
Hydroxy-β-diketones	Odd C_{27} - C_{33}	C_{31}, C_{33}
Cyclic components		
Sterols		β-Sitosterol, stigmasterol, campesterol
Steryl ester		β-Sitosteryl esters
Triterpenols		α-Amyrin, β-amyrin, lupeol
Triterpenoid acids		Ursolic acid, oleanoic acid
Triterpenoid ketones		Taraxerone, lupen-3-one, oleanen-3-one, ursen-3-one

1.5 Biosynthetic pathway of plant cuticular wax

1.5.1 Biosynthetic pathway

The first stage of the biosynthetic pathway of plant cuticular wax begins by the synthesis of C_{16} and C_{18} fatty acids *de novo* in the plastids in the epidermal cells. The synthesis is carried out when the growing acyl chain attaches to the ACP in the FAS complex and is illustrated in Figure 1.9.[81] Acetyl-CoA and malonyl-CoA are both activated by ACP to form acetyl-ACP **(A)** and malonyl-ACP **(B)** which can then form an extending malonyl-ACP **(C)** via a **(a)** condensation reaction by KAS.[82, 83] Fatty acids are then synthesised by a series of reactions which result in the addition of C_2 moiety to form palmitoyl-CoA. The reactions involve the **(b)** reduction of β-ketoacyl-ACP by β-ketoacyl reductase, **(c)** dehydration of β-hydroxyl-ACP by β-hydroxylacyl dehydratase and **(d)** reduction of *trans* double bond in enoyl-ACP by enoyl reductase.[82, 83] The **(e)** reaction cycles continue until seven C_2 moieties have been added to form a total of C_{16} or C_{18} acyl chain. There are three different KAS which are acyl chain length specific (KASIII: C_2 – C_4, KASI: C_4 – C_{16} and KASII: C_{16} – C_{18}) during the synthesis process which dictates whether hexadecanoic or octadecanoic acid **(G)** is synthesised.[84] The termination of fatty acid synthesis occurs by the **(f)** deactivation of the palmitoyl-ACP **(D)** or stearyl-ACP by acyl-ACP thioesterase and to **(f)** activate to CoA thioesters by acyl-CoA synthase which results to hexadecanoic acid **(E)** and coenzyme A **(F)**.[83]

The C_{16} and C_{18} fatty acids can then undergo an **(h)** elongation process which involves the extension of the acyl chains to very long chain fatty acids once **(g)** esterified back to CoA **(H)** and translocated to the ER.[85] These are the basic components for the synthesis of the different wax constituents. The **(h)** elongation process is catalysed by other multi-enzyme complexes FAE.[82] Similar to fatty acids synthesis **(a)** condensation of acyl-CoA and malonyl-CoA, **(b)** β-ketoacyl reduction, **(c)** β-hydroxyl dehydration and **(d)** enoyl reduction. Usually the repeated elongation process extends to about 20 – 34 carbons with the addition of C_2 moiety and the termination of the process is dependent on the specificity of the FAE forming a range of C_{20} - C_{34} fatty acids.[82, 85]

The fatty acids of various chain lengths can convert back to CoA **(H)** or ACP and undergo one of the two downstream biosynthetic pathways as shown in Figure 1.9.[85] First is the acyl reduction pathway in which a **(i)** reduction reaction occurs to form aldehyde intermediates **(J)** and can further **(j)** reduce to primary fatty alcohols **(K)**. The aldehydes usually have similar carbon number to primary alcohols.[85] The primary alcohols are then either exported directly to the plant cuticle or **(k)** esterify with the pool of fatty acids **(I)** available to form wax esters **(L)**.[86] Second is the alkane pathway which explains the existence of odd numbered carbon chains which were extensively reported in plant cuticular waxes.[87, 88, 89] A typical **(i)** reduction reaction occurs to give the aldehyde intermediates **(J)** in the acyl reduction pathway followed by **(l)** decarbonylation to form odd-numbered alkanes **(M)**.[85] In many species, this is the end product of the pathway but in some cases, the alkanes can carry out further downstream conversions as the corresponding secondary alcohols and ketones have been observed.[85] The alkanes can undergo a **(m)** hydroxylation to secondary alcohols **(N)** which can then follow by an **(n)** oxidation into ketones **(O)**.[85]

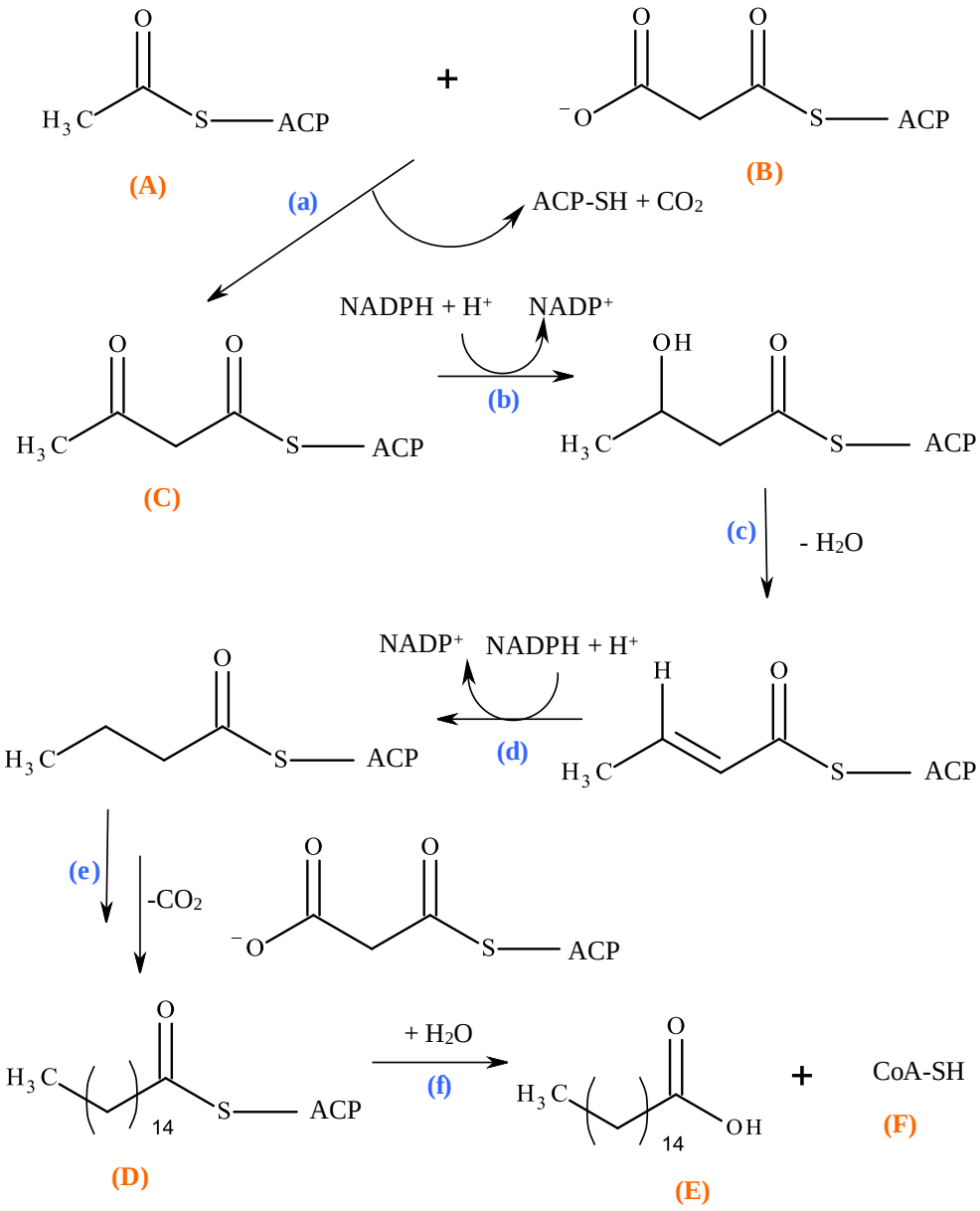


Figure 1.9: Fatty acids *de novo* synthetic pathway[85] (originally in colour)

The biosynthesis of fatty acids, fatty alcohols, wax esters, aldehydes and alkanes are widely accepted. Odd-numbered β-diketones and hydroxy β-diketones have also been reported in various plant species.[90, 91, 92] As the diketones observed are odd-numbered, it was suggested that the synthesis of the β-diketones and hydroxy β-diketones must derive from the alkane pathway of the wax biosynthesis similar to the synthesis of secondary alcohols and ketones.[92] Wettstein-Knowles *et al.* investigated the origins of

β-diketone specifically hentriacontan-14,16-dione in barley spike wax by radioactively labelling the acetate.[93] The study concluded that the two carbonyl groups were built into the carbon skeleton during the elongation rather than downstream reactions after the termination like secondary alcohols and ketones as shown in Figure 1.10.[94] This was supported by the lack of abundance correlation between alkanes and β-diketones.[94] The β-keto group is introduced into the acyl chain when a C_2 moiety is added to the palmitoyl-CoA but in this case, the β-keto group is not reduced like the fatty acid elongation pathway.[93] The group is **(P)** protected by a metal ion e.g.: copper and this could be a metal ion from an enzyme. Further elongation would occur by the **(m)** addition of another C_2 moiety and the protecting metal ion would remain until the number of carbon units have been added to the chain.[93] A **(n)** decarboxylation of the β-keto acyl chain and a **(o)** deactivation process occurs which results in an **(Q)** odd-numbered β-diketone.[93] The **(R)** β-diketone precursors can then further react and synthese hydroxyl β-diketones via a reduction reaction in one of the carbonyl groups.[94]

The biosynthetic pathway for cyclic compounds such as sterols is different as it derives from the isoprenoid pathway where IPP and DMAPP are the main building blocks. These two molecules react to form FPP by FPP synthetase which is an important precursor for sterol synthesis that occurs in the plastid.[95] More than 30 enzymes and steps are reported to be involved in the sterol synthetic pathway but this is controlled by a key enzyme, squalene synthetase.[96] Squalene **(R)** forms and then converts to squalene oxide **(S)** where cyclisation occurs to create cycloartenol **(T)** then cycloeucalenol **(U)** and followed by obtusifoliol **(V)**.[95] A series of different reactions then occur for the synthesis of various sterols but studies showed that sitosterol is the first sterol to be made in the sequence.[95] Various chemical reactions further downstream lead to a matrix of very complex sterols. Sterols are an important class of molecules that regulate the fluidity of membranes.[95] Sterols can also exist in bound form as the reactive 3' OH group can react and interact with other molecules such as fatty acids to form steryl fatty acid esters and sugars to form sterol glycosides.[95] These are also components that are commonly found in plant waxes both as free and bound forms.[95] The biosynthetic pathways for the aliphatic and cyclic wax constituents give a good explanation in the various wax classes that have been reported in the literatures.

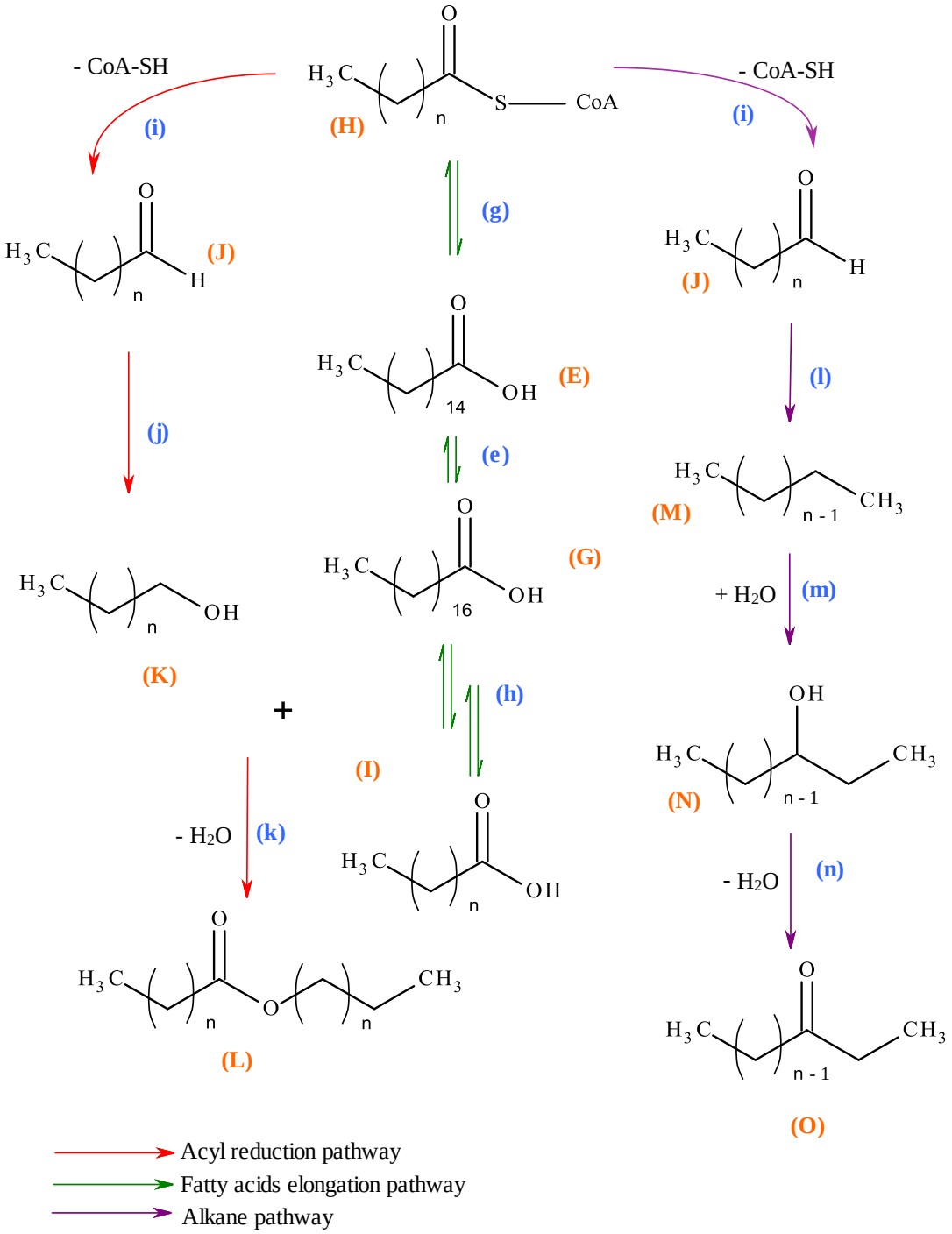


Figure 1.10: Wax biosynthesis including acyl reduction, fatty acids elongation and alkane pathways[85] (originally in colour)

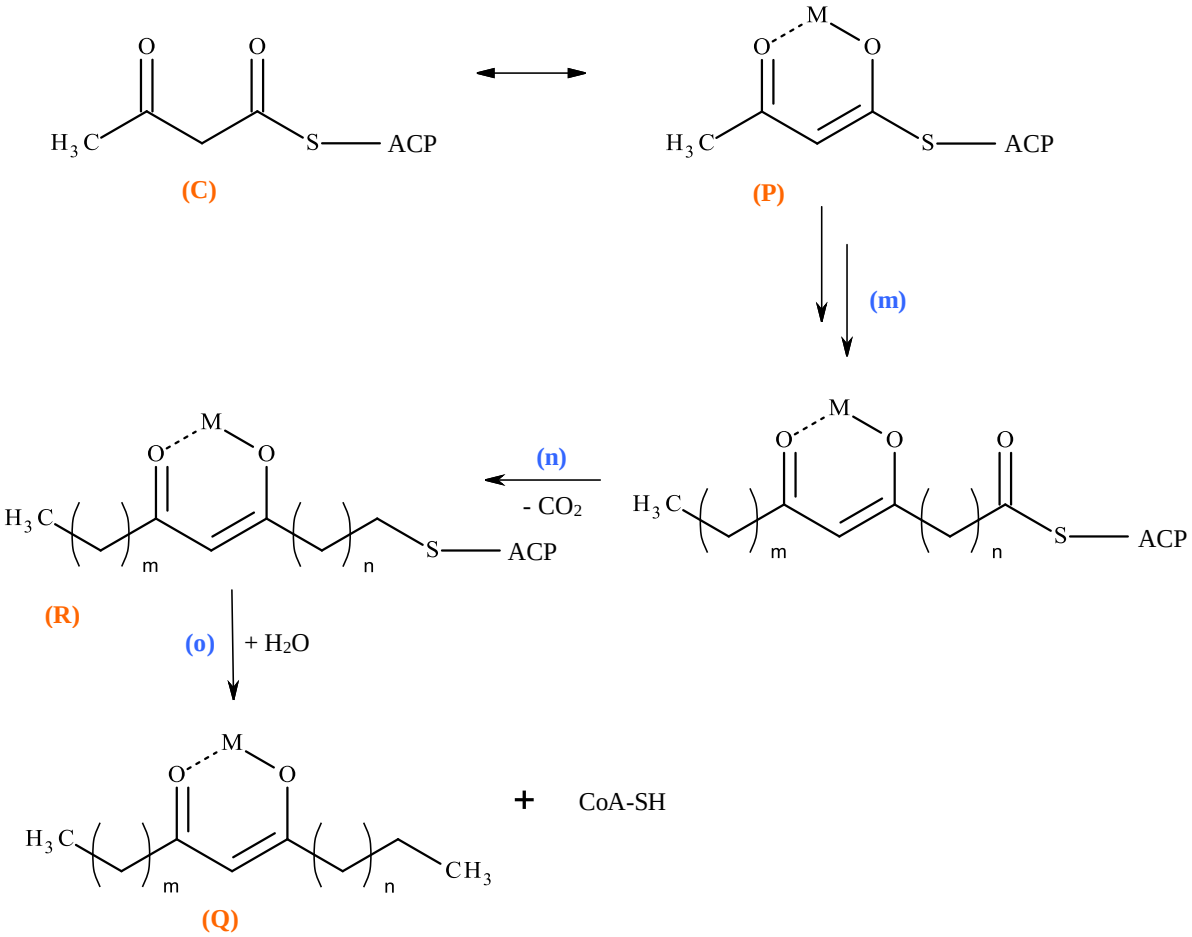


Figure 1.11: Beta-diketone biosynthetic pathway[93] (originally in colour)

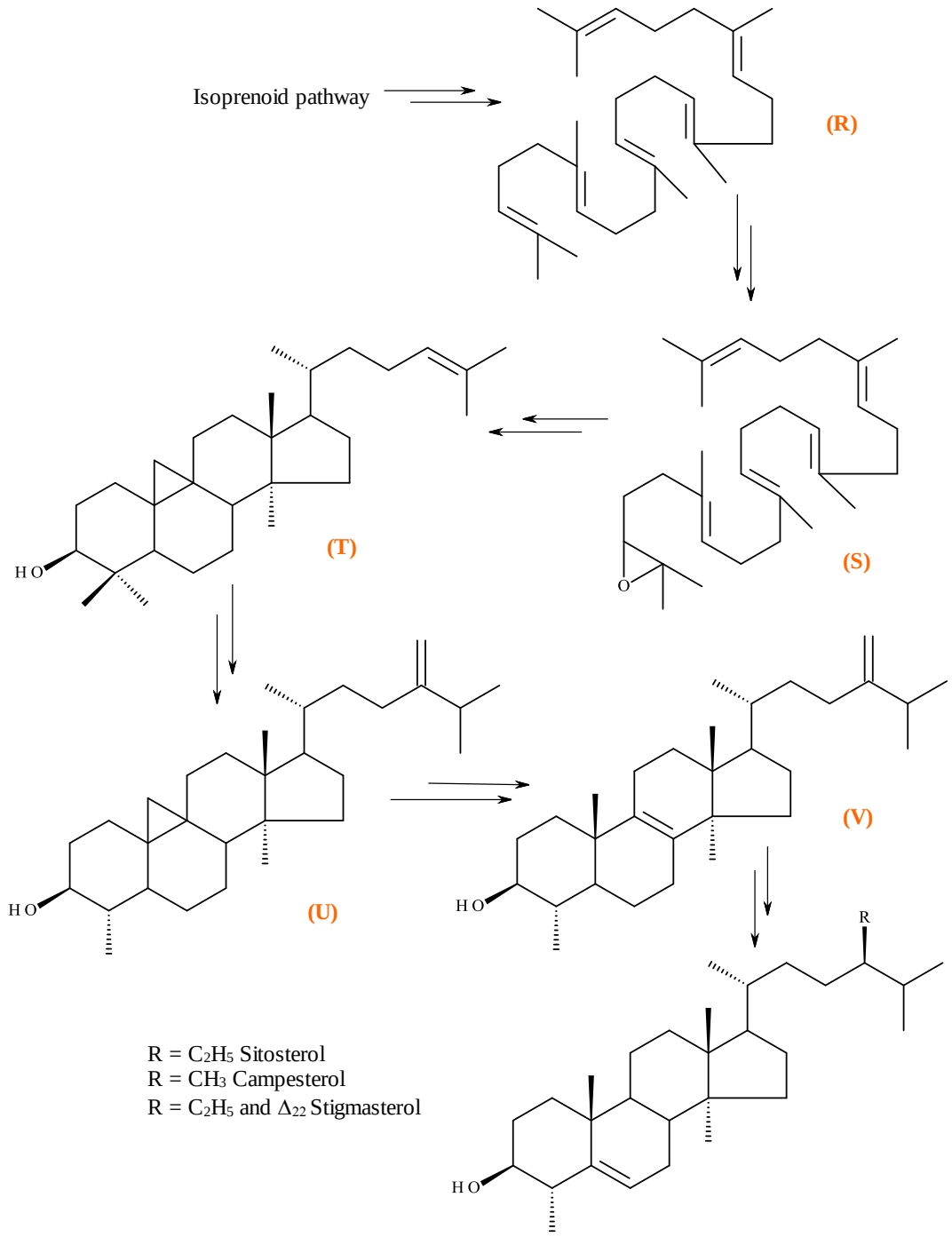


Figure 1.12: A simplified sterol biosynthetic pathway from the isoprenoid pathway[95]

1.5.2 Cereal waxes

Extensive research was carried out by Tulloch and Bianchi between late 1960s to mid-1980s on the epicuticular wax of cereal crops as shown in Table 1.2.

Table 1.2: Epicuticular wax of cereal plants

Reference	Cereal (Species)	Investigation
Tulloch *et al.* (1969)[97]	Wheat (*Triticum compactum*)	Six varieties (Little Club) of young wheat (weeks before maturity) dipped using cold light petroleum ether: composition quantification of one variety
Tulloch *et al.* (1971)[98]	Wheat (*Triticum durum*)	Two varieties (Pelissier and Stewart 63) of young wheat (weeks before maturity) dipped using cold light petroleum ether: composition comparison
Tulloch *et al.* (1973)[99]	Wheat (*Triticum aestivum*)	Two varieties (Selkirk and Manitou) of young spring wheat (65 days after germination) dipped using cold light petroleum ether : composition quantification
Tulloch *et al.* (1974)[100]	Oat (*Avena sativa*)	Mature oat (Kelsey) dipped using cold light petroleum ether: composition quantification
Bianchi *et al.* (1977)[91]	Wheat (*Triticum aestivum*)	Three stages of growth (30, 130 and 190 days after germination) of young wheat (Demar 4) plants dipped using cold chloroform: composition comparison
Bianchi *et al.* (1978)[101]	Sorghum (*Sorghum vulgare*)	Two varieties (Alliance A and SD 102) of sorghum (tasseling stage) dipped using chloroform: composition quantification
Bianchi *et al.* (1979)[102]	Rice (*Oryza sativa*)	Early boot stage rice (Ribe) dipped using cold chloroform: composition quantification
Bianchi *et al.* (1980)[103]	Wheat (*Triticum aestivum*)	Chromosomal deficiencies on matured wheat (Chinese Spring) dipped using chloroform: composition quantification
Bianchi *et al.* (1984)[104]	Sorghum (*Sorghum bicolor*)	Two stages of growth (panicle and fourth-fifth leaf) of mature sorghum (SD 102) and (tassling) sorghum (Alliance A and Martin A) dipped using chloroform: composition quantification
Bianchi *et al.* (1986)[105]	Wheat (*Triticum durum*)	Glaucous and non-glucous matured wheat dipped using cold chloroform for 1 minute: composition quantification

Most of the research tends to focus on the age and genetics of the cereal plants. Only a small number of cereal plants have been studied and, as shown in Table 1.3, the most studied was wheat. This could be due to the countries of study or availability of raw materials. Column chromatography was used frequently as a mean of separating the

complex mixtures for compound identification and quantification with GC.[97, 98, 99] Major classes of wax compound have been clearly identified as fatty acids, fatty alcohols, alkanes, wax esters, sterols, aldehydes and β-diketones, however most of the data collected was based on young plants and there is a lack of data on mature plants.[91, 97, 99, 100] Due to the high molecular weight of many wax compounds, a lot of the studies only showed partial characterisation. High temperature GC-MS tends to be the technique of choice when analysing the wax components.[18] The wax samples are sometimes derivatised (usually silylation) prior to analysis to make the compounds more volatile, improve peak shapes, reduced adsorption losses and shorten retention times.[18]

In the 1990s, there was a lack of interest in the field of cereal waxes and limited research took place until the 2000s. The raw materials of interest then were cereal wastes and not young plants. As shown in Table 1.2, a lot of research was carried out on cereal straws. This could be part of sustainable development and adding value to waste products. In the early 2000s, organic solvent extractions were studied on various cereal straws and full characterisation was carried out. Physical properties of these valuable extracts became an interest not only in the academic field but also in the commercial field. A lot of physical properties (particularly thermal properties) were determined for the extracts and compared to existing commercial waxes as part of the sustainable development and bio-refinery concept. [107, 108] Alternative plant waxes were needed to replace some of the existing synthetic and petroleum waxes so there was a real drive in the research. From the mid-2000s until 2012, industries were not only trying to secure a more sustainable wax source but also attempting to modify the existing extraction method. Supercritical CO_2 is being introduced as a green alternative solvent for the extraction of cereal straw wax.[18, 88, 90] Supercritical CO_2 is not a new technology; it has been used extensively in commercial extraction such as decaffeination of coffee and extraction of hops.[106, 107] Despite this, the solubility of wax compounds in CO_2 is not well understood compared to tradition organic solvents so many attempted to compare $scCO_2$ with organic solvent properties. Optimisation of CO_2 conditions for extraction had been modelled using various established equations, however none have yet to apply to cereal wax.[90] Using CO_2 to fractionate the wax extracts has been developed so that extraction and fractionation can be carried out as a one step process.[16]

Table 1.3: Epicuticular wax from cereal waste

Reference	Cereal (Species)	Investigation
Sun *et al.* (2001)[108]	Rice (*Oryza sativa*)	Rice straw extracted in six solvents (toluene/ethanol (2/1, v/v), chloroform, petroleum ether (bp: < 318 K), DCM, hexane and MTBE, Soxhlet): physical properties and composition quantification
Sun *et al.* (2001)[89]	Barley (*Hordeum vulgare*)	Barley straw extracted in four solvents (toluene/ethanol (2/1, v/v), chloroform, MTBE, hexane/acetone (2/1, v/v) and DCM, Soxhlet): physical properties and composition quantification
Sun *et al.* (2001)[109]	Rye (*Secale cereale*) and rice (*Oryza sativa*)	Rye and rice straw extracted with MTBE (Soxhlet): physical properties and composition quantification
Weller *et al.* (2002)[110]	Sorghum (*Sorghum bicolor*)	Sorghum straw dipped in three organic solvents (light petroleum ether, hexane, chloroform) for 1 minute: physical properties and composition quantification
Sun *et al.* (2003)[111]	Wheat (*Triticum aestivum*)	Wheat straw extracted in four solvents (toluene/ethanol (2/1, v/v), toluene/ethanol/ methanol (1/1/1, v/v/v), MTBE and chloroform/methanol (2/1, v/v), Soxhlet): physical properties and composition quantification
Morrison *et al.* (2005)[112]	Flax/linseed (*Linum usitatissimum*)	Flax dusk from flax fiber processing extracted with hexane (Soxhlet), $scCO_2$ and $scCO_2$ with ethanol (333 K and 55.2 MPa and sequential extraction with addition of ethanol at 1%, 5% and 10%): composition quantification
Deswarte *et al.* (2006)[18]	Wheat (*Triticum aestivum*)	Wheat straw extracted in five solvents (hexane, toluene, MeTHF, ethanol and acetone, Soxhlet), $scCO_2$ (313 K and 10/30 MPa) and lCO_2 (283 K and 6 MPa) : physical properties and composition identification
Mazza *et al.* (2009)[88]	Flax/Linseed (*Linum usitatissimum*)	Three varities of flax straw (AC, McDuff, NorLin and Flanders) extracted with hexane (Soxhlet) and $scCO_2$ (343 K and 30 MPa): physical properties and composition quantification
Mazza *et al.* (2010)[90]	Triticale (*Triticosecale wittmack*)	Triricale straw (AC Ultima) extracted with hexane (Soxhlet) and $scCO_2$ (343 K and 25/30/35/40 MPa): physical properties and composition quantification

From the literature review, it is clear that optimisation of CO_2 and solvent extraction of cereal waxes is needed. Alternative purification and fractionation techniques must be adopted instead of traditional column chromatography for a greener process. The influence of chemical composition on physical properties must also be understood so that the extraction conditions can be optimised for commercialisation and replacement of existing commercial waxes.

1.6 Commercial waxes

The word "wax" is actually originally derived from the Anglo-Saxon "waex" for the material found in the honeycomb of the bee. However, beeswax is not the only type of commercial wax available. The competition in the wax business is high with many traditional waxes as well as new waxes and they can be natural or synthetically produced. There are broadly five different categories of waxes: animal, insect, vegetable, mineral and synthetic.

1.6.1 Manufacturing of commercial waxes

Waxes from different sources can be extracted by various methods depending on many factors such as chemical compositions, yield and location of the wax. The most common animal waxes are beeswax and lanolin and they are both by-products of other industries.[113] Beeswax is a valuable wax that is projected from the wax glands under the abdomen of the honey bee (usually European bees *Apis mellifera* in UK) for the application of honeycomb.[114] The wax can be recovered by melting when the honey is removed from the honeycomb and is classed as a by-product from honey production.[113] It is then filtered through a steam-jacketed filter press and washed with water to remove impurities.[113]

Wool grease is another common type of animal wax which is a by-product from the textiles industries.[115] The shorn wool tends to have wool wax from 5 – 16% by weight of the fleece depending on the quality and breed.[115] The wax is secreted from the sebaceous gland of the sheep to protect the animal against wetting.[116] The Australian or New Zealand wool is washed in a mixture of cold and warm water with detergents for the textiles industries.[117] Heavy solids such as sand are removed to give clean wool. The wool grease can be recovered from the "dirty" washings with a vortex and centrifuge step.[117] Project sponsor Croda Europe Limited is a global leader in lanolin supply and the manufacturing process described is based on communications with the company during a site visit. The wool grease from scouring mills arrives at Croda site for lanolin processing.

The wool grease is then recovered by the addition of sulphuric acid to about pH 2.5 – 3.5 to remove any pesticides and sodium hexametaphosphate added to remove any metal salts and for bleaching purposes.[117] The wax is further bleached by hydrogen peroxide to a specified Gardner colour and then followed by an alkaline wash to remove any free acids. Any soap created during the free acid removal process would be removed by washing in isopropanol and water. At this stage of the process, the wax can undergo various processing routes to create different forms and grades of lanolin. The wax can be steam deodourised to give the "undistilled" lanolin, molecular distilled to yield the "distilled" lanolin, winterised using ethyl acetate to create "hard" and "liquid" lanolin fractions or saponified to give the lanolin acid and lanolin alcohol.[117] A few types of the lanolin can then be super-refined using flash chromatography to give super-refined lanolin.[117]

Other important waxes include candelilla and carnauba waxes, which are two of the key vegetable waxes and both are grown in South and Central America.[113] Carnauba wax originates from carnauba palms (*Copernicia cerifera*) in Brazil but as the Brazilian government is protective and tightly controls their production, the manufacturing processes are carried out only in that country.[113] The wax is removed by threshing when the leaves are cut and dried. Approximately 5 – 8% of wax can be retrieved depending on the size of leaf, age, growing conditions etc.[118] Candelilla wax is also derived from a regional specific candelilla shrub (*Euphorbia antisiphilitica*, *Euphobia cerifera* and *Pedilanthus pavonis*) which is grown predominately in Mexico. The wax is extracted out by heating the plant in boiling water with 1% sulphuric acid which is added to enhance the wax recovery and reduce foaming.[113] The waxy layer located on top of the aqueous layer is removed and strained and gently heated to remove the excess water.[113] The yields vary from 1.5 – 2.5% depending on age, growing conditions etc.[119]

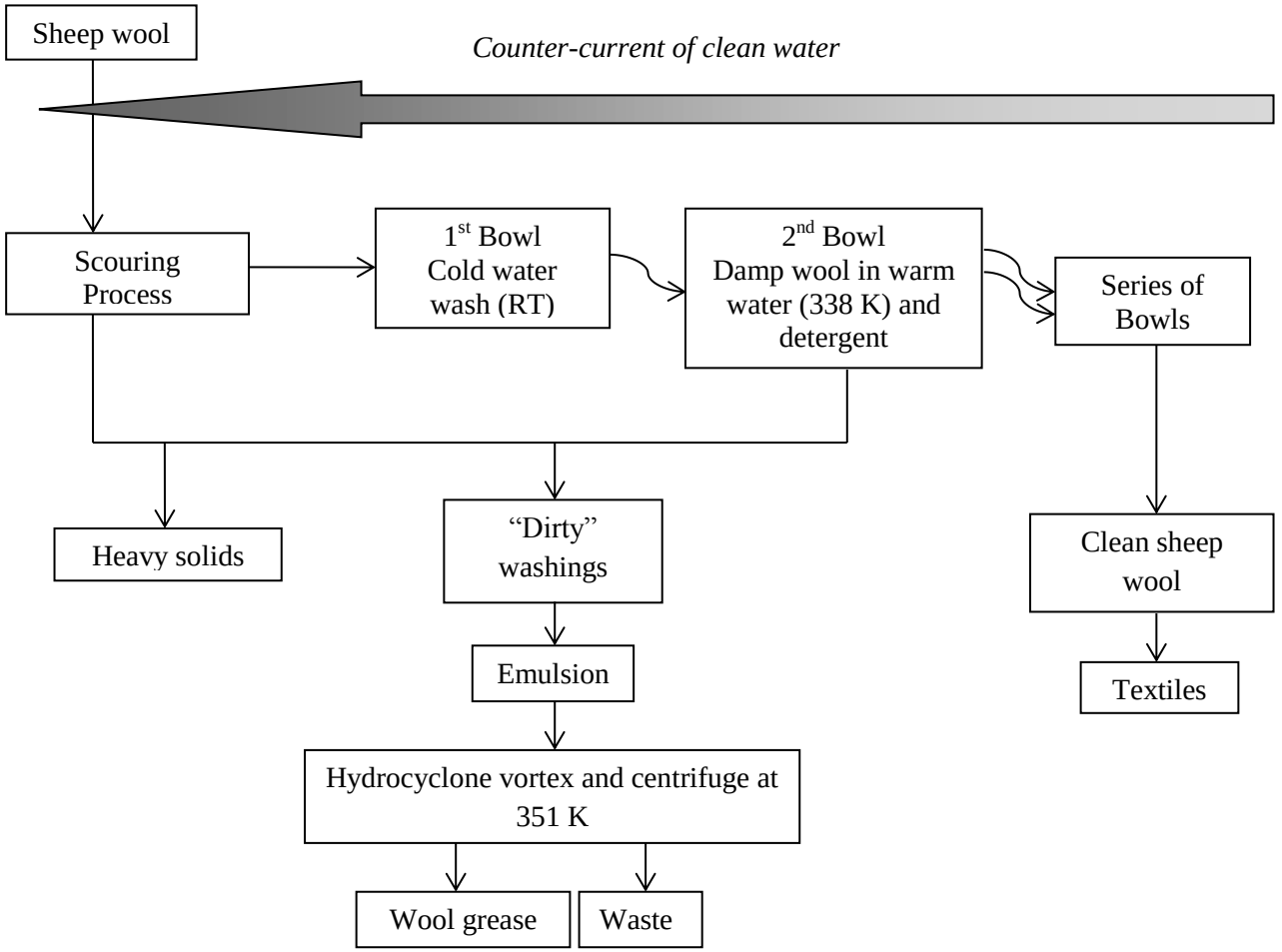


Figure 1.13: Wool grease retrieval from sheep wool[115, 117]

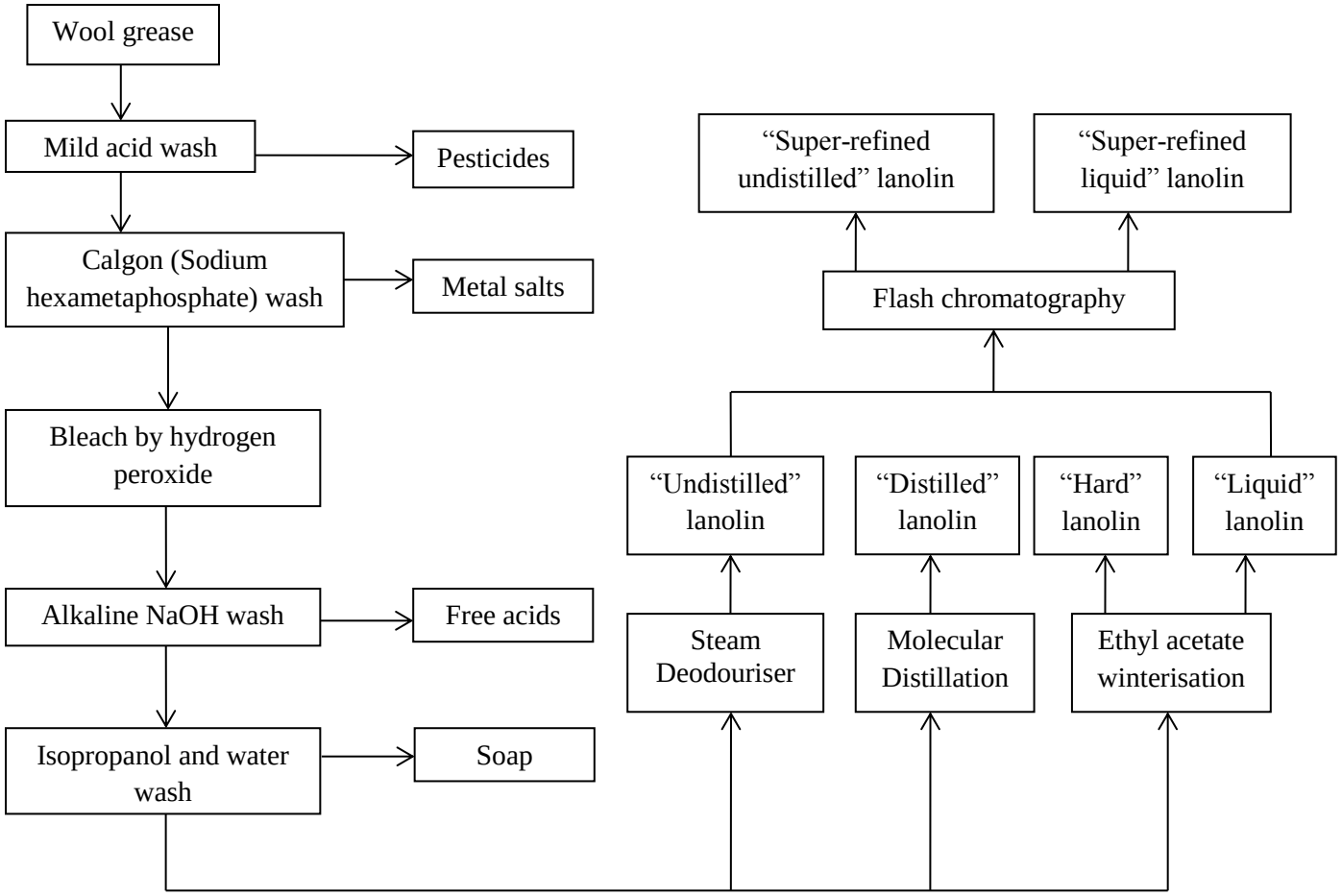


Figure 1.14: Wool grease processing at Croda

1.6.2 Chemical composition and physical properties

The four waxes consist of predominately esters and hydrocarbons. Beeswax, carnauba wax and lanolin are ester-based with at least 80% in its composition. Candelilla wax is distinctly different to the other two commercial waxes as it has a high content of hydrocarbon. Wheat straw wax had been reported to contain aliphatic wax esters, alkanes, free fatty acids, free fatty alcohols, sterols and β-diketones but the quantity of each lipid class has not been identified.[16] The identification of the chemical compounds in the wax is important as it is directly related to its physical properties which can influence its applications. Commercial waxes are graded, characterised by a number of properties and tested according to various pharmacopeia guidelines depending on its application. The colour, odour and taste of waxes are tested and usually decolourisation and deodorisation steps are carried out to fit with the wax application. For applications such as cosmetics, it is undesirable for the wax to be heavily coloured or have strong odour. Beeswax is commonly bleached by hydrogen peroxide to a satisfying colour.[120] The unpleasant sheep odour is removed from lanolin by vacuum deodorisation at 0.2 – 0.5 KPa and 373 – 393 K with steam purging.[115] The waxes are usually taste-free after purification and processing and this is particularly important for candelilla wax as one of the major applications is as a binder for chewing gums.[113] Thermal properties such as melting and softening points are necessary for the functionality of certain applications such as depilatory waxes.[121] Water absorption levels for emulsion formation in cosmetics and waterproofing properties in coatings must be balanced for applications performance.[122, 123] Other physical characteristics that are also important include penetration, shrinkage, drop point and density.[113, 117] Chemical constants such as acid value, saponification value and iodine value are commonly determined for waxes as they give an overview into the chemical components. Acid value is the number of mg of KOH needed to neutralise 1 g of the wax, saponification value is the number of mg of KOH required to hydrolyse 1 g of wax and iodine value is the amount of iodine that is reacted with the double bonds in the wax so it would give an indication of the degree of unsaturation.[113] Some of the key physical properties for the commercial waxes are expressed in Table 1.4. The physical properties for the commercial waxes are distinctively different to serve different applications.

Table 1.4: Chemical composition and physical properties of four commercial waxes[113, 116]

	Beeswax	Carnauba wax	Candelilla wax	Lanolin
Lipid classes				
Aliphatic wax esters	70 - 80%	84 - 85%	28 -29%	48 - 59%
Steryl esters	-	-	-	32 - 35%
Hydrocarbons	10 - 15%	1.5 - 3%	50 - 51%	0.4 - 2%
Free fatty acids	12 - 15%	3 - 3.5%	7 - 9%	1 - 4%
Fatty fatty alcohols	-	2 - 3%	12- 14% (Total)	-
Resins	-	4 - 6%		-
Sterols	-	-		0.8 - 1.5%
Triterpene alcohols	-	-	-	4.5 - 5.5%
Lactides	-	2 - 3%	-	-
Lactones	-	-	-	6 - 6.6%
Physical properties				
Acid value (mg of KOH per g)	17 - 36	2.9 - 9.7	12 - 22	7 - 15
Iodine value (g iodine per 100 g)	7 - 16	7 - 14	19 - 45	15 - 30
Saponification value (mg of KOH per g)	90 - 149	79 - 95	43 - 65	100 - 110
Melting point (K)	335 - 338	351 - 358	339 - 344	308 - 315

1.6.3 Wax market and applications

Waxes are the basis of many different applications and are important raw materials for many industries. In 2010, there was a global demand of 4.35 million tonnes of waxes of which 85% was petroleum waxes, 11% was synthetic waxes and 4% animal or vegetable waxes.[124] Synthetic and vegetable waxes are the fastest growing products in the 2010 wax market with a massive increase in production from Asia.[124] Petroleum wax is by far the most important in the wax market as the main application for this wax is candles which accounts for 43% of the application of waxes.[113] The second biggest application is board sizing which holds 15% of the wax market. Impregnation of petroleum wax consisting C_{30} - C_{50} hydrocarbons are typical for the paper industries.[113] Another major application for waxes is coatings and this includes coatings for wires, food packaging, fruits, paper, tablets and wrapping as some waxes are very hydrophobic and can provide water proofing of the products.[113] This holds about 14% of the global wax market as shown in Figure 1.15. Waxes are also used in adhesives as a laminator and protective coating and a number of different polishes

from shoes to cars.[113] A large number of cosmetics also have waxes in their formulations for different purposes including lip-sticks, skin cream, depilatory wax etc.[113, 121] Lanolin can self-emulsify into a stable water-in-oil emulsion and has emollient properties which are ideal as a cosmetic ingredient for skin creams.[115] Waxes can also be blended into different products for improved performance. Paraffin wax is added to rubber to increase the stiffness of the product; carnauba wax is blended into candles at a low percentage as a hardener and beeswax is incorporated into coatings to increase flexibility.[113] Many formulations or applications involve more than one wax as there are advantages and disadvantages to each wax. In a typical lip-stick formulation, a combination of waxes is formulated into the stick for various functions.[125] Candelilla wax is formulated in as a harder; lanolin serves as an emollient and helps the stick to remain as an homogeneous mass whereas carnauba wax is added in order to increase the toughness of the stick.[125]

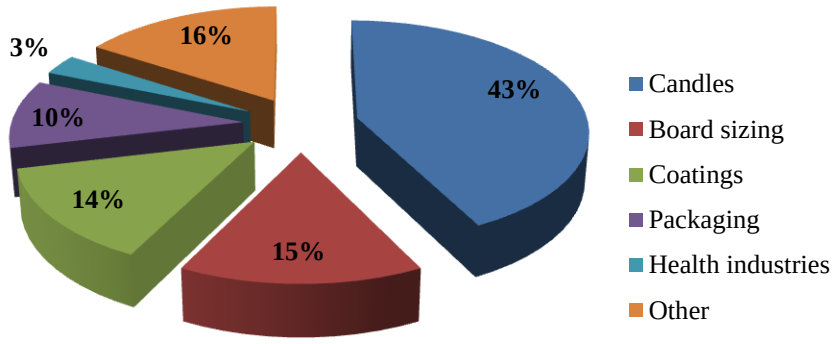


Figure 1.15: Global wax applications[124] (originally in colour)

1.6.4 Wax market outlook and opportunities

The demand for wax is predicted to grow from 4.35 million tonnes in 2010 to 5.46 million tonnes in 2020 with an average annual growth of 2.3%. However, the wax supply will not be growing at the same rate. Wax supply is forecasted to grow at just 1.4% which gives a predicted wax supply of only 5 million tonnes so as a result there would be 0.47 million tonnes of wax shortage as shown in Figure 1.16. This is mainly due to a zero increase in petroleum waxes from the shortage of crude oil. By 2015, the

market will experience uneven demand and supply of wax despite the increased production of synthetic and natural waxes. This market research data shows a huge gap in the wax supply and as a result increases the opportunities for new waxes in the market.

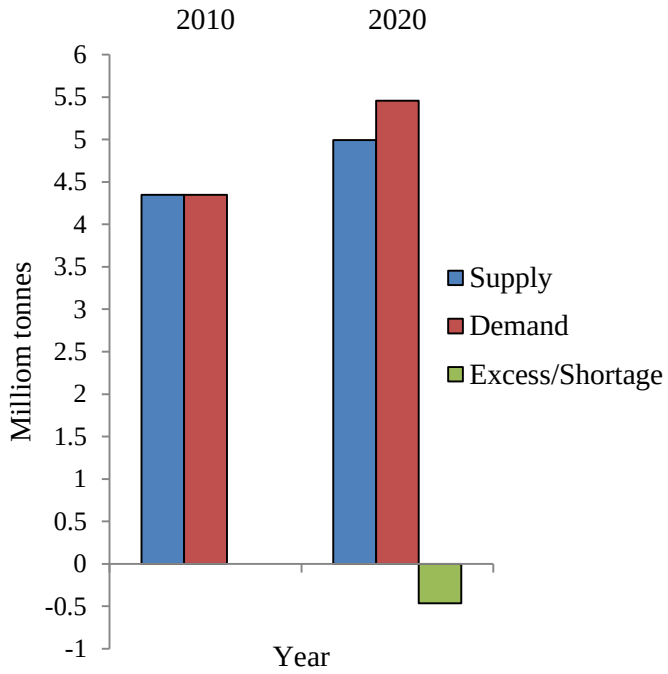


Figure 1.16: Wax market outlook in 2020 (originally in colour)

Wheat straw wax has been highlighted as a potential renewable wax with many attractive characteristics to replace some existing commercial waxes. In 2006, Deswarte carried out a successful pilot scale $scCO_2$ extraction of wheat straw yielding 770 g of wax and various physical properties were tested and showed promising results for the replacement of commercial waxes.[16] It had been concluded that wheat straw wax could potentially be useful as a raw material in cosmetics formulations. This new wax could potentially be a real competitor in the cosmetics market as there is a big drive from consumers for natural cosmetics. In order to brand a product "natural", the processing and the product must follow tight guidelines. Figure 1.17 shows a picture of a B&T Company poster taken at the international cosmetics exhibition "In-Cosmetics®" in 2010 as an example of industrial interest in green products. Companies are researching and developing new green raw materials and processing to meet the consumers' needs; many of which have developed their own

in-house “green” guides when formulating and are branding their products “natural” according to their in-house guidelines which usually involve a “banned” list.

Figure 1.17: A poster by B&T Company at In-Cosmetics® 2010 (Picture taken at the exhibition) (originally in colour)

1.7 Introduction to work in thesis

The literature showed there is a large quantity of under-utilised straw and this renewable agricultural by-product has been reported to be covered with a layer of valuable semi-crystalline wax which clearly indicates a great opportunity for the extraction of these high value chemicals from this waste. Only a small amount of work had been done in regards to characterisation of straw cuticle waxes so therefore it would be interesting to carry out a biomass screen for extraction yields and important wax molecules so potential biomass for sources of waxes can be identified. Typical key wax classes identified in straw cuticle waxes include fatty acids, fatty alcohols, sterols, wax esters, β-diketones and aldehydes. Traditionally, these wax molecules are extracted using organic solvents but due to demand for more sustainable extraction methods, supercritical CO_2 as an environmentally friendly alternative to traditional organic solvents are being explored. However, minimal

optimisation of solvent and $scCO_2$ extractions had been carried out. In solvent extractions, mathematical modelling can be applied on natural product extractions so a relationship between solvent parameters and extraction yields can be established. The different wax components can be characterised and quantified and develop extraction models for specific wax classes. Optimisation of CO_2 extraction on straw waxes carried out previously was limited so it would be interesting to further develop the optimisation using different $scCO_2$ models. Extraction and fractionation methods can alter the chemical compositions of the wax products so it is crucial to examine the physical properties and performance of the novel renewable wax products. This would give an indication on the similarities and differences in physical properties between existing commercial waxes so the potential applications can be identified. With the research gaps highlighted, the work in this thesis had been split into four main areas:

Chapter 2 describes the extraction of wheat straw wax using various organic solvents and mathematical modelling was carried out to relate the solvent parameters to selectivity. Characterisation of the chemical composition of the wheat straw wax was described and highlighted potential applications for the compounds identified. Quantification of the extracts was studied and mathematical modelling for different wax classes was investigated.

Chapter 3 examines the use of carbon dioxide as an environmentally friendly alternative to traditional organic solvents. Carbon dioxide was compared with the organic solvents and the similarities and differences were highlighted. The effect of temperature and pressure on $scCO_2$ extraction was investigated extensively. Optimisation of temperature and pressure was carried out using two different methods. Qualitative and quantification analysis of the wheat straw wax were completed for different extraction conditions so conclusions can be drawn on optimal extraction conditions for both total crude extraction yields and specific wax classes. The influence of chemical composition on physical properties was considered.

Chapter 4 investigates the potential biomass for wax sources by carrying out a straw screening using hexane and ethanol extractions. The extraction yields and new molecules found were highlighted. The chemical composition of the different types of straw wax was compared and the potential biomass for further investigation was selected. Selected cereal straws were scaled up to production scale and the chemical compositions of the straw waxes were compared and analysed. Comparison between laboratory and production scale was drawn.

Chapter 5 focuses on downstream processing after $scCO_2$ extraction. The co-extraction of water with the waxes was the first initial processing step and was noted as new potential waste materials for different markets. Other processing steps such as fractionation and saponification were discussed. The physical properties such as melting point were determined so potential applications can be selected for formulation trials. Economic analysis was carried out based on the production scale trial and was used to conclude for further scale ups and potential commercialisation.

Chapter 2

Solvent extraction of wheat straw wax

V.L. Budarin, P.S. Shuttleworth, J.R. Dodson, A.J. Hunt, B. Lanigan, R. Marriott, K.J. Milkowski, A.J. Wilson, S.W. Breeden, J. Fan, E.H.K. Sin and J.H. Clark, *Energy Environ. Sci.*, 2011, **4**, 471-479

Poster given at 6th International Conference on Renewable Resources and Biorefineries 6 (RRB6), Düsseldorf, Germany, June 2010

Poster given at Vice Chancellor visit to Department of Chemistry, University of York, August 2010

Oral presentation given at 1st NORthern Sustainable Chemistry meeting (NORSC), York, UK, October 2010

Poster given at Dechema's Green Solvents for Synthesis conference, Berchtesgaden, Germany, October 2010

Poster given at Shell poster competition, University of York, January, 2011

Oral presentation given at 7th International Conference on Renewable Resources and Biorefineries (RRB7), Bruges, Belgium, June 2011

2. SOLVENT EXTRACTION OF WHEAT STRAW WAX

2.1 Introduction

A semi-crystalline layer of valuable wax is located on the surface of plants which can be removed mechanically or by solvent extraction. In this Chapter, a number of solvents (heptane, ethanol, diethyl ether, 2-methyl tetrahydrofuran, acetone, toluene, ethyl acetate, propanol, butan-2-ol, dimethyl carbonate and methanol) were investigated for their abilities to selectively Soxhlet extract waxes from winter wheat straw (Viscount 09). Chemical compositions of the wheat straw wax extracted were characterised using KI and GC-MS. The main components in the extracts were quantified using GC FID. Potential applications of the key high value wax groups are discussed. LSER modelling was applied to the extraction crude yield and individual wax groups for future solvent extractions. The environmental impact of the solvents is considered when identifying the "ideal" solvent.

2.2 Solvent selection and extraction

2.2.1 Solvent parameters and linear solvation energy relationships (LSER)

Solvent is a liquid substance that serves the purpose of dissolving chemicals in applications such as extractions and can be removed unchanged after the applications (unless solvent is one of the reactants).[126] In extractions, the purpose of the solvents is to solubilise solutes selectively and effectively as well as easily separate and recover solvents and solutes. The dissolution of a compound in a solvent can only occur when the attractive forces between solute and solvent outweighs both solvent-solvent and solute-solute interactions.[127] The extent of solubility is dependent on the intermolecular interactions between solvent and solute molecules giving the overall mutual solubility. Typically molecules of "similar" structures and properties would have greater solvent-solute interactions resulting in high dissolution. The forces that determine this are the dipole-induced dipole forces, instantaneous dipole-induced dipole forces and hydrogen bonding.[128] Solvent is a major part of extractions with most of the 8 million tonnes of oils isolated per year from seeds and grain

commodities being extracted by hexane in the US.[129] Solvents must be selected in consideration of solvent properties, environmental impact, economic issues and associated hazards. Solvents can be classified by different ways and in an extraction; it is the solvent-solute interaction that is considered the main driver of the outcome. A list of physical constants namely melting point, boiling point, heat of vapourisation, index of refraction, density, viscosity, molar volume, surface tension, dipole moment, relative permittivity, polarisability, etc. are compiled which can describe the behaviour of each solvent.

Solvent polarity is a measurement to describe the strength of the solvent including all the solvent-solute interactions and cannot be defined by a single physical parameter alone.[128] Researchers attempted to measure solvent polarity quantitatively and introduced empirical scales of solvent polarity.[128] In 1951, Brooker was first to have thought of the idea of the use of solvatochromic dyes to measure solvent polarity but it was Kosower who was the first to establish the first polarity scale (Z values) in 1958.[130, 131] In 1963, Reichardt devised a normalised polarity scale using the solvatochromatic pyridinium-*N*-phenoxide betaine dye (non-protonic dye) from 0 (TMS) to 1 (water).[132] Kamlet and Taft developed a comprehensive set of solvatochromic parameters α, β and π* using various solvatochromic dyes (e.g.: *N*, *N*-diethyl 4-nitroaniline (non-protonic indicator), 3, 5 dinitroaniline (protonic indicator), 4-amino 7-methyl coumarine (hydrogen bond donor indicator), where α is the hydrogen bond donating ability, β is the hydrogen bond accepting ability and π* is the polarisability of the solvent.[133, 134, 135, 136] The π* scale was derived from the shift of the wavelength absorption maximum in the dyes that was caused by the solvent-solute interactions which was normalised to cyclohexane (π* = 0) and DMSO (π* = 1).[132, 134] The α and β scales were also derived using a similar method but π* interactions tend to occur as well as α or β interactions so the π* interactions must be subtracted from all solvent-solute interactions in order to give only the α or β values. The β scale was normalised to cyclohexane (β = 0) and hexamethylphosphoramide (β = 1).).[132, 134] Table 2.1 shows the parameters of selected solvents for extraction.

Linear solvation energy relationships have been proposed to correlate physical and chemical properties of solvents and can relate to the solvent-solute interactions.[136] Many have been proposed but the LSER had been one of the most successful as

shown in Equation 2.1, where XYZ describes the ln(K) of the process, XYZ_0, where K is the equilibrium constant, a, b and s are experimental dependent and α, β and π* are the Kamlet-Taft parameters.[137] Other physical and chemical properties of the solvents can also be included into the expression in order to fully describe the solvent and solute relationship.[137]

Table 2.1: List of selected solvents and their Reichardt's number (E_T^N) and Kamlet-Taft polarity parameters (α, β and π*)

Solvent	Reichardt's[132]	Kamlet- Taft parameters[133, 134, 135]		
	E_T^N	α	β	π*
Heptane	0.012	0	0	-0.08
Toluene	0.100	0	0.11	0.54
Diethyl ether	0.117	0	0.47	0.27
2-MeTHF	0.180	0	0.45	0.51[138]
Ethyl Acetate	0.228	0	0.45	0.46
DMC	0.232	0	0.43	0.55
Acetone	0.360	0.08	0.43	0.71
Butan-2-ol	0.506	0.69	0.8	0.4
Propan-1-ol	0.617	0.84	0.9	0.52
Ethanol	0.652	0.86	0.75	0.54
Methanol	0.762	0.98	0.66	0.6

$$XYZ = XYZ_0 + a.\alpha + b.\beta + s.\pi^* + \cdots$$

Equation 2.1: Kamlet-Taft linear solvation energy relationships

2.2.2 Solvent green guides

As large quantities of solvents are used and disposed of during natural product extraction, its environmental impact must be considered carefully during the selection process. Over the years, both the government and industries have attempted to eliminate, replace, recycle and minimise the use of solvents across all different process. Researchers and industries have developed solvent "green guides" to highlight the environmental issues associated with individual solvents and aim to use it as guidance to switching to alternative greener solvents. Most of the solvent guides

broadly take account of three main areas: environmental, health and safety impacts associated with each solvent. In 1999, SmithKline Beecham (now known as GSK) conducted solvent selection guidelines based on a number of key categories (incineration, recycle, bio-treatment, VOC, environmental impact to water, environmental impact to air exposure potential and safety hazard) on thirty-five different solvents.[139] The guides consider each of the categories and apply a score system for four categories (waste, impact, health and safety) from 1 (major issues) to 10 (no issues).[139] The environmental impact of distillation of solvents was compared. In 2004, GSK improved the solvent guide and expanded its solvent list to forty seven solvents and reviewed the reported solvents.[140] A similar scoring system applied but an extra category of LCA has been introduced. The guide was also more user friendly by introducing a colour coding system of red (Unacceptable), yellow (average) and green (acceptable). Fischer *et al.* developed an environmental assessment of twenty six solvents through EHS (identify potential substance hazards) and LCA (quantify of emissions and resource use over the whole life cycle) method in 2007. By considering nine different categories (water hazard, air hazard, persistency, chronic toxicity, irritation, acute toxicity, reactivity, potential for fire or explosion and release potential), an EHS score has been allocated and added together to indicate the overall environmental impact of each solvent. In 2008, Pfizer produced a solvent selection guide which clearly divided the solvents into three groups as green (preferred), yellow (useable) and red (undesirable). This guide not only clearly indicated the problems of "undesirable" solvents but it also highlighted the solvents that can be used to replace them e.g.: heptane can be used to replace hexane. GSK also produced a similar but more comprehensive solvent guide in 2011. The solvents are grouped into three categories as green (few issues), yellow (some issues) and red (major issues).[1] Like the previous versions of the GSK guides, a scoring system from 1 – 10 was adopted on a more extensive list of categories (waste, environmental impact, health, flammability and explosion, reactivity or stability and life cycle score) as shown in Table 2.2.[1] This solvent guide will be used when comparing the solvents used for the wheat straw extractions as it is the most recent and detailed.[1]

Table 2.2: List of selected solvents and their environmental, health and safety issues[1] (originally in colour)

Solvent	Waste	Environmental impact	Health	Flammability & Explosion	Reactivity/ Stability	Life Cycle Score
Heptane	6	3	8	3	10	7
Toluene	6	3	4	4	10	7
Diethyl ether	4	4	5	2	4	6
2-MeTHF	4	5	4	3	6	4
Ethyl Acetate	4	8	8	4	8	6
DMC	4	8	7	6	10	8
Acetone	3	9	8	4	9	7
Butan-2-ol	4	6	8	7	9	6
Propan-1-ol	4	7	5	7	10	7
Ethanol	3	8	8	6	9	6
Methanol	4	9	5	5	10	9

Solvents are colour coded according to the GSK solvent guide[1]

2.2.3 Biomass pre-treatment techniques

A large number of pre-treatment processes have been developed for lignocellulosic biomass such as wheat straw to enhance the extraction efficiency and lower processing costs.[141] The pre-treatment steps aim to cause physical and/or chemical change to the biomass.[142] Pre-treatments are either physical or chemical and in some cases, both are incorporated for added positive effects. Physical pre-treatments tend to be applied to make raw materials easier to handle and reduce particle size for better solvent diffusivity to achieve better extraction efficiency. These include comminution, steam explosion and hydrothermolysis.[143, 144, 145] Comminution is carried out to reduce particle size by dry, wet or vibratory milling.[142]

Chemical treatments are commonly used to help with the recovery of targeted molecules by chemical modification or disruption of the biomass structure. Acids and bases such as sulphuric acid and sodium hydroxide can be added to improve the extraction yield by enhancing the recovery of targeted molecules.[146, 147] As well as acid and base treatment, ammonium-based solvents (e.g.: ammonia), aprotic solvents (e.g.: DMSO), metal complexes (e.g.: ferric sodium tartrate) and water oxidation are

also commonly used as a mean to disrupt the cellulose structure of biomass.[148, 149, 150, 151] For the extraction of wheat straw, physical pre-treatment is necessary to increase the extraction efficiency so hammer milling was the chosen technique to reduce the particle size.

2.2.4 Solvent extraction techniques

Waxes can be extracted using various solvent extraction techniques such as Soxhlet. It is important to select a solvent technique for this investigation. Soxhlet extraction is a traditional continuous extractor that is originally designed for the extraction of lipids. The pre-treated sample is filled into a porous cellulose thimble which is then slotted into the Soxhlet extractor as shown in Figure 2.1. During the extraction, the solvent was heated to boiling point and the Soxhlet extractor then slowly filled with condensed solvent. When the solvent has reached the siphon top, the filled Soxhlet extractor empties back into the solvent reservoir directly below. During this process, the extracted solvent carries the materials extract with it. This cycle is repeated continuously until heating is stopped. Typically, the extraction uses a large amount of solvent and needs to be run for several hours.

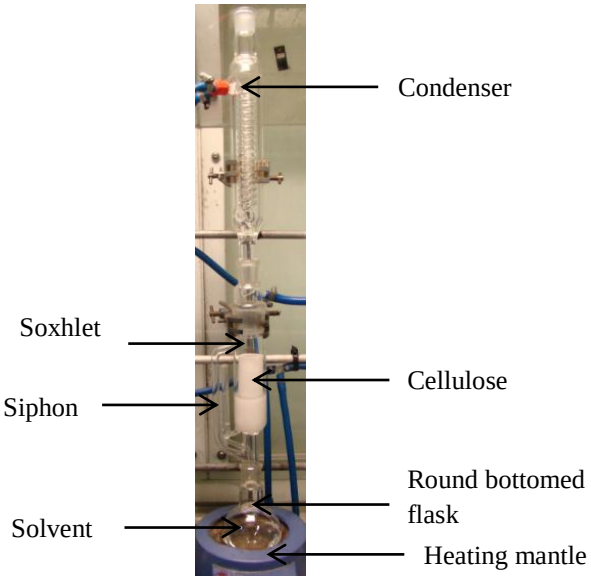


Figure 2.1: Soxhlet extractor (originally in colour)

FexIKA® extraction is designed to tackle the main disadvantages of Soxhlet such as the large volume of solvent usage and long extraction times. It is a multi-point solid-

liquid extractor (Figure 2.2) that is based on the fluidised bed extraction principle with the aim of the design to reduce solvent and extraction time. IKA® claim that this technique can reduce the extraction times by 90%. The sample is loaded in to the extractor where the solvent vapour penetrates the filter, permeates through the sample and comes into contact with the cooling finger. The condensed solvent remains in the extraction tube whilst the solvent level in the base vessel is continuously being reduced as the heating proceeds. This enables electronic online monitoring of temperature changes and feedback controls over the entire process. When the heating is switched off after a set time, the cooling water enters the device to cool the system. The induced cooling and condensing creates a vacuum and the resulting differential pressure and atmospheric pressure pulls the solvent along with the extract through the filter and into the base vessel. This process can be repeated continuously.

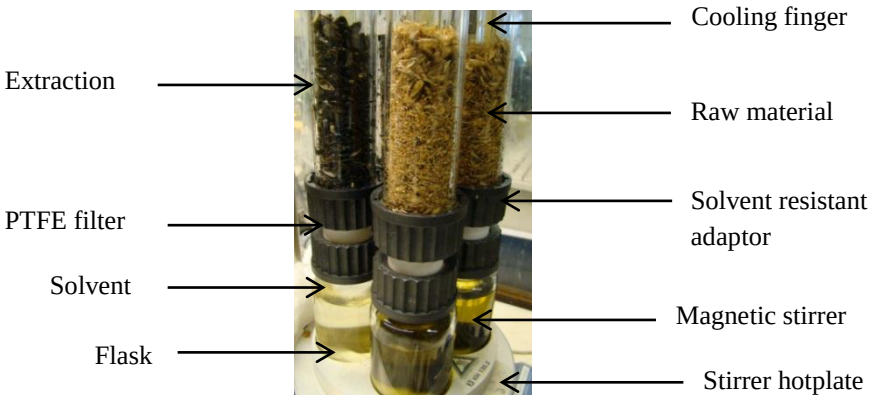


Figure 2.2: FexIKA® Vario Control Series Extractor (originally in colour)

ASE® is also a common extraction technique which is designed to extract at an elevated temperature and pressure in order to reduce the solvent usage and minimise extraction time. This technique enables the solvent extractions to be carried out at the same temperature with elevated pressure. With this technique, solvent consumption can be reduced by 90% making it both cheaper and more environmentally friendly. It is an automated multi-cell extractor which makes it ideal for sequential extractions and extraction of a large number of samples. Ultrasound-assisted extraction is an alternative method for solid-liquid extraction. It is very similar to Soxhlet extraction with the assistance of ultrasound for the enhancement of solubility of solutes in solvents. The advantage is that the extraction time is shortened by this enhancement, and ultrasound can help with cell disruption if a biological sample is used which could

enable better penetration of solvent through the sample. All the techniques described rely on conventional heating but microwave-assisted extraction is an efficient extraction process without conventional heating. The extraction can be carried out under pressure with controlled internal temperature. This advanced technology is more environmentally friendly compared with traditional Soxhlet extraction with shorter extraction times and 90% less solvent usage. After careful consideration, Soxhlet extraction method was chosen for the investigation as it is a traditional method that is quick and easy to set up.

2.3 Optimisation of solvent extraction using LSER

The wheat straw (Viscount 09) was physically pre-treated, extracted and moisture determined using methods in Sections 6.2.2, 6.2.3 and 6.2.1 respectively. The water content of the milled wheat straw was checked to ensure it is less than 10% prior to extraction. The percentage crude yields were calculated using Equation 2.2 and found to lie between 1.22 – 5.13% (with a ±0.11% error) depending on solvent used.

$$\%\ Crude\ yield = \frac{Mass\ of\ extract\ (g)}{Mass\ of\ biomass\ (g)} \times 100$$

Equation 2.2: Calculation of percentage crude yield

Solvent extractions tend to be very unselective and can lead to the extraction of many different unwanted molecules. It had previously been reported that phenolic acids, resin acids, glycerides, phospholipids, pigments, low molecular weight carbohydrates, ash and salts were found in plant extracts.[152, 153, 154, 155] The extracts were partitioned using DCM and water to retrieve just the lipophilic fraction of the extract. Table 2.3 and Figure 2.3 show a graph of the average percentage crude yields from different organic solvents (with DCM and water soluble fractions).

Table 2.3: Percentage crude yield with percentage of DCM and water soluble fractions

Solvent	Crude Yield (%)	DCM soluble (%)	Water soluble (%)
Methanol	5.13	1.07	4.06
Propan-1-ol	4.07	1.22	2.85
Ethanol	3.87	1.01	2.87
Butan-2-ol	3.38	1.32	2.06
MeTHF	2.53	1.79	0.73
DMC	2.36	1.30	1.06
Ethyl Acetate	2.31	1.89	0.42
Toluene	2.16	1.51	0.65
Acetone	1.80	0.62	1.18
Heptane	1.26	0.96	0.30
Diethyl ether	1.22	0.46	0.76

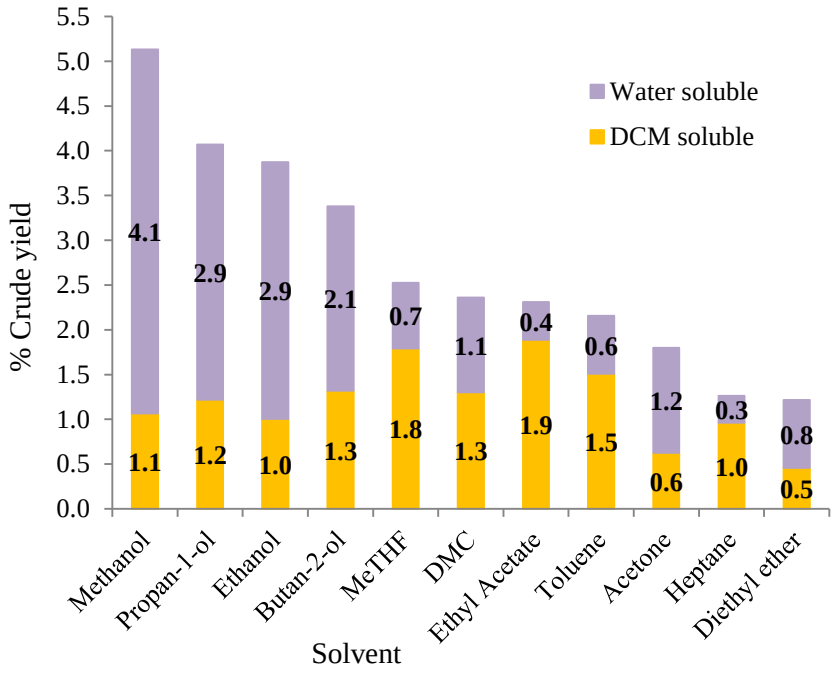


Figure 2.3: Percentage crude yield with percentage of DCM and water soluble fractions (originally in colour)

From Figure 2.3, methanol has the highest percentage crude yield for wheat straw extraction and can be considered as the solvent with the most extracting ability for the various molecules that are presented in the biomass. On the other extreme, diethyl

ether has the lowest percentage crude yield indicating poor compatible properties with molecules in wheat straw or alternatively, this could be due to the low boiling point of only 308 K so the amount of molecules that can extracted are limited. Methanol appeared to have strong solvent power but it is important for the solvent to have high selectivity so downstream processes can be minimised.

It had been reported that the content of the wax in the straw is low and only comprised of about 0.5 – 1.0% of the dry weight.[49] Previously, Deswarte recovered the lipophilic fraction of wheat straw waxes from 5 different solvents (hexane, acetone, toluene, Methyl-THF and ethanol) using silica gel column chromatography with (85:15 v/v) hexane: diethyl ether.[16] Results show a range of 0.8 – 1.2% wax yields were collected and hexane was the most selective with 69.2% of the extracts as wax. A different purification procedure using biphasic (DCM: water) extraction had been adopted in this case. Figure 2.4 shows the selectivity of the different organic solvent extractions. The percentage selectivity was calculated using Equation 2.3.

$$\%\ Selectivity = \frac{\%\ DCM\ soluble\ fraction}{\%\ Crude\ yield} \times 100$$

Equation 2.3: Calculation of percentage selectivity

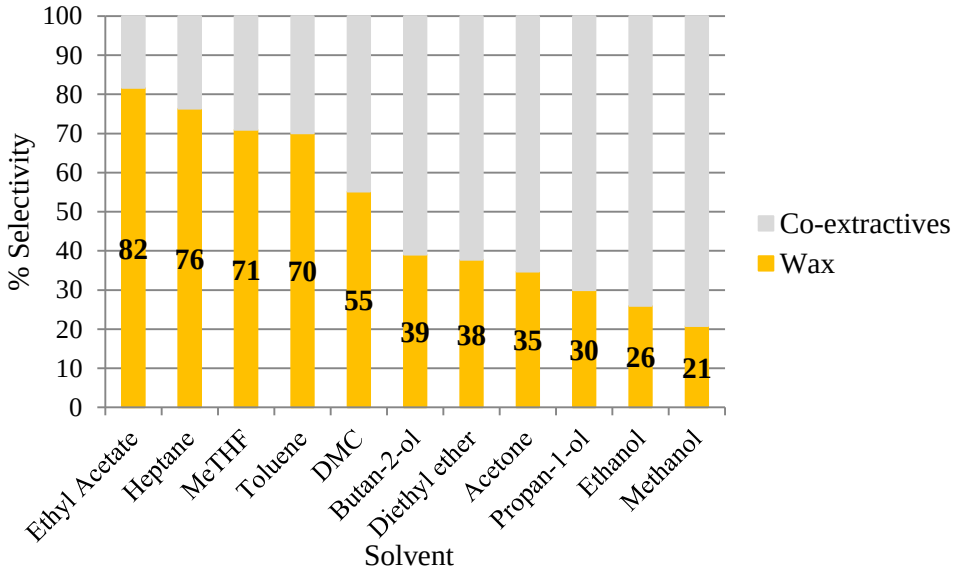


Figure 2.4: The selectivity of different organic solvent extractions (originally in colour)

From Figure 2.4, ethyl acetate shows exceptional selectivity as over 80% of ethyl acetate was lipophilic waxy material. Heptane also indicated high selectivity of 76% for wax which is in agreement with the high selectivity seen with hexane by Deswarte.[16] Heptane and hexane are compared as the polarity of the solvents (E_T^N: 0.012 and 0.009 respectively) are very similar and researchers have proposed that trends of extraction yields are mainly dependant on the polarities of solvent.[16, 152]

Table 2.4: Comparison of data with literature values[16]

Solvent	Crude Yield (%)	Lipophilic extract (%)
Ethanol (n = 3)	3.87 ± 0.24	1.01
	3.29[a]	1.20[a]
MeTHF (n = 3)	2.53 ± 0.12	1.79
	3.10[a]	1.12[a]
Toluene (n = 3)	2.16 ± 0.17	1.51
	1.97[a]	0.91[a]
Acetone (n = 3)	1.80 ± 0.14	0.62
	1.90[a]	0.87[a]
Heptane (n = 3)	1.26 ± 0.04	0.96
Hexane	1.17[a]	0.81[a]

[a] Published data from Deswarte

Table 2.4 shows a comparison of novel data with literature values of similar extraction work on wheat straw. Both sets of data are in agreement and show the same trends in terms of percentage crude yield in the order ethanol, methyl-THF, toluene, acetone and heptane/hexane. The variation in results could be due to a number of factors e.g.: different varieties, age, growing conditions etc. The influence of natural variations on the compositional differences is discussed in Chapter 3. The yield data for methyl-THF appeared to be distinctly different to other solvents as the percentage selectivity almost doubled (Deswarte: 36% compared to 71%). Methyl-THF is a solvent of high interest as it is bio-derived and can be used to replace some of the petroleum-derived ethers.[156] Methyl *tert*-butyl ether is also recommended in the Pfizer green guide as a greener replacement.[157] Sun *et al.* extracted wheat straw using methyl *tert*-butyl ether without any further purification steps which resulted in a yield of 1.12%. A greater amount of wax esters, sterols and triglycerides were extracted with ether compared to more polar solvents (e.g.: toluene: ethanol: methanol, 1/1/1, v/v/v) in the study.[152] The

higher percentage of lipophilic compounds in methyl-THF extracts could be due to the extra wax esters, sterols and triglycerides extracted.

To fully understand how the solvent properties influence the extraction of wheat straw, LSER was proposed to describe the percentage crude yield. Initially, only 10 solvents (all except DMC) were carried out and DMC was the chosen solvent for the validation of any LSER deduced because it is a bio-solvent and is listed as a green solvent in the most recent solvent guide. Linear regression was applied and related to a list of solvent parameters such as the Reichardt's number, Kamlet-Taft polarity parameters and molar volume on the percentage crude yield. Usually a single polarity parameter is introduced into the LSER to generate an expression to describe the process. Other physical parameters can also be included in the expression in order to enhance the effectiveness of the expression. Assuming the extraction has reached equilibrium, the equilibrium constant (K) is expressed as the percentage total compounds extracted over percentage total compounds not extracted. This equilibrium constant is used as a substitute as mole fractions of the extraction are not available. A Kamlet-Taft LSER expression was used successfully as shown in Equation 2.5 and discussed previously in Section 2.2.1. This LSER can be used to describe the standard Gibbs free energy of extraction as a new LSER expression for extraction (Equation 2.4). Equation 2.5 had been normalised to 323 K (T_{expt} = extraction temperature and T_{ref} = normalised temperature) because of the different extraction temperature from the various solvents. Table 2.5 shows the extraction temperature and its boiling point for each solvent. The Gibbs free energy of extraction is shown in Equation 2.4, where K is the equilibrium constant, $\Delta G^{\ominus}$ is the change in Gibbs free energy under standard conditions ($Jmol^{-1}$), R is the gas constant ($JK^{-1}mol^{-1}$) and T is the temperature (K).

$$\ln(K) = \frac{-\Delta G^{\ominus}}{RT} \times \frac{T_{Expt}}{T_{Ref}}$$

Equation 2.4: New Gibbs free energy equation

$$\ln\left(\frac{\%\ Crude\ yield}{100\% - \%\ Crude\ yield}\right) \times \frac{T_{Expt}}{T_{Ref}} = -3.15 + 0.635\beta - 0.0128V_m$$

Equation 2.5: New normalised temperature LSER equation with percentage crude yield

In this study, Equation 2.5 was developed and showed strong correlation with two important solvent parameters V_m and Kamlet-Taft parameter β (R^2 = 0.936). Table 2.5 shows the V_m and β of the solvents under investigation. The equation was used to predict the percentage crude yield with DMC. The model developed has proven fairly accurate as the experimental yield was 2.4% and the predicted was 2.9% which results in only a difference of 0.5%. Kamlet-Taft parameter β describes the hydrogen bond accepting ability of the solvent and is a key solvent polarity parameter.

Table 2.5: Solvent parameters in LSER Equation 2.5

Solvent	V_m at 298K	Extraction temperature (K)	β[134]
Methanol	40.7	337	0.66
Propanol	74.7	370	0.90
Ethanol	58.7	351	0.75
Butan-2-ol	92.4	371	0.80
MeTHF	101.7	350	0.45
DMC	84.7	361	0.43
Ethyl Acetate	98.4	350	0.45
Toluene	106.9	384	0.11
Acetone	74.1	329	0.43
Heptane	147.6	372	0
Diethyl ether	104.7	308	0.47

Figure 2.5 shows a 3D plot of the percentage crude yield of the solvents (including DMC) with β and its environmental impact (using latest GSK green solvent guide). A rough (without normalised temperature) trend with β is reflected in the plot: as the hydrogen bond accepting ability increases, the percentage crude yield also increases. As shown in Figure 2.5, the greener solvents e.g.: butan-2-ol can achieve similar percentage crude yield, though it is necessary to identify and quantify the extractives for comparison.

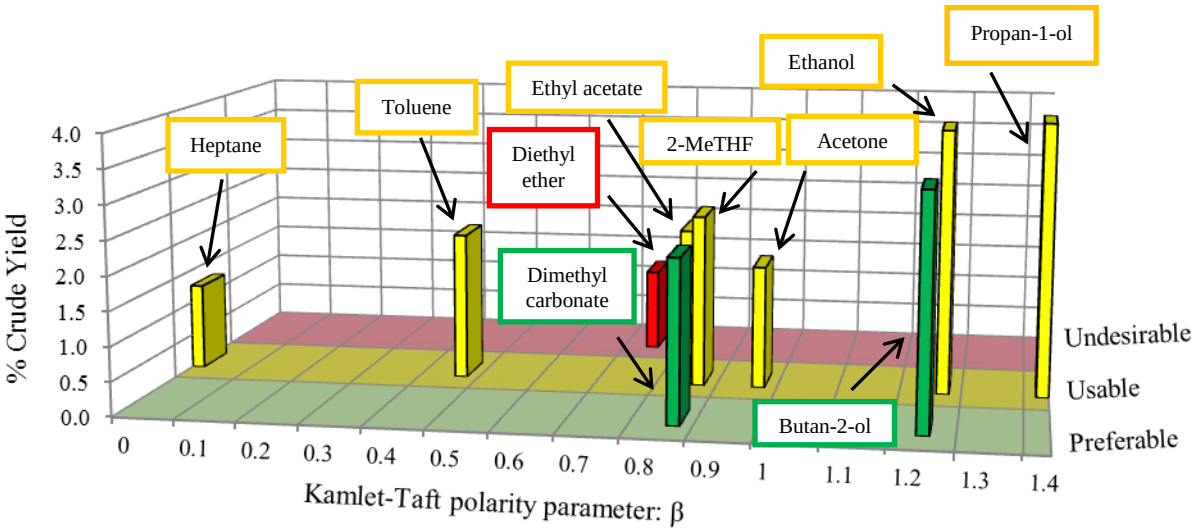


Figure 2.5: A 3D plot of the percentage crude yield of the solvents (originally in colour)

Welton *et al.* shows by that using ionic liquids the anion basicity which can be described by Kamlet-Taft parameter β correlated with the ability to expand pine lignocellulose. The anions in ionic liquids are the most important determinant for β.[158] It was also reported that the anion in ionic liquids has an impact on the ability to dissolve cellulose.[159]

Wheat straw is high in lignocellulose, it comprised of 35 – 40% cellulose, 20 – 30% hemicellulose and 8 – 15% lignin.[160] It is possible that as the hydrogen bond basicity (β) increases, the lignocellulose in the wheat straw behaves similarly to pine and expands, therefore enabling the embedded wax in the plant cuticle to be extracted out into the solvent. Alternative argument is that the lignocellulosic molecular structure of the straw breaks down as β increases so the solvent molecules can penetrate through the straw more easily therefore leading to enhanced extraction. The diffusivities of molecules in and out of the straw would be easier hence for solvents of higher β, the selectivity will be lower due to the extraction of other unwanted molecules e.g.: low molecular weight carbohydrates. If the lignocellulose structure is expanded, solvent with higher V_m will be able to reach the inner structure of the straw and more wax molecules would be extracted as penetration through the biomass is easier. The extraction of the epicuticular wax on the surface of straw is also inversely proportional

to V_m. As molar volume is the volume of one mole of solvent and the lower the V_m, the more the solvent molecules in a specific volume so more β interactions would occur. Both the V_m and β are cooperative and together they describe the relationship of the solvent to solutes in wheat straw as the new LSER expression Equation 2.5. Figure 2.6 shows a plot of experimental data and expected data from the LSER described in Equation 2.5 and the axis represents the y term (left) of the Equation. DMC was added onto the plot as a validation of the result and it showed that the experimental data of DMC are similar to predicted data. This indicated that the LSER developed can be used to predict the percentage crude yield on a wide range of solvents with known V_m and β. To achieve highest percentage crude yield sustainably, the solvent must have a low V_m and a high β and these parameters are usually found in small aliphatic alcohols.

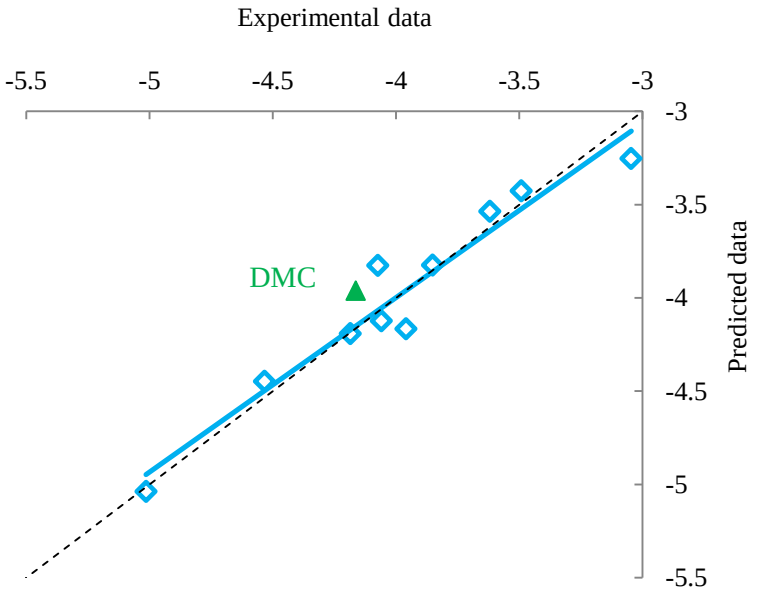


Figure 2.6: A comparison of experimental and predicted percentage crude yield (originally in colour)

The same linear regression and normalised temperature were also applied to the percentage aqueous yield and a similar relationship between V_m and β was identified. Equation 2.6 shows a newly established relationship between percentage aqueous yield with V_m and β (r^2 = 0.89). DMC was also used for data validation and the experimental and predicted data were compared. The experimental and predicted

aqueous yields were 1.3% and 1.1% respectively giving only a 15% error. The residual errors for the correlation of experimental and predicted data are calculated using Equation 2.7. The residual error plot is shown in Figure 2.7 and the data was evenly distributed both positively and negatively which means there is no bias towards over or under estimation. Figure 2.8 shows a comparison of the experimental and predicted data using the new LSER Equation 2.6.

$$\ln\left(\frac{\%\ Aqueous\ yield}{100 - \%\ Aqueous\ yield}\right) \times \frac{T_{Expt}}{T_{Ref}} = -3.52 + 1.41\beta - 0.0228V_m$$

Equation 2.6: New normalised temperature LSER equation with percentage aqueous yield

$$Residual\ error = Predicted\ value - Experimental\ value$$

Equation 2.7: Calculation for residual error

As a new LSER equation had been established, the percentage of aqueous soluble components can be minimised and the selective solvents can be identified. A high V_m and a low β parameter are required to minimise the aqueous soluble extraction which means the selectivity of the solvent can be increased and further downstream processing steps can be reduced.

The residual error was also calculated for the aqueous soluble yield using Equation 2.7 which is shown on Figure 2.9. The data was evenly distributed both positively and negatively as previously which means there is no bias over or under estimation.

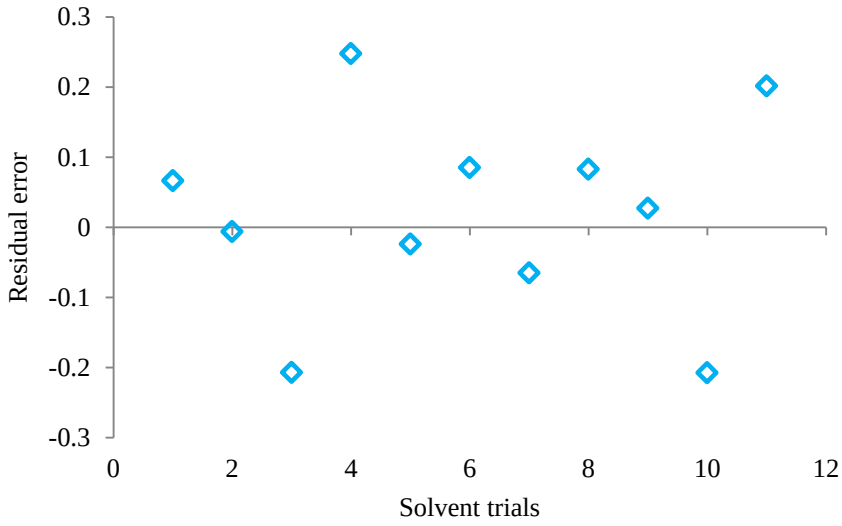


Figure 2.7: Residual error plot for experimental and predicted percentage crude yield (originally in colour)

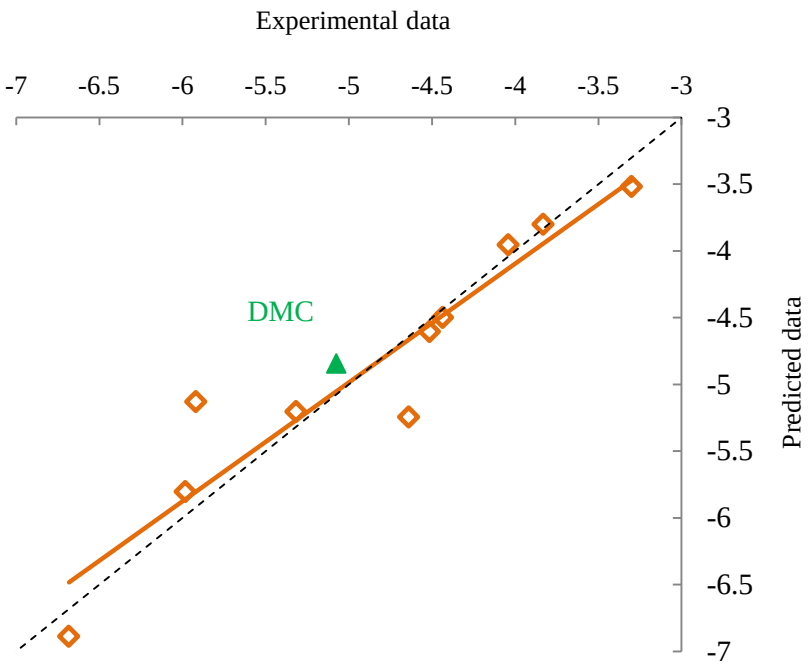


Figure 2.8: A comparison of experimental and predicted percentage aqueous soluble yield (originally in colour)

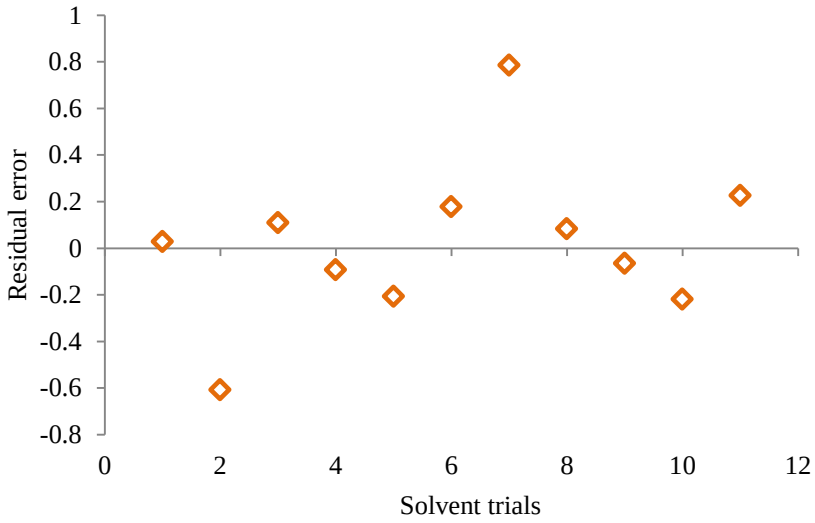


Figure 2.9: Residual error plot for experimental and predicted percentage aqueous soluble yield (originally in colour)

The same linear regression was also applied to the percentage DCM soluble yield, however no correlation was found as expected. Both newly established LSER equations for percentage crude yield and aqueous soluble yield have similar coefficients for β and V_m suggesting lipophilic fraction extraction had gone to completion as shown in Figure 2.10 (with all eleven solvents). A horizontal trend line for the different solvents (saturation) is displayed with percentage DCM soluble yield. It can be concluded that the same trends were observed from percentage crude yield and aqueous yield and the newly established aqueous yield equation (Equation 2.6) would be useful in highlighting the highly selective solvents for lipophilic fraction of the extracts. Figure 2.10 is plotted using the values derived from linear regression and the values used are shown in Equations 2.8, 2.9 and 2.10. Equations 2.8 and 2.9 show strong correlation with $r^2 = 0.92$ and 0.89 respectively.

$$\underbrace{\ln\left(\frac{\%\ yield}{100\% - \%\ yield}\right) + \frac{T_{Expt}}{T_{Ref}}}_{\text{y term}} = \underbrace{a.\beta + b.V_m + c}_{\text{x term}}$$

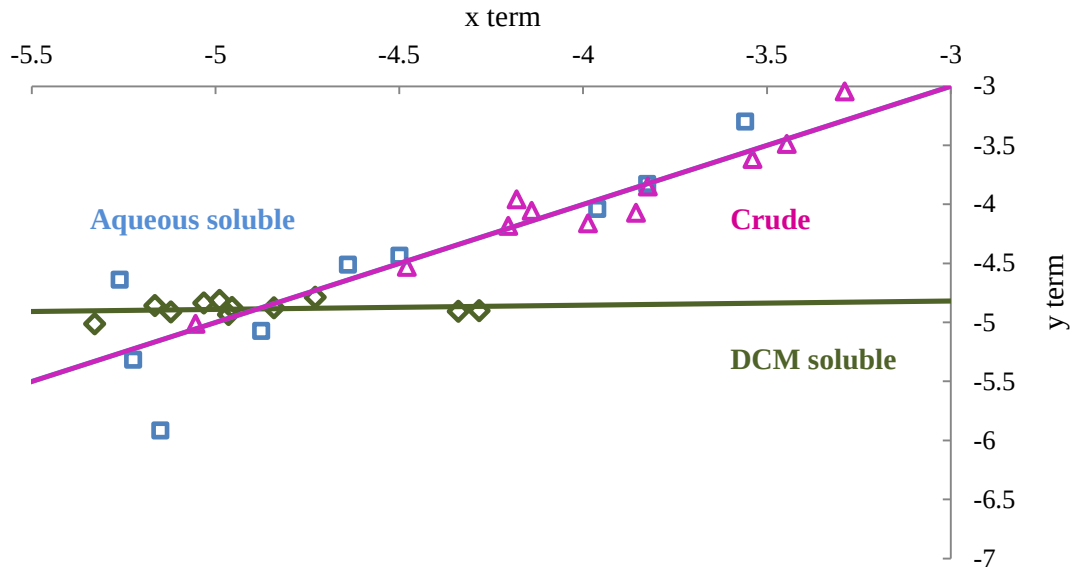


Figure 2.10: A comparison of DCM soluble, aqueous soluble and crude yield (originally in colour)

$$\ln\left(\frac{\%\ crude\ yield}{100\% - \%\ crude\ yield}\right) + \frac{T_{Expt}}{T_{Ref}} = 0.692\beta - 0.0122V_m - 3.25$$

Equation 2.8: LSER equation for percentage crude yield

$$\ln\left(\frac{\%\ DCM\ soluble\ yield}{100\% - \%\ DCM\ soluble\ yield}\right) + \frac{T_{Expt}}{T_{Ref}} = 0.0476\beta - 0.00181V_m - 4.75$$

Equation 2.9: LSER equation for percentage DCM soluble yield

$$\ln\left(\frac{\%\ aqueous\ soluble\ yield}{100\% - \%\ aqueous\ soluble\ yield}\right) + \frac{T_{Expt}}{T_{Ref}} = 1.47\beta - 0.0222V_m - 3.63$$

Equation 2.10: LSER equation for percentage aqueous yield

2.4 Chemical composition identification using GC and GC-MS

Extensive research had been carried out on the characterisation of waxes from young wheat plants and only limited composition data were found on wheat straw extracts. Sun *et al.* explored the compositional differences between different solvent extractions which included the use of solvent mixtures. Deswarte also successfully identified the different wax components in wheat straw wax, though no quantification data were available so the relative abundance of each compound from different solvent extractions was not compared.[16]

Initially, each of the extracts was analysed using GC-FID and GC-MS using the method described in Sections 6.3.1 and 6.3.2. The GC chromatograms for all the different solvent extractions showed similar patterns indicating that the chemical compositions are comparable so therefore only selected extracts were analysed using GC-MS. This suggests that the differences between the extracts are mostly due to the relative abundance of each component present. A representative GC chromatogram of heptane wheat straw extract is shown in Figure 2.11.

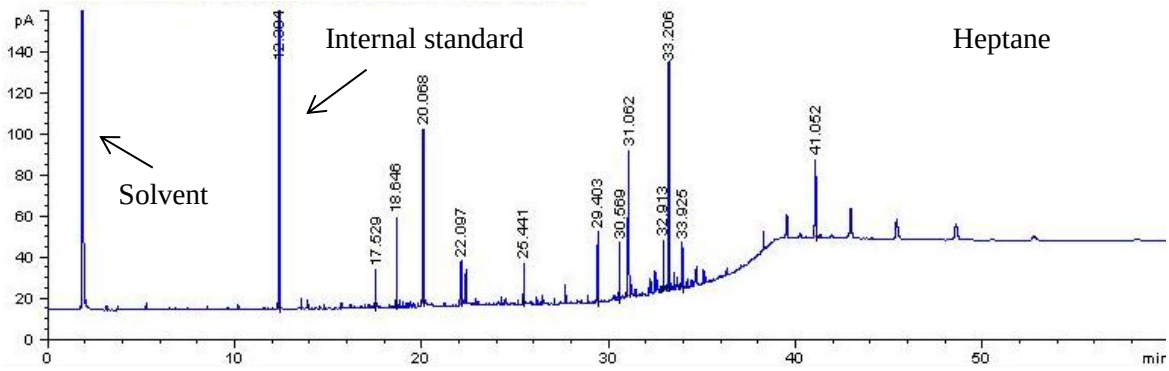


Figure 2.11: GC chromatogram of whole heptane wheat straw extract (originally in colour)

The DCM soluble extracts were also compared with the full extracts using GC. Figure 2.12 shows that the full ethanol extract consisted of highly similar components as the DCM soluble fraction so full extracts were identified using GC-MS.

The retention index system (known as the Kovats Index) was originally developed by Kovats in 1958 using *n*-paraffinic series as reference materials for gas chromatography. Kovats used the logarithms of the retention times and identified a linearity relationship with

ramp temperature for programmed GC. In 1963, Van Den Dool and Dec Kratz modified the equation to also include linear temperature programmed GC. Equation 2.11 shows the Kovats Index calculation as modified by Van Den Dool and Dec Kratz, where KI is Kovats Index, n is the number of carbons in the smaller reference alkane, N is the number of carbons in the larger reference alkane, t_N is the retention time for larger reference alkane, t_n is the retention time for smaller reference alkane and $t_{unknown}$ is the retention time of the compound of interest.

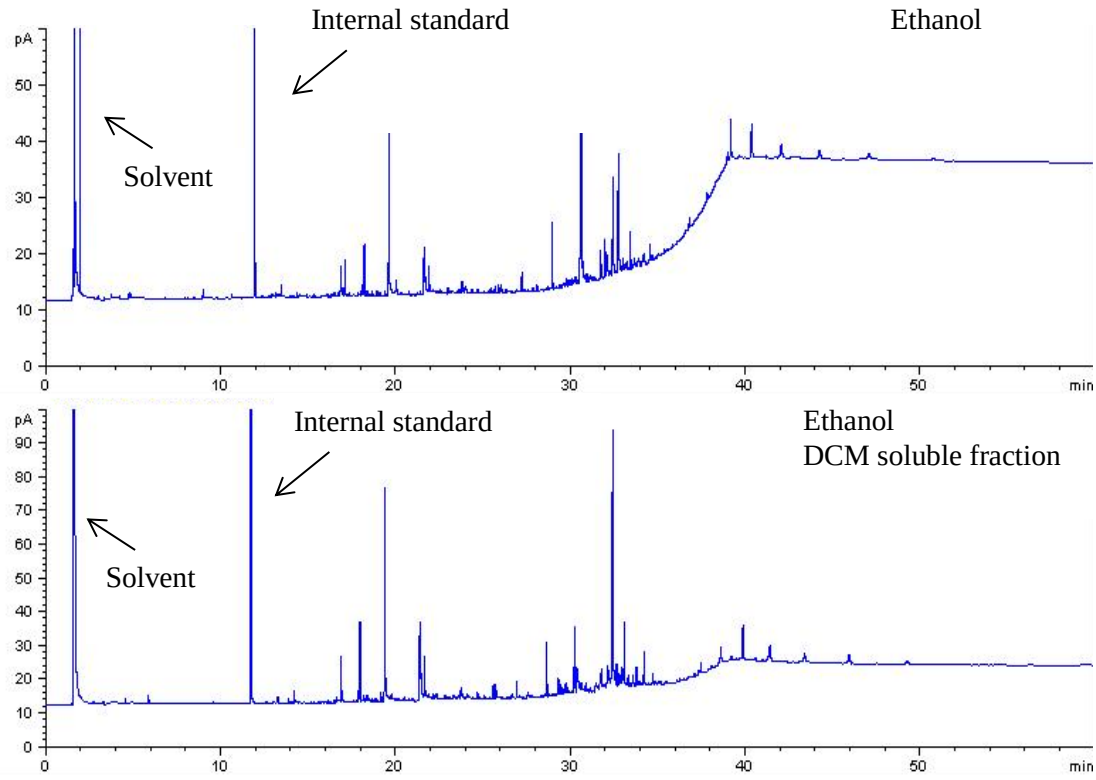


Figure 2.12: GC chromatogram of ethanol wheat straw extract (originally in colour)

The compounds in the wheat straw extracts were identified using the combination of the fragmentation pattern from the mass spectra, NIST mass spectra library, authentic standards and the Kovats Index.

$$KI = 100\left[n + (N - n)\left(\frac{t_{unknown} - t_n}{t_N - t_n}\right)\right]$$

Equation 2.11: Kovats Index (KI) calculation as modified by Van Den Dool and Dec Kratz[161, 162]

Two different capillary columns (ZB-5HT, 30 m x 0.25 mm I. D. X 0.25 µm film thickness, Phenomenex and ZB-5 HT, 15 m x 0.25 mm I.D. x 0.25 µm film thickness Phenomenex) were used to analyse the wax extracts. The 15 m column was used for the identification of larger molecules (up to 60 carbons) as these molecules would struggle to elute off the 30 m column in a reasonable retention time. Figure 2.13 shows differences in retention times between the two columns for reference alkane mix C_{12} - C_{60}.

The NIST Library is a database of mass spectra where the chemical structures of the different components can be predicted by the mass fragmentation. The Library was used to search for the best possible match mass spectra which can help with the identification. As many of the EI spectra do not show the molecular ion, FI was used to obtain some molecular ions to confirm the identification. Calculated KI was compared with literature KI for the calculation of the chain length of the aliphatic molecules. The quantification of the wax components were discussed in Section 2.5.

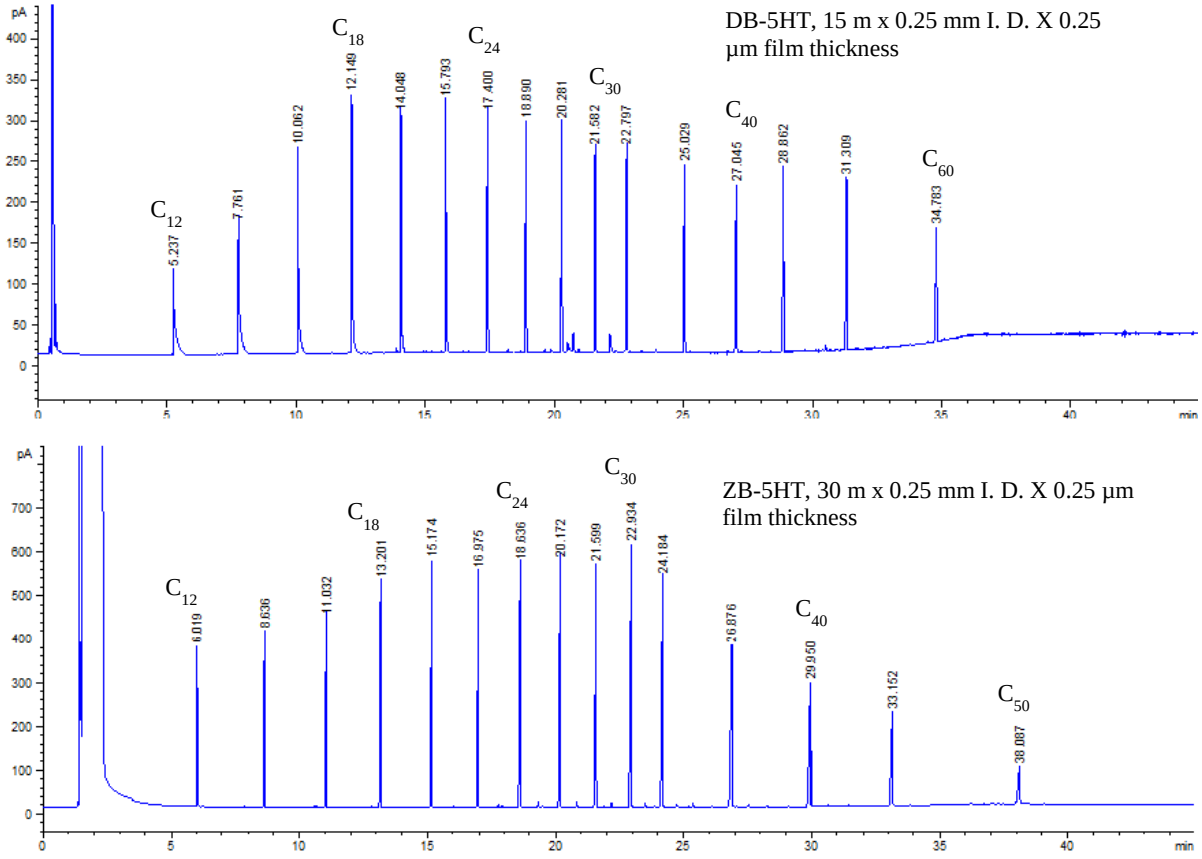


Figure 2.13: Reference alkane mix analysed by two different capillary columns (originally in colour)

2.4.1 Free fatty acids

Free fatty acids identified in wheat straw waxes by GC-MS were exclusively *n*-alkanoic acids of chain length ranging from C_{14} – C_{18} with different degrees of unsaturation which is in agreement with past literature.[16, 152] Three saturated free fatty acids were identified: tetradecanoic acid ($C_{14:0}$), hexadecanoic acid ($C_{16:0}$) and octadecanoic acid ($C_{18:0}$). Figure 2.15a shows the EI spectrum of hexadecanoic acid. The other fatty acids all have a similar EI fragmentation pattern. Figure 2.15b shows the NIST library match of hexadecanoic acid to Figure 2.15a.

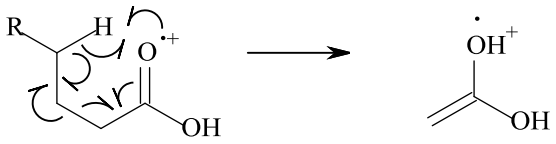


Figure 2.14: Formation of EI fragment ion m/z = 60

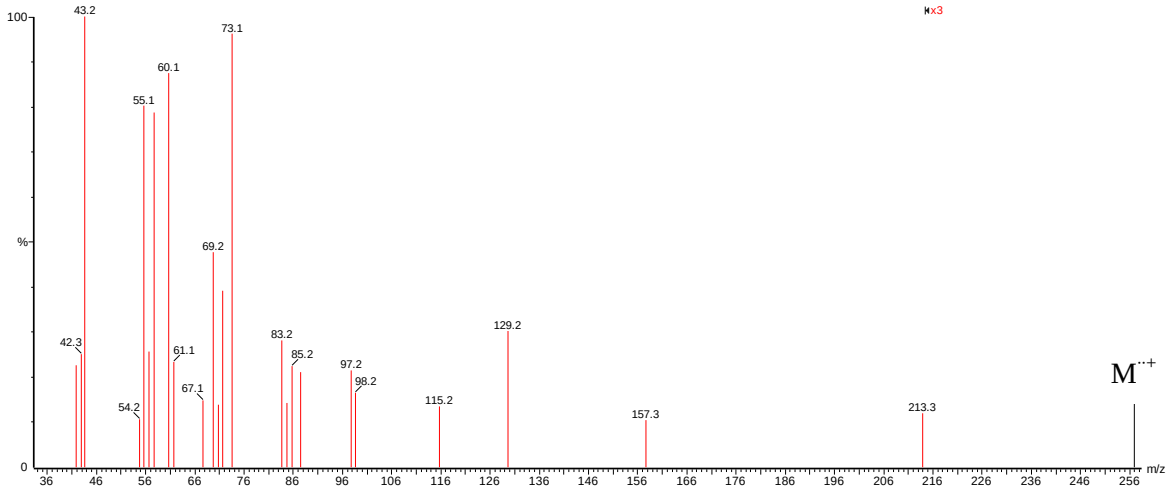


Figure 2.15a: EI mass spectrum of hexadecanoic acid ($C_{16:0}$) (magnified three times from m/z 213 – 260) (originally in colour)

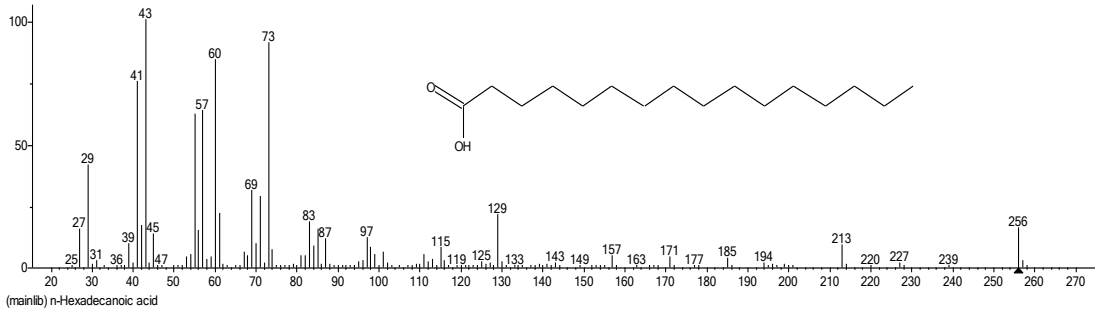


Figure 2.15b: EI mass spectrum of hexadecanoic acid ($C_{16:0}$) from the NIST library

Main distinct EI fragment m/z = 60 is formed as a result of McLafferty Rearrangment as shown in Figure 2.14. The abundant fragment ion m/z = 73 is generated through the migration of a hydrogen atom. This fragment ion is part of the series of oxygen-containing fragments of m/z = 45, 59, 73 and 87. Table 2.6 shows a list of fragment ions and its percentage base peak intensity as well as calculated and published KI values that assist in the identification.

Due to the nature of EI mass spectra, a weak molecular ion of the fatty acids is formed, and sometimes, the ion is not detected so therefore the molecular weight was assigned using calculated KI. Other abundant series of ions in the saturated free fatty acids series include m/z = 43, 55, 57 and 71. This series of ions are indication of alkyl chain with the difference of 14 mass units in the molecule as shown in Table 2.7. The ions at m/z = 55 and 69 occur due to the elimination of two hydrogens in the alkyl chain.

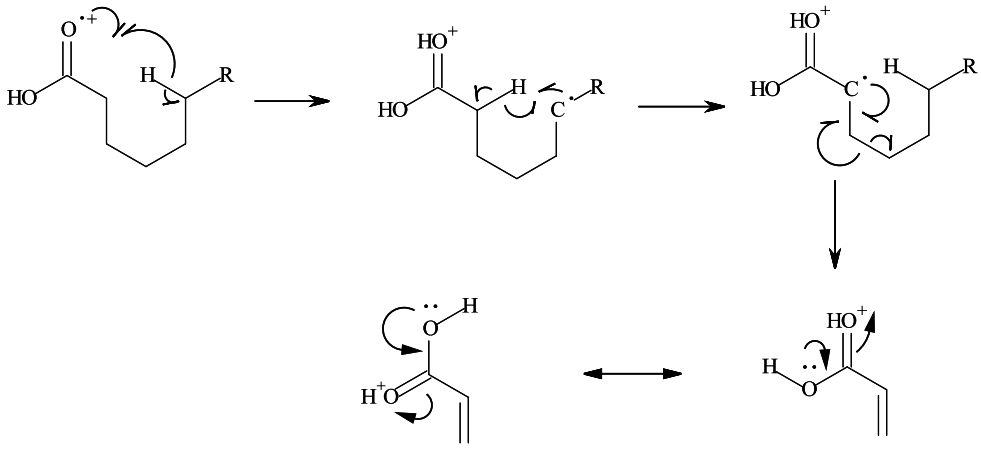


Figure 2.16: Formation of EI fragment ion m/z = 73

Table 2.6: Identification of free fatty acids presented in solvent extracts including the EI fragmentation pattern with percentage base peak intensity and KI values

Identification	M^+	EI Fragmentation	Calculated KI	Literature KI
Saturated free fatty acids				
Tetradecanoic acid ($C_{14:0}$)	228.4	43(100), 73(89), 60(81), 57(60), 55(60), 71(30), 69(30), 129(23), 42(21), 83(21)	1750	1751[163]
Hexadecanoic acid ($C_{16:0}$)	256.4	43(100), 60(87), 57(87), 73(80), 55(75), 69(39), 71(38), 83(28), 41(27), 129(24)	1951	1950[164]
Octadecanoic acid ($C_{18:0}$)	284.5	43(100), 55(91), 73(81), 60(80), 57(79), 69(47), 71(35), 83(31), 129(29), 85(28)	2148	2168[165]
Unsaturated free fatty acids				
Octadecenoic acid ($C_{18:1}$)	282.5	55(100), 69(64), 43(49), 79(41),83(39), 97(33), 57(32), 67(27), 70(23), 84(20)	2128	2137[166]
Octadecadienoic acid ($C_{18:2}$)	280.5	67(100), 81(70), 54(56), 68(55), 95(50), 82(34), 55(31), 96(25)	2126	2132[165]

Table 2.7: Aliphatic alkyl chain fragmentation peaks in mass spectra

m/z	Structure
43	$[CH_3(CH_2)_2]^+$
57	$[CH_3(CH_2)_3]^+$
71	$[CH_3(CH_2)_4]^+$
85	$[CH_3(CH_2)_5]^+$
55	$[CH_3CH_2CH{=}CH]^+$
69	$[CH_3(CH_2)CH{=}CH]^+$

Deswarte previously identified free saturated fatty acids from nonanoic acid ($C_{9:0}$) to dotricontanoic acid ($C_{32:0}$) containing both odd and even-numbered carbon atoms in variety Consort.[16] Odd-numbered free fatty acids are very uncommon as this group of molecules are formed by the addition of C_2 moiety during an elongation process.[82] The free acids arise from lipid biosynthesis and possibly from hydrolysis of

triglycerides or wax esters as the plant enter senescence.[82] Sun *et al.* reported a range of free saturated fatty acids in wheat straw ranging from decanoic acid ($C_{10:0}$) to tetracosanoic acid ($C_{24:0}$).[111] Both researchers show the presence of both odd and even numbered of free saturated fatty acids, though only three predominate: tetradecanoic acid ($C_{14:0}$), hexadecanoic acid ($C_{16:0}$) and octadecanoic acid ($C_{18:0}$).[16, 111] The results from wheat straw (Viscount 09) are in agreement with previous literature. Limited free saturated fatty acids were identified as the extracts were analysed crudely without any separation. Unsaturated free fatty acids hexadecenoic acid ($C_{16:1}$), octadecenoic acid ($C_{18:1}$), octadecadienoic acid ($C_{18:2}$), octadecatrienoic acid ($C_{18:3}$) and eicosenoic acid ($C_{20:1}$) were identified in previous studies.[16, 111] Initially tetradecanoic acid ($C_{14:0}$) is formed in the fatty acids *de novo* biosynthetic pathway then followed by hexadecanoic and octadecanoic acids. Deswarte was able to identify such a wide range of fatty acids as the crude extracts were fractionated by column chromatography so GC method can be tuned to analyse free fatty acids exclusively e.g.: the use of a more polar column to aid separation. It is not necessary to fractionate the fatty acids from these crude extracts in this case as the aim of the study was to compare the selectivity of various solvents. Fatty acids can be used in potential applications such as soaps, detergents, lubricating oils and cleaning polishes.[167, 168]

2.4.2 Hydrocarbons

Hydrocarbons have been identified in all wheat straw extracts as straight chain *n*-alkanes with chain lengths between C_{27} to $C_{33.}$ Odd-numbered *n*-alkanes were predominately found which is in agreement with the wax biosynthesis in which the alkanes are formed by the decarbonylation of the aldehydes in the alkane pathway. Previous research by Tulloch *et al.* on young wheat plant showed that the wheat extracts contain odd-numbered *n*-alkanes with chain lengths from $C_{21\text{-}35}$.[97, 98] However, Sun *et al.* reported absence of hydrocarbons completely in mature wheat straw which is in disagreement with the results presented. This is unusual as *n*-alkanes are commonly found in many plants and not just cereal crops. [169] The range of the *n*-alkanes tends to vary widely even within species.[169] In the case of wheat, the most abundant alkanes were highlighted to be nonacosane (C_{29}) and hentriacontane (C_{31}) which is exactly what had been observed in these wheat straw extracts.[91, 97, 98, 99, 105] The identification of *n*-alkanes was relatively simple as the fragmentation pattern is

very unique but it can be challenging when some of the alkanes co-eluted with other wax components in the complex mixture. Previously, Deswarte managed to separate an alkane rich fraction by column chromatography over silica which can aid in the identification process.[16]

A distinct fragmentation pattern for *n*-alkanes is shown in Figure 2.17 where in the low m/z region of the spectrum, there is a series of peaks regularly spaced by 14 mass units indicating the loss of CH_2 shown in Table 2.8. Figure 2.18 shows the zoomed in regions from m/z of 120 – 410 and magnified higher m/z regions that showed the regularly spaced 14 m/z units continued through the spectrum and the presence of m/z 409 which is the molecular ion for nonacosane. The largest peak in each cluster of fragment ions usually occur with C_nH_{2n+1} at m/z = 14*n* + 1 where n is the number of atom. Other smaller peaks are also observed are C_nH_{2n} and C_nH_{2n-1} fragments. It can be observed in Figure 2.17a that the most abundant fragments are at C_3, C_4 and C_5 and the fragment ions decrease in a skewed curve to the large fragment ions. Figure 2.17a is compared with Figure 2.17b which is the identification from the NIST library. The molecular ion is commonly lost in an EI spectra so the chain length of the alkanes was determined by the KI and standards as shown in Table 2.8. The table also shows the major ions and its base peak intensity in the EI spectrum for alkanes with the chain lengths C_{27} to C_{33}.

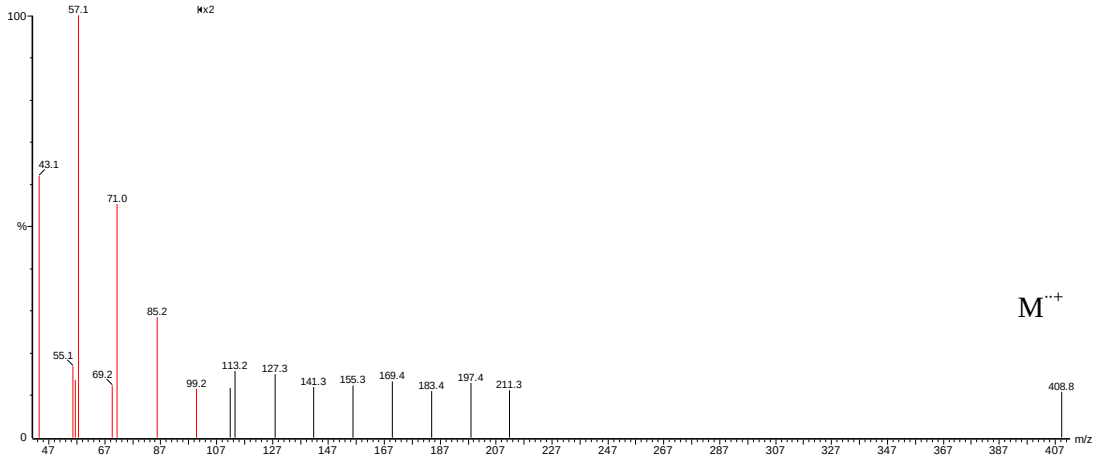


Figure 2.17a: EI mass spectrum of nonacosane (C_{29}) (magnified two times from m/z 100 – 410) (originally in colour)

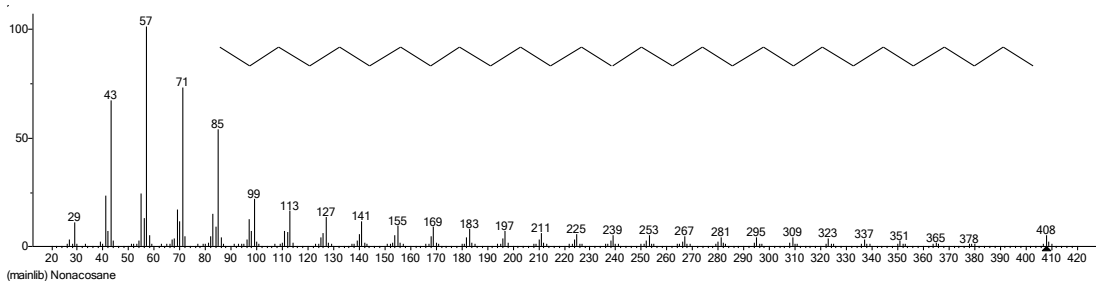


Figure 2.17b: EI mass spectrum of nonacosane (C_{29}) from the NIST library

Table 2.8: Identification of *n*-alkanes presented in solvent extracts including the EI fragmentation pattern with percentage base peak intensity and KI values

Identification	M^+	EI Fragmentation	Calculated KI	Literature KI
***n*-Alkanes**				
Heptacosane (C_{27})	380.7	57(100), 43(81), 71(62), 85(41), 55(35), 69(27), 56 (23)	2685	2700[161]
Nonacosane (C_{29})	408.8	57(100), 71(69), 43(59), 85(36), 55(25), 56(20)	2884	2900[161]
Hentriacontane (C_{31})	436.9	57(100), 71(72), 43(68), 85(39), 55(22), 56(21)	3083	3100[161]
Triatriacontane (C_{33})	464.9	57(100), 43(55), 71(49), 85(34), 55(28)	3282	3300[161]

Due to these long hydrocarbon chain structures, hydrocarbons are very hydrophobic and can be used to replace existing paraffin waxes and various coatings applications.[170] This distinct group of odd-numbered alkanes have been reported to be involved with semiochemical functions on different insects.[171, 172, 173] Semiochemicals (from Greek *simeon* as "a mark or signal") are biochemical compounds that contain "messages" which are used by insects and other organisms to communicate and these chemicals can be divided into two groups: pheromones and allelochemics.[174] Pheromone (from Greek *phereum* as "to carry" and *horman* as "to stimulate") involved in intraspecific interactions and allelochemics (from Greek *allelon* as "one another" involved in interspecific interactions.[174] In 2009, Tina Han (student in green chemistry group) separated a high alkane fraction and carried out aphid control tests in collaboration with Rothamsted Research. It was concluded that the alkane fraction in wheat straw wax can be used as an effective aphid semiochemical.

2.4.3 Free fatty alcohols

Free fatty alcohols of exclusively *n*-alkanols with chain lengths from C_{20} to C_{30} in wheat waxes in young plants have been extensively reported in the literature. [91, 98, 99] Octacosanol (C_{28}) was defined as the most abundant homologue in young wheat plants. [91, 98, 99] The range of free fatty alcohols identified in the young plants is in agreement with the composition in wheat straw extracts carried out by Deswarte. Deswarte identified *n*-alkanols with chain length from C_{22} to C_{34} in wheat straw after fractionation. Only octacosanol (C_{28}) was identified in the wheat straw in the crude extracts and in many cases, there is co-elution of octacosanol (C_{28}) with hentriacontane (C_{31}) which made identification more difficult. Fractionation of free fatty acids appeared to be an important step for identification and quantification.

Figure 2.18a shows an EI spectrum for octacosanol (C_{28}) which is compared with the EI spectrum from the NIST library (Figure 2.18b). As EI is a relatively harsh ionisation technique, molecular ions are rarely observed so in order to calculate the number of carbons in the aliphatic chain, the KI was calculated and compared with literature value. The octacosanol standard was also analysed to confirm the identification. Table 2.9 shows the fragments and its percentage base peak intensity and from both Figure 2.19 and Table 2.9, the fragmentation pattern appeared to be extremely similar to standard *n*-alkanes. The regular pattern of 14 mass units differences indicated the progressive loss of CH_2 meaning there is no unsaturation in the molecule and the assignment for the aliphatic alkyl chain is shown in Table 2.7. Due to the harsh ionisation technique used, most of the time, the larger ions are fragmented and therefore have low peak intensity and for this reason the region around the molecular ion was magnified as shown in Figure 2.18a. Sometimes a distinct prominent peak of $[M - 18]^+$ can be observed from the loss of water. The loss of the H_2O occurs when a δ-hydrogen is lost as shown in Figure 2.20 and in the case of octacosanol (C_{28}), a fragment ion of m/z = 393 would be observed. As well as just the elimination of water, the elimination of alkene from primary fatty alcohols also tends to happen which will result in a peak of $[M - 18 - 28]^+$. Octacosanol (C_{28}) would show a peak at m/z = 364 which represents the elimination processes shown in Figure 2.20.

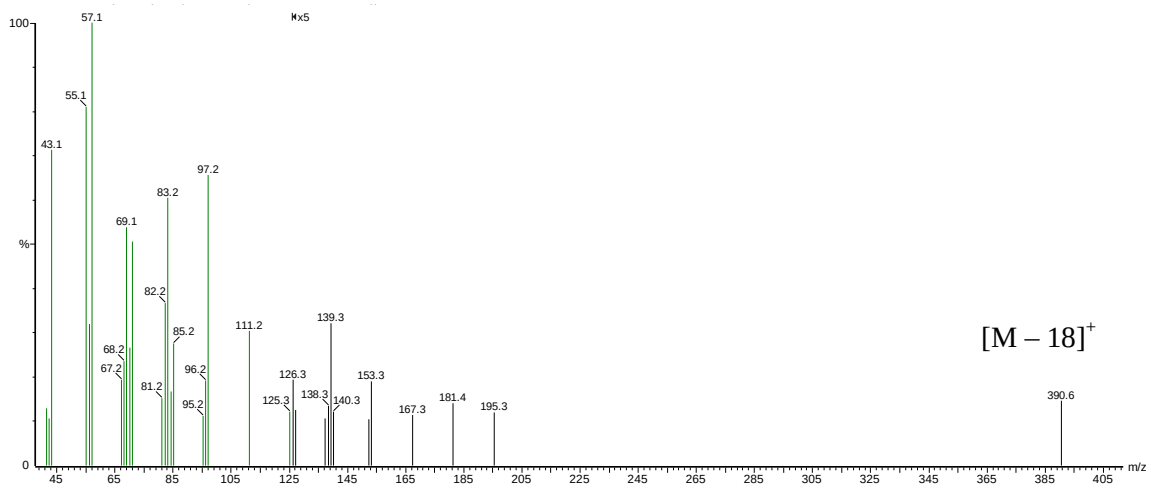


Figure 2.18a: EI mass spectrum of octacosanol (C_{28}) (magnified five times from m/z 126 – 410) (originally in colour)

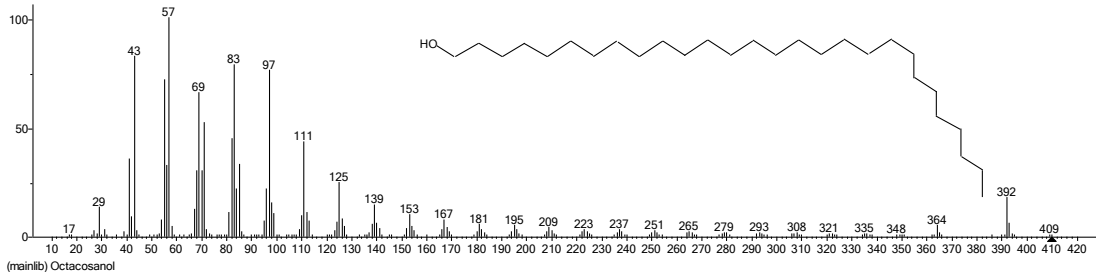


Figure 2.18b: EI mass spectrum of octacosanol (C_{28}) from the NIST library

Table 2.9: Identification of octacosanol in solvent extracts including the EI fragmentation pattern with percentage base peak intensity and KI values

Identification	M^+	EI Fragmentation	Calculated KI	Literature KI
Free fatty alcohol				
Octacosanol (C_{28})	410.8	57(100), 43(82), 55(76), 97(65), 69(64), 83(57), 71(50), 56(39), 111(30), 85(27)	3083	3118[175]

Octacosanol is not just an ordinary component identified on plant waxy surfaces as it has many nutritious and health benefits such as cholesterol lowering effects, anti-aggregatory properties, cytoprotective uses and ergogenic properties.[176] Due to the

low amount that is actually ingested, policosanol (natural mixture of primary fatty alcohols) containing mainly octacosanol has been made into dietary supplements.[177] Policosanols supplied derived from sugarcane wax, grasses, cereal grains and beeswax.[178] There has been much research on the extraction of wheat leaf wax and the isolation of octacosanol. In 1933, Pollard *et al.* first reported the isolation of octacosanol from wheat wax and Tulloch *et al.* also identified this valuable group of molecules in young wheat plants.[97, 179] Dunford *et al.* have studied the policosanol composition in many wheat grains of different varieties and have identified a policosanol range with chain lengths from C_{20} to C_{30}, predominantly tetracosanol (C_{24}) and hexacosanol (C_{26}).[180] The data reported for wheat grains are different to wheat straw where octacosanol (C_{28}) is the predominant fatty alcohol. Different fractions of the wheat grain have also been investigated and wheat bran has been highlighted as the highest policosanol containing fraction which shows that the level of policosanols vary between different parts of the wheat plant.[180]

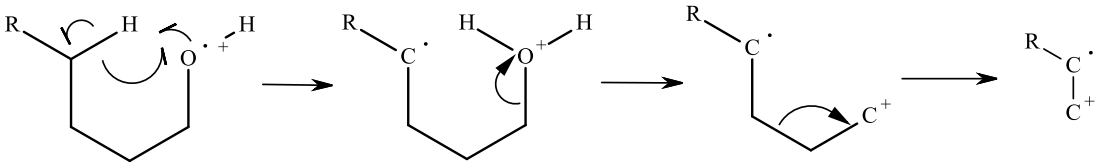


Figure 2.20: Formation of EI fragment ion m/z = 393 and m/z = 365 for octacosanol (C_{28})

2.4.4 Aldehydes

Aldehydes have also been previously identified and in this particular variety of wheat, only the aldehyde octacosanal (C_{28}) was identified which is in agreement with the biosynthetic route.[82] Aldehydes are the intermediates between alcohols and fatty acids precursors and as octacosanol (C_{28}) is the only fatty alcohol identified, this suggests that the octacosanal (C_{28}) may be present due to partial oxidation of the alcohol.[82] Aldehydes can be used as flavourings but many have reported that aldehydes are only minor constituents in wheat extracts.[16, 98, 99, 181] A few have also reported a complete absence of aldehydes in the extracts which means that any purification steps for applications exclusively for aldehydes will probably be not viable.[111, 152] Figure 2.21 shows the EI mass spectrum of octacosanal (C_{28}) and in this case molecular ion was not observed due to the harsh EI ionisation technique used for analysis but when the

region was magnified, the $[M - 18]^+$ ion can be observed. The fragmentation pattern for aliphatic aldehydes are very similar to aliphatic primary fatty alcohols as both compounds would undergo a dehydration step giving $[M - 18]^+$ ion. The fragment ion of $[M - 28]^+$ ion can also be observed due to the loss of CO with aliphatic aldehydes but not primary fatty alcohols. Other characteristic ions $[M - 1]^+$, $[M - 29]^+$ and $[M - 44]^+$ might be observed due the cleavage of the C – H bond and cleavage of C – C bond to release just the functional group CHO and CH_3CHO respectively as shown in Figure 2.22.

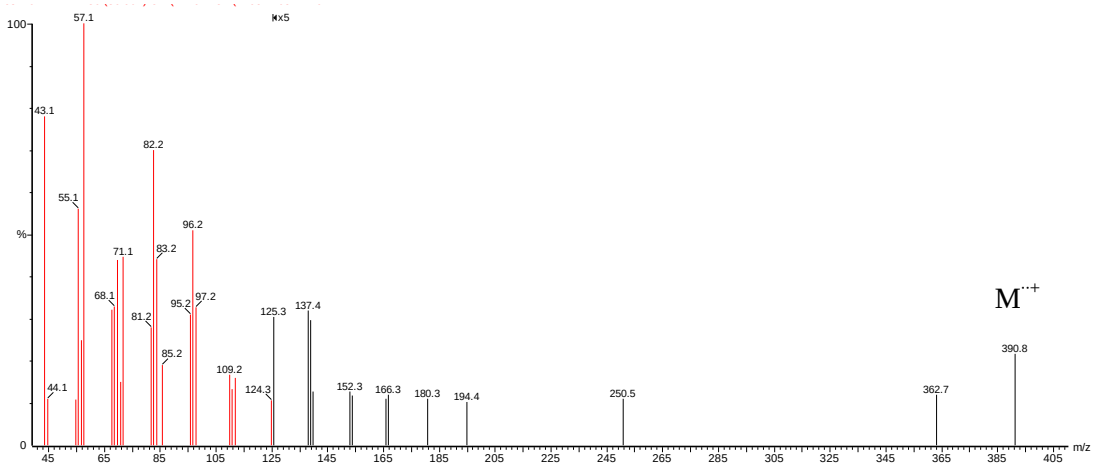


Figure 2.21: EI mass spectrum of octacosanal (C_{28}) (magnified five times from m/z 125 – 410) (originally in colour)

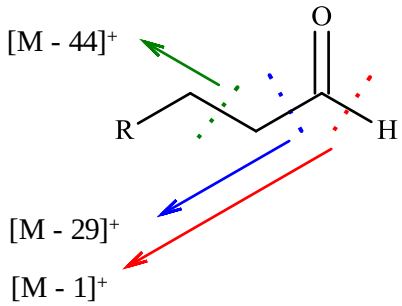


Figure 2.22: Mass fragment ion of aliphatic aldehyde (originally in colour)

A softer FI technique was used for the mass spectrometry to give less fragmentation is shown in Figure 2.24 to confirm the identification.

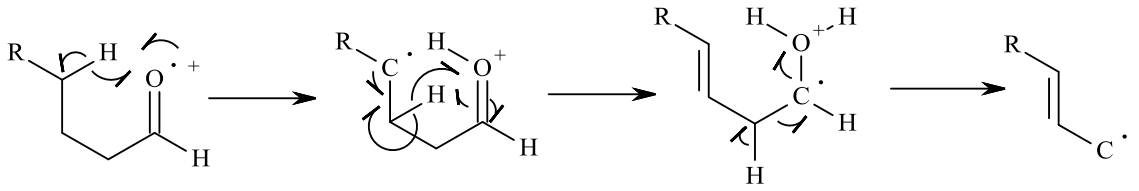


Figure 2.23: Formation of mass fragment ion m/z = 390

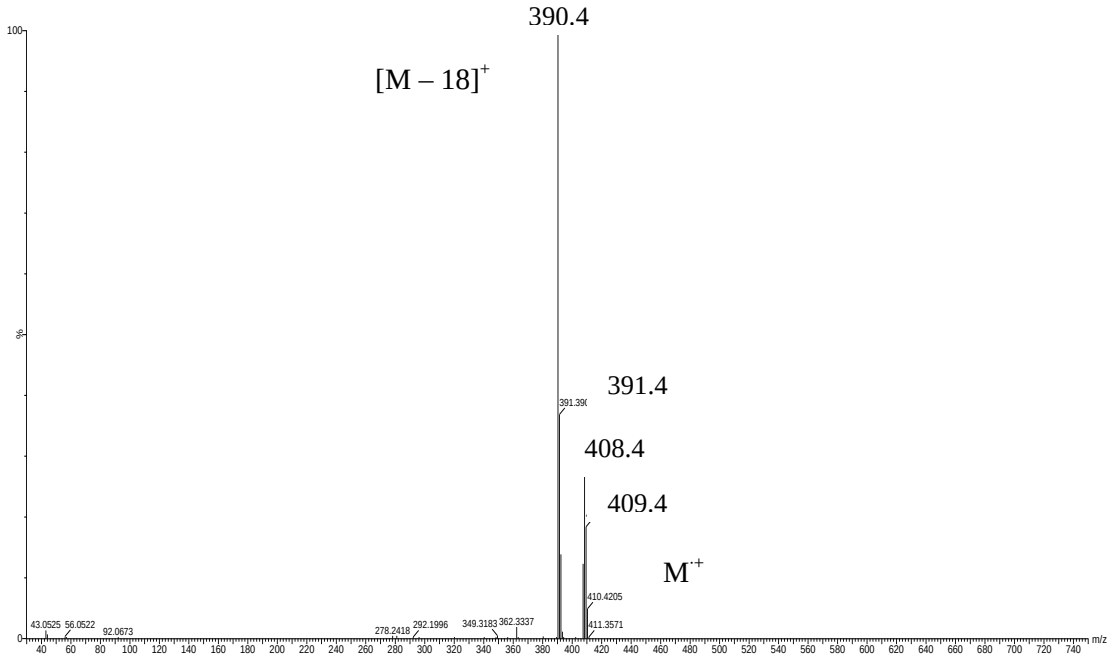


Figure 2.24: FI mass spectrum of octacosanal (C_{28})

The molecular ion and fragment ion $[M - 18]^+$ can be identified for octacosanal. The $[M - 18]^+$ ion corresponds to the loss of water as shown in Figure 2.23. The extra information gathered from the FI mass spectrum confirmed the identification of aliphatic aldehyde and the number of carbons on the alkyl chain. Table 2.10 shows the EI fragment ions with the percentage base peak and the calculated KI but unfortunately, there are no published literature KI for comparison. The base peak ion is a result of a combination of fragment ion from the alkyl chain $[CH_3(CH_2)_3]^+$ and McLafferty rearrangement and then followed by an α-cleavage as shown in Figure 2.25.

Table 2.10: Identification of octacosanal in solvent extracts including the EI fragmentation pattern with percentage base peak intensity and calculated KI

Identification	M^+	EI Fragmentation	Calculated KI
Aldehyde			
Octacosanal	408.7	57(100), 43(99), 82(97), 55(66), 96(63), 69(57), 71(52), 83(51), 81(48), 97(45)	3024

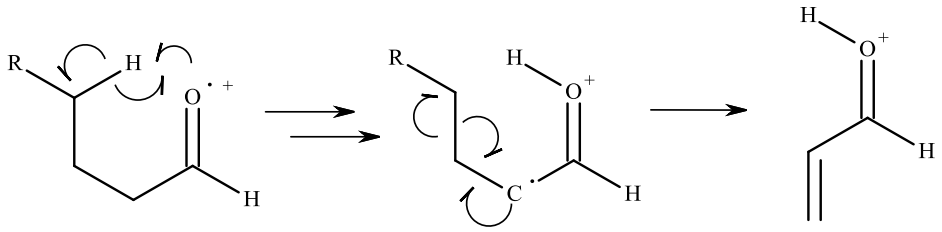


Figure 2.25: Formation of base peak ion m/z = 57 for aliphatic aldehydes

2.4.5 Wax Esters

Wax esters are one of the most important groups of molecules found in plant waxes as the high molecular weight molecules have many valuable applications such as cosmetics, hard wax polishes, lubricants, coatings and plasticisers.[182] Structures of wax esters in wheat are derived from esterification of saturated primary even-numbered chain fatty alcohol and fatty acids to give the ester chain lengths from C_{40} to C_{56}. The analysis of high molecular weight compounds using GC-FID and GC-MS is difficult because of the low volatility so wax esters are usually analysed by hydrolysis of the wax esters into fatty alcohols and fatty acids. The benefit is that this would ensure that all the high molecular weight wax esters are eluted off the column although the original wax ester structures are lost. To combat this problem, the wheat straw wax extracts were analysed using a shorter column (DB-5HT, 15 m x 0.25 mm I. D. X 0.25 µm film thickness) as described in Section 6.3.1 for the identification of the wax esters. The method enabled carbon chain lengths of up to 60 with the KI of 6000 to be analysed. Figure 2.26 shows the GC chromatogram of the identified wax esters from wheat straw wax. The trace displays that even- numbered saturated wax esters predominately found in wheat straw wax tend to consist of hexadecanoic acid

($C_{16:0}$) and octacosanol (C_{28}). These two compounds were identified as a "free" form in the extracts which is in agreement with the biosynthetic pathway of wax esters.[82]

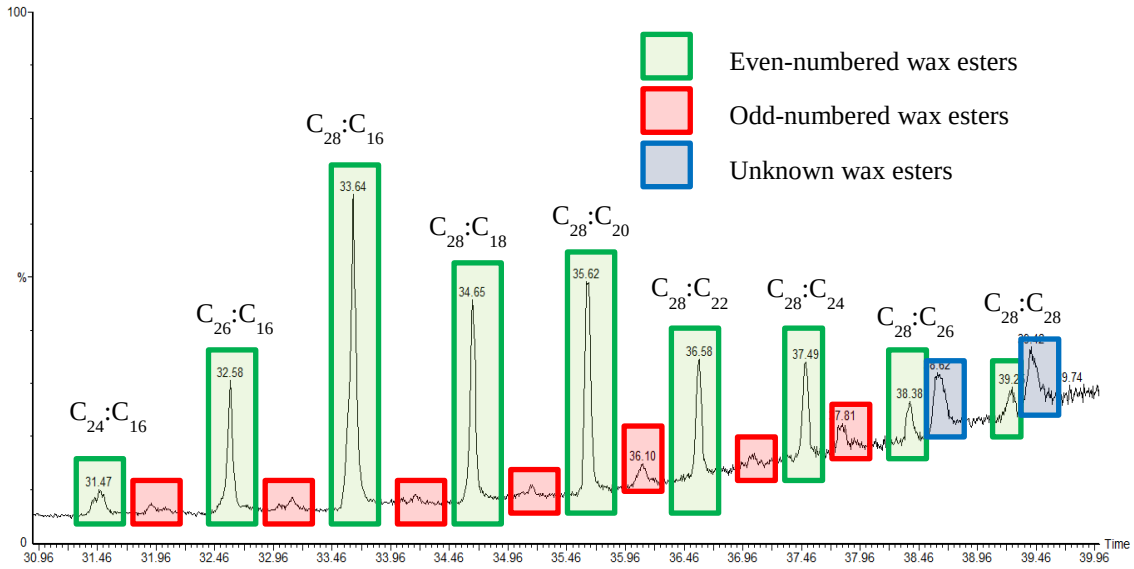


Figure 2.26: GC chromatogram of wheat straw wax esters (originally in colour)

As can be observed from Figure 2.26, the most abundant wax ester is octacosanyl hexadecanoate (C_{28}:C_{16}). The EI mass spectrum of octacosanyl hexadecanoate (C_{28}:C_{16}) as a representative of the saturated wax ester group is shown in Figure 2.28. A small molecular ion is observed and distinct characteristic ion of $[R2CO_2H_2]^+$ was generated by the β-cleavage at the oxygen on the ester group and McLafferty rearrangement with double hydrogen transfer which is shown in Figure 2.27. In the case of hexadecanoate esters, the fragment ion $[R2CO_2H_2]^+$ with m/z = 257 is generated and sometimes this is the base peak ion. Figure 2.27 also shows that another important ion of $[R2CO_2H]^+$ could also be identified and is also believed to be generated by McLafferty rearrangement. Weak ions of $[R1CO_2]^+$ and $[R1 - 1]^+$ arising from the fatty alcohol moiety can sometimes be observed. Table 2.11 shows a list of distinct characteristic fragment ion for the even-numbered wax esters identified. Other fragment ions found in a long chain aliphatic wax ester have a regularly spaced 14 m/z units apart and this is due to the long alkane chain and the corresponding ions are identified in Table 2.7.

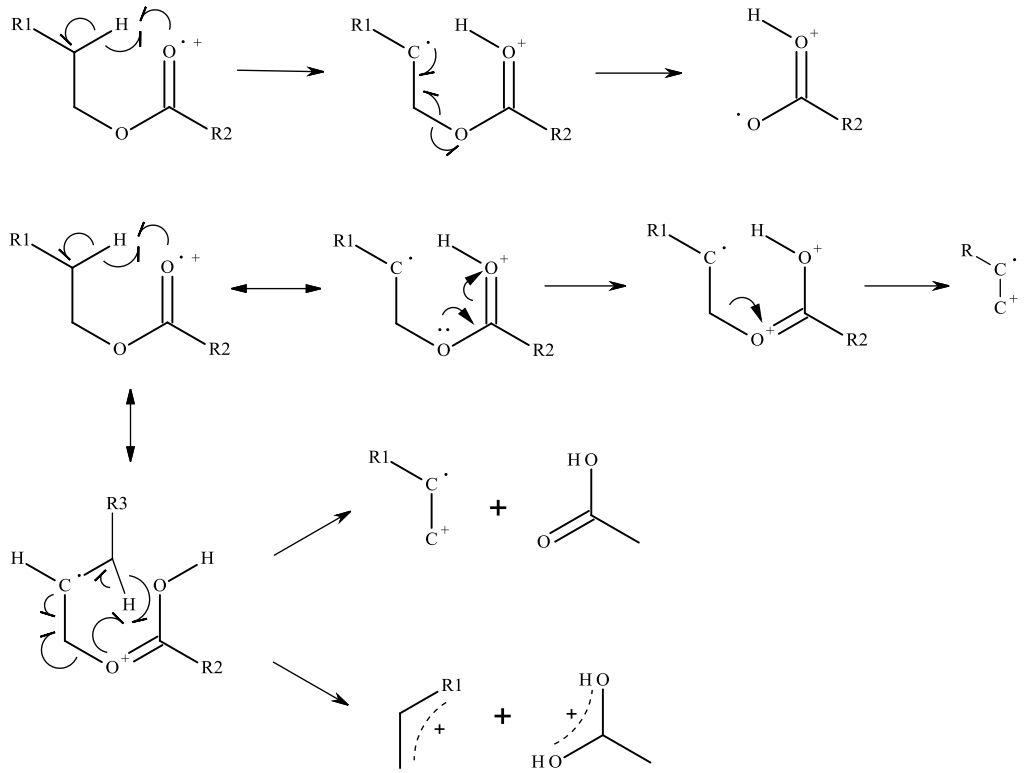


Figure 2.27: Formation of EI fragment ions m/z = 393, 257 and 256 for octacosanyl hexadecanoate (C_{28}:C_{16})

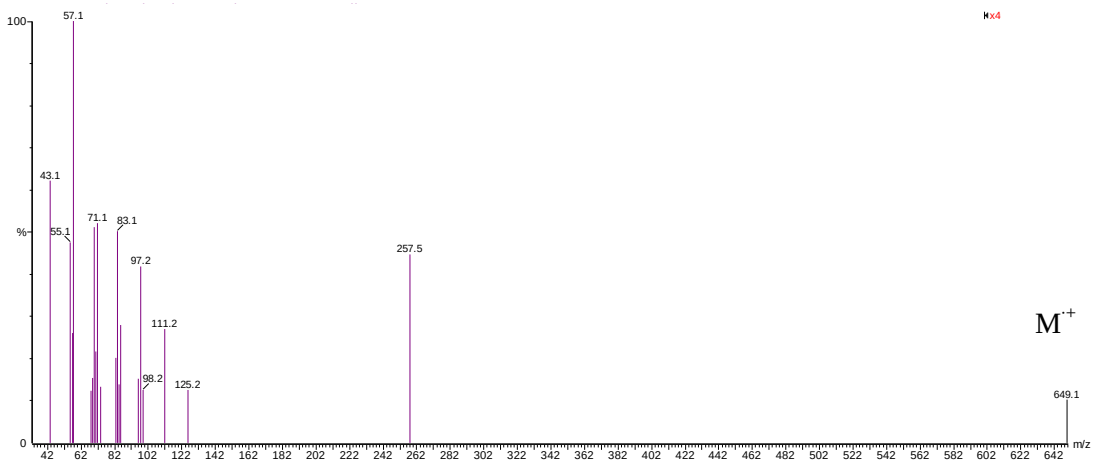


Figure 2.28: EI mass spectrum of octacosanyl hexdecanoate (C_{28}:C_{16}) (magnified four times from m/z 600 – 650) (originally in colour)

Table 2.11 shows a list of saturated wax esters identified, corresponding EI fragmentation and percentage base peak intensity. It was apparent from the KI that it

is a series of wax esters with increasing two carbons in chain length. However, the difficulties of identifying the exact structure arise as the observed molecular ions are very weak so therefore the distinct fragment ion $[R2CO_2H_2]^+$ was used to identify the length of the fatty acid moiety. The chain length of the fatty alcohol can be calculated by the known fatty acid chain length so therefore the wax esters can be identified.

All the wax esters identified in wheat straw are saturated which is in agreement with literature.[16, 111, 152] Limited identification work had been carried out on the wax esters due to the high molecular weight so therefore only Deswarte had managed to also identify wax esters of up to C_{56}. As shown in Figure 2.26, odd-numbered aliphatic esters were also found in the wheat straw waxes. It was reported that odd chain wax esters found in barley had carbon chains in the range C_{31} - C_{37} where the alcohol moieties are secondary alcohols of C_{11} - C_{17} and wheat straw waxes may possess the same homologous series.[183] Odd-numbered wax esters in wheat straw have also previously been reported by Deswarte.[16]

Table 2.11: Identification of wax esters in solvent extracts including EI fragmentation pattern with percentage base peak intensity and calculated KI

Identification	M^+	EI Fragmentation	Calculated KI
Wax esters			
Tetracosanyl hexadecanoate (C_{24}:C_{16})	592.6	43(100), 57(88), 69(64), 55(59), 71(58), 83(44), 97(43), 68(34), 85(30), 56(29),	4137
Hexacosanyl hexadecanoate (C_{26}:C_{16})	620.6	57(100), 43(83), 69(67), 71(64), 55(61), 97(55), 83(55), 85(37), 257(34), 70(30)	4335
Octacosanyl hexdecanoate (C_{28}:C_{16})	648.7	57(100), 43(82), 71(65), 69(62), 83(54), 55(54), 97(49), 85(30), 111(27), 70(27)	4513
Octacosanyl octadecanoate (C_{28}:C_{18})	676.7	57(100), 43(80), 71(70), 69(61), 83(53), 55(50), 97(49), 85(31), 70(28), 111(26)	4699
Octacosanyl eicosanoate (C_{28}:C_{20})	704.7	57(100), 43(69), 71(61), 83(51), 69(51), 97(47), 55(45), 85(33), 70(27), 111(25)	4953
Octacosanyl docosanoate (C_{28}:C_{22})	732.8	57(100), 43(78), 71(70), 69(58), 83(53), 55(52), 97(47), 85(34), 82(28), 111(26)	-
Octacosanyl tetracosanoate (C_{28}:C_{24})	760.8	57(100), 43(76), 71(64), 69(57), 55(50), 83(46), 97(43), 85(32), 73(30), 96(30)	-
Octacosanyl hexacosanoate (C_{28}:C_{26})	788.8	57(100), 43(78), 71(54), 83(46), 96(46), 97(43), 55(40), 85(36), 70(27), 73(26)	-
Octacosanyl octacosanoate (C_{28}:C_{28})	816.9	57(100), 71(68), 43(46), 83(43), 97(40), 85(34), 55(33), 111(25), 70(23)	-

Table 2.12: List of characteristic fragment ions for even-numbered wax esters

Identification	M^+	$[R2CO_2H_2]^+$	$[R2CO_2H]^+$	$[R1 - 1]^+$	$[R2CO_2]^+$
Wax esters					
Tetracosanyl hexadecanoate (C_{24}:C_{16})	592.6	257.4	256.4	336.6	381.7
Hexacosanyl hexadecanoate (C_{26}:C_{16})	620.6	257.4	256.4	364.7	364.7
Octacosanyl hexdecanoate (C_{28}:C_{16})	648.7	257.4	256.4	392.8	437.8
Octacosanyl octadecanoate (C_{28}:C_{18})	676.7	285.5	284.5	392.8	437.8
Octacosanyl eicosanoate (C_{28}:C_{20})	704.7	313.5	312.5	392.8	437.8
Octacosanyl docosanoate (C_{28}:C_{22})	732.8	341.6	340.6	392.8	437.8
Octacosanyl tetracosanoate (C_{28}:C_{24})	760.8	369.7	368.6	392.8	437.8
Octacosanyl hexacosanoate (C_{28}:C_{26})	788.8	397.7	396.7	392.8	437.8
Octacosanyl octacosanoate (C_{28}:C_{28})	816.9	425.8	424.8	392.8	437.8

R1 and R2 are side chains indicated in Figure 2.27

2.4.6 Sterols

Sterols and 3'-ketosteroids were identified in the wheat straw wax. They are closely related in both functionally and structure. Both groups of molecules consist of the four cycloalkane carbon skeleton rings as shown in Figure 2.29. Sterols are paramount in membrane function as the hydroxyl group in the 3' position can form hydrogen bonds with various ester carbonyl moieties from the fatty acids of phospholipids to regulate the fluidity of the membrane.[184] 3'-Ketosteroid also have important roles in destabilisation of the phospholipid bilayer membrane structure and when incorporated into erythrocytes, the permeability and fragility of the membranes are greatly increased.[185] The abundance of sterols and 3'-ketosteroids must be highly regulated in wheat in order to maintain the appropriate fluidity and permeability of the membrane.

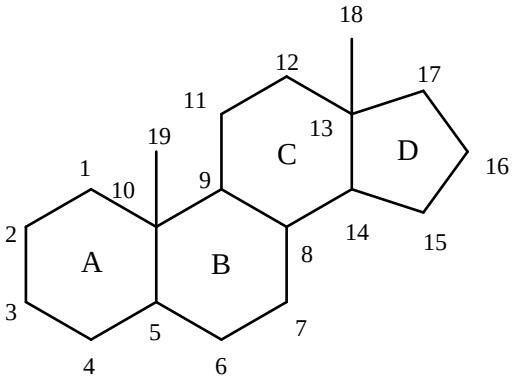
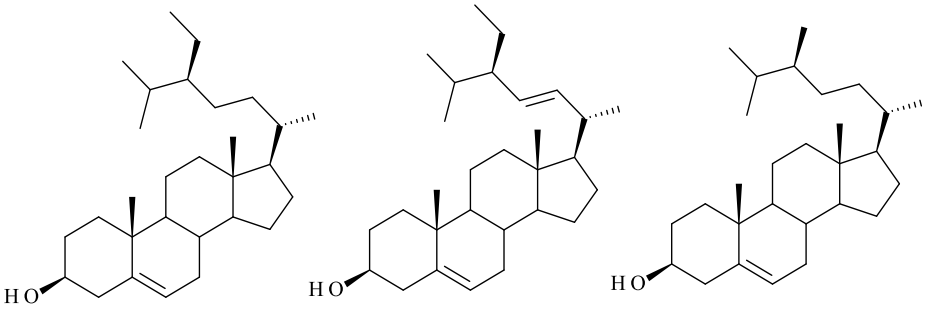


Figure 2.29: Four cycloalkane carbon skeleton rings found in sterols and 3'-ketosteroids

Sterols such as stigmasterol, campesterol and β-sitosterol were identified in wheat straw wax and these compounds are structurally similar as they are all derived from β-sitosterol.[82] These are three predominant cereal phytosterols that have been identified in wheat and other cereal crops such as barley and oat in previous studies.[186, 187] Cholesterol, ergosterol, and stigmastanol were also identified in wheat straw but these phytosterols were not found in the organic solvent extracts. [16, 111, 152] Phytosterols have been reported to be found in other wheat by-products such as wheat bran and germ.[69] As well as the three common sterols, brassicasterol, avenasterol and campestanol were also identified in the wheat by-products.[69] The wheat straw wax extracts were analysed directly without further purification and fractionation so there is a chance that the sterols may co-elute with other wax components as the extracts consist of many compounds of around 30 carbons. Phytosterols are an important class of compounds and have nutritional benefits and are used as food supplements for reducing blood cholesterol level.[188, 189] As highlighted previously, polycosanols are capable of reducing blood cholesterol level even though the mode of action for both phytosterols and policosanols are very different. Policosanols can lower total and LDL cholesterol by inhibition of cholesterol synthesis from activation of AMP kinase.[190] Phytosterols reduce the total and HDL cholesterol level by minimising intestinal cholesterol absorption and increase faecal excretion of cholesterol.[191, 192] Due to the different lowering cholesterol mechanisms, it had been reported that a combination of both policosanols and phytosterols can give an enhanced effect.[193, 194] However, Jones

et al. showed that there is not a significant amount of benefit when both of these groups of molecules are applied together in hamsters.[195] Extensive research has taken place in order to find new potential sources and to separate these high value sterols.[69, 196] It would be interesting to fractionate the phytosterols and policosanols and test for cholesterol lowering effects. Previous studies indicated that sterols can also exist as bound forms such as steryl esters within wheat straw however, no steryl esters were found in this case.[183]



β-Sitosterol Stigmasterol Campesterol

Figure 2.30: Structures of sterols present in wheat straw wax

The three predominant sterols were identified using a combination of NIST library 2008, standard compounds, EI fragmentation pattern and published KI data. The EI mass spectrum of stigmasterol is shown in Figure 2.31a which is compared with the EI spectrum from the NIST library (Figure 2.31b). Other sterols have a similar fragmentation pattern and the molecular ion is weak but can be observed which is extremely useful when distinguishing between different sterols.

The ion m/z = 57 forms from the β-cleavage of the ring, followed by the formation of a diene. Characteristic ion m/z = 357 for β-sitosterol followed the same β-cleavage of the ring then a hydrogen migration occurred to give a 3-rings ion. Sterols can also undergo the cleavage of three rings giving a stable tertiary cation m/z = 272. The 3' OH group on the sterols can also undergo a dehydration step with carbon 4 on ring A giving a stable conjugated cation as indicated in Figure 2.32.

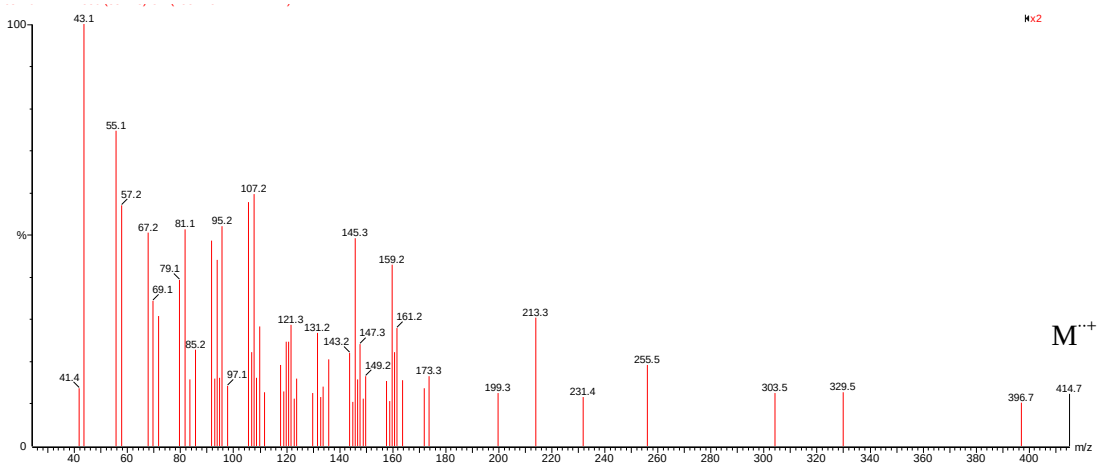


Figure 2.31a: EI mass spectrum of stigmasterol (magnified two times from m/z 400 – 415) (originally in colour)

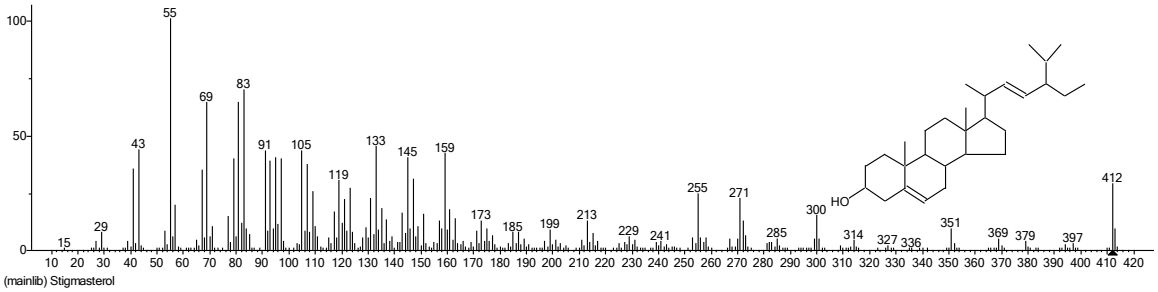


Figure 2.31b: EI mass spectrum of stigmasterol from the NIST library

Table 2.13 shows the characteristic fragment ions for the three important sterols in wheat straw. A total of five sterols are found in the wheat straw wax but sterol 1 and 2 were not identified as it is not present in all the different solvent extracts. The presence and relative abundance of sterols between various solvent extracts are discussed in Section 2.5.7.

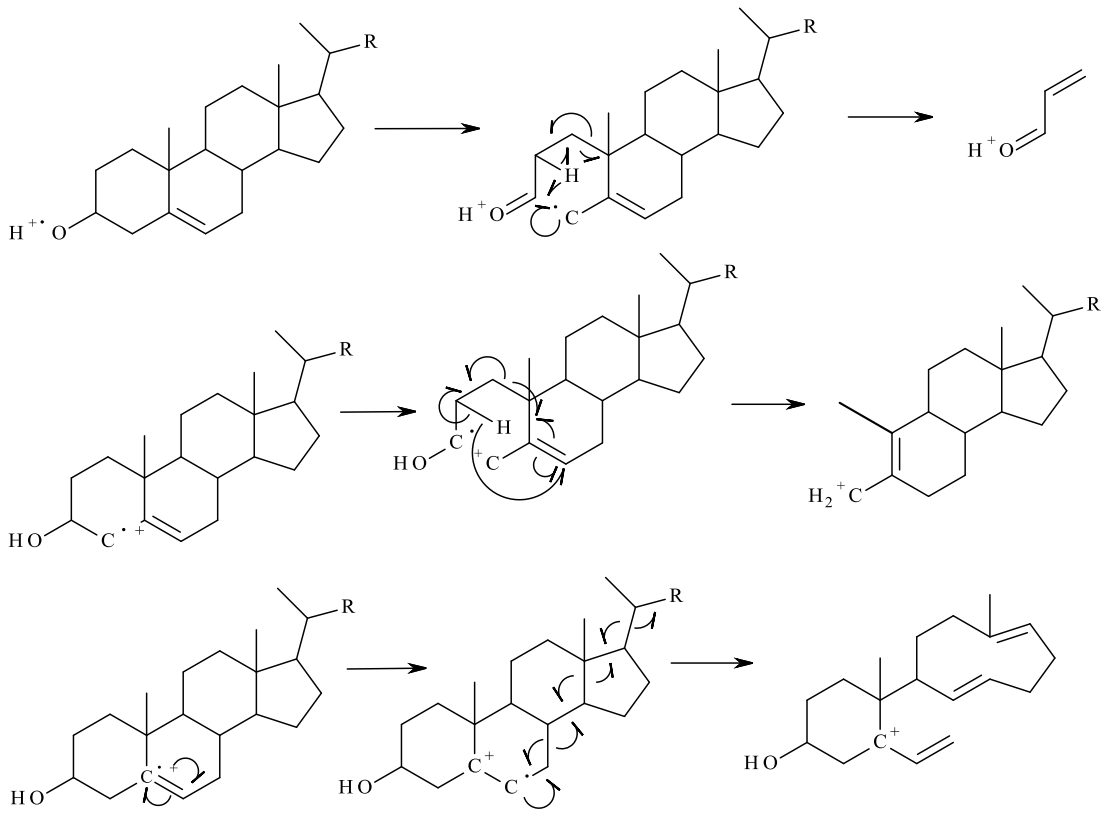


Figure 2.32: Fragmentation of a sterol to give characteristic mass ions

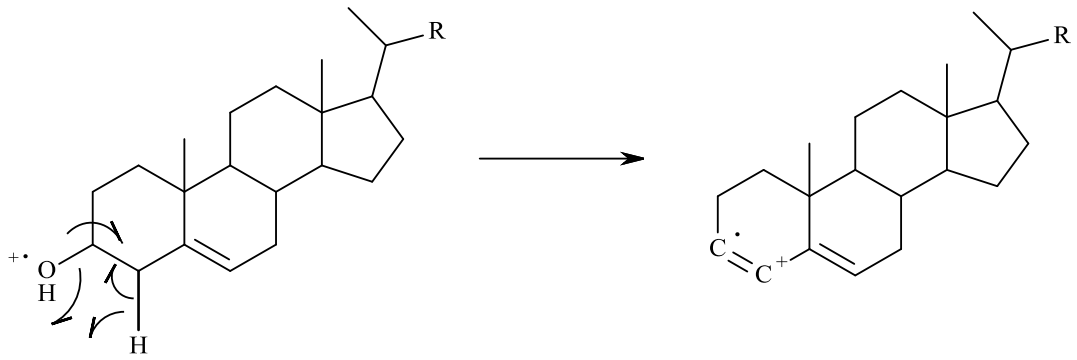


Figure 2.33: Dehydration step of a sterol to give characteristic ion

Table 2.13: List of characteristic fragment ions for sterols

Identification	M^+	$[M - H_2O]^+$	$[M - CH_3]^+$	$[M - R - OH]^+$	[M - C1, C3 - OH]$^+$	[C1, C3 + OH]$^+$
Sterols						
Campesterol	400.7	382.7	385.7	255.2	343.3	57.0
Stigmasterol	412.7	394.7	397.7	255.2	355.3	57.0
β-Sitosterol	414.7	396.7	399.7	255.2	357.3	57.0

An interesting 3'-ketosteroid was identified in wheat which is in contradiction to all the previous studies on both plants and straw.[16, 87, 97, 98, 99, 103, 152] The 3'-ketosteroid found was confirmed to be Δ^4-sitosten-3-one using a combination of the EI fragmentation pattern in Figure 2.35, the molecular ion information from the FI mass spectrum in Figure 2.36 and the NIST library 2008. The EI fragment ions and peak intensities are shown in Table 2.14. The base peak ion m/z = 124 is generated by two different mechanisms as shown in Figure 2.37. One of which is the fission of the carbon 9 – 10 bond with the cation remaining on carbon 5 then followed by the migration of hydrogen on carbon 11 (left mechanism). Alternatively, the base peak ion can be generated by the migration of two hydrogen atoms from carbon 11 and 6 so the charge remains on the oxygen (right mechanism). This base peak ion is unique and characteristic of a Δ^4-sterone. Other important fragment ions include m/z = 149 which is the consequence of the fission of the 9 – 10 bond which is similar to the generation of m/ z = 124 shown in the left mechanism. Both proposed mechanisms for the generation of m/ z = 149 ion involve the migration of a hydrogen atom on carbon 8. On the left mechanism, the formation of m/z = 149 ion is driven by the fission of the allylically labilised carbon 6 – 7 bond whereas the right mechanism is driven by the formation of the ionised carbon 8 – 9 bond. In Figure 2.38, a mechanism for the formation of m/z = 230 ion was proposed, this ion was not observed initially but the ion can be found when the higher m/z region was magnified. The fragment ion is formed by a four-membered ring formation in ring A of the steroid skeleton.

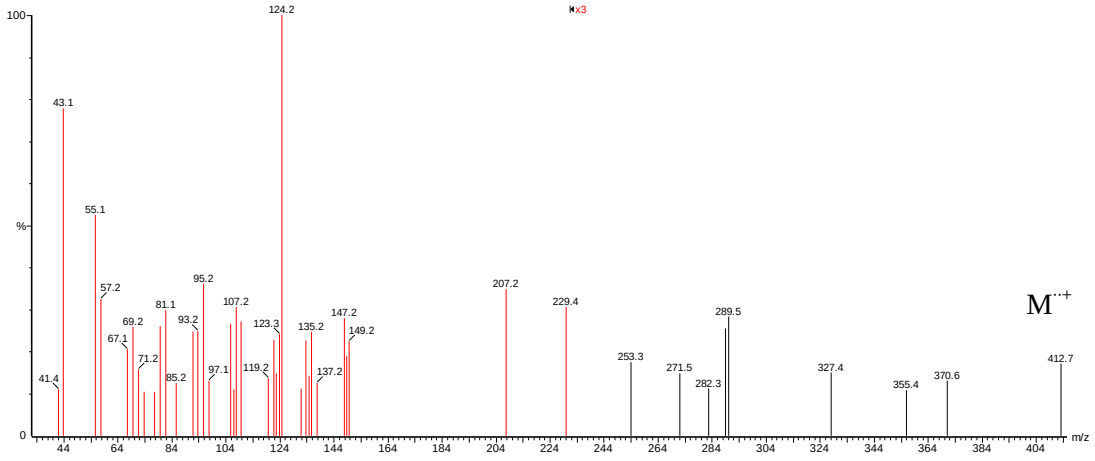


Figure 2.35: EI mass spectrum of Δ^4-sitosten-3-one (magnified two times from m/z 230 – 415) (originally in colour)

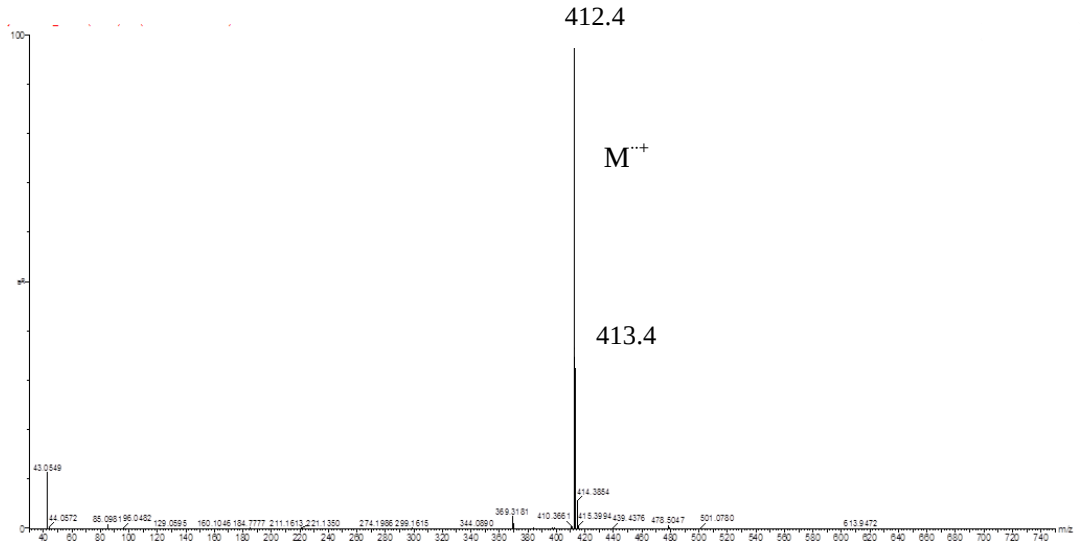


Figure 2.36: FI mass spectrum of Δ^4-Sitosten-3-one

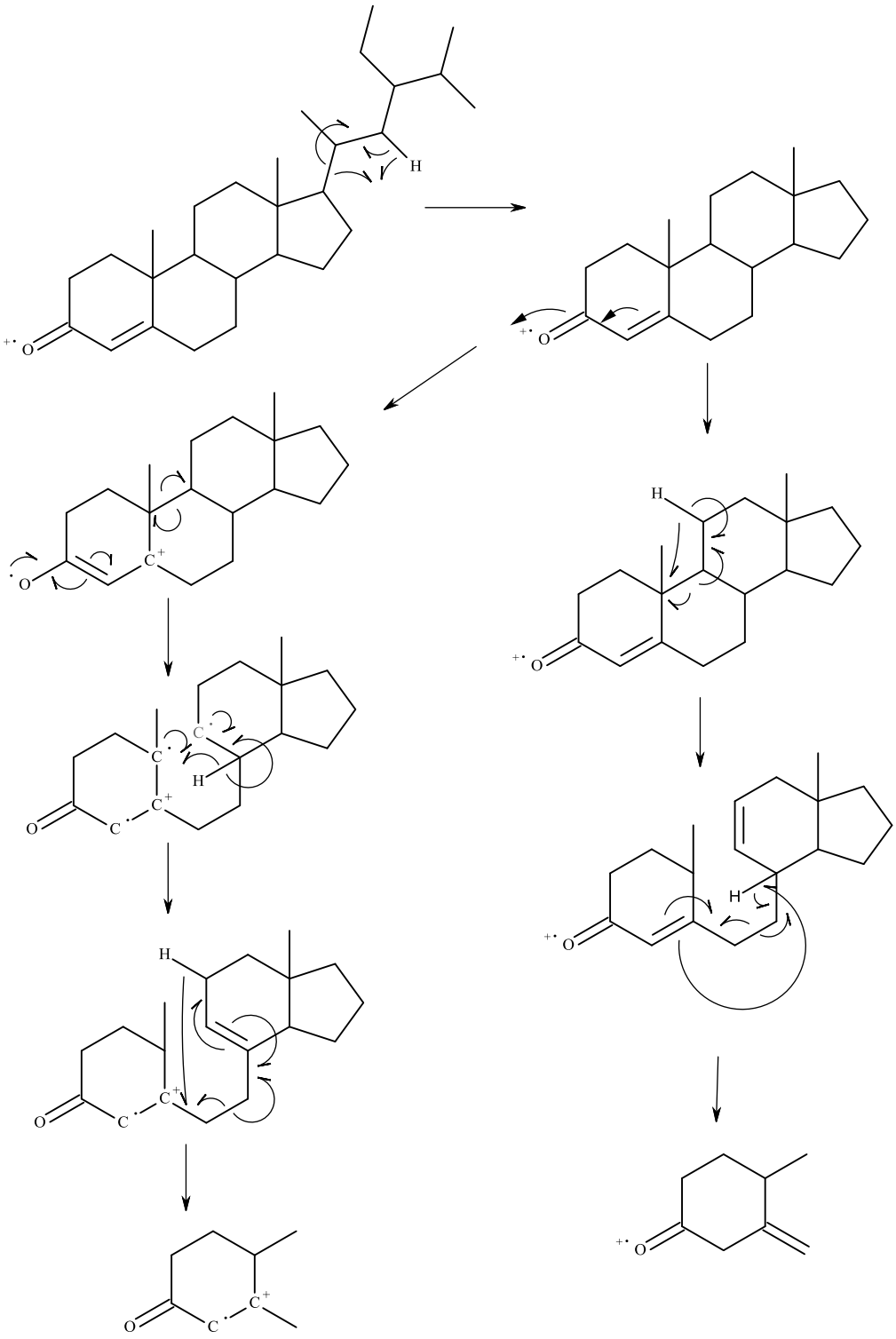


Figure 2.37: Formation of base peak ion m/z = 124[197]

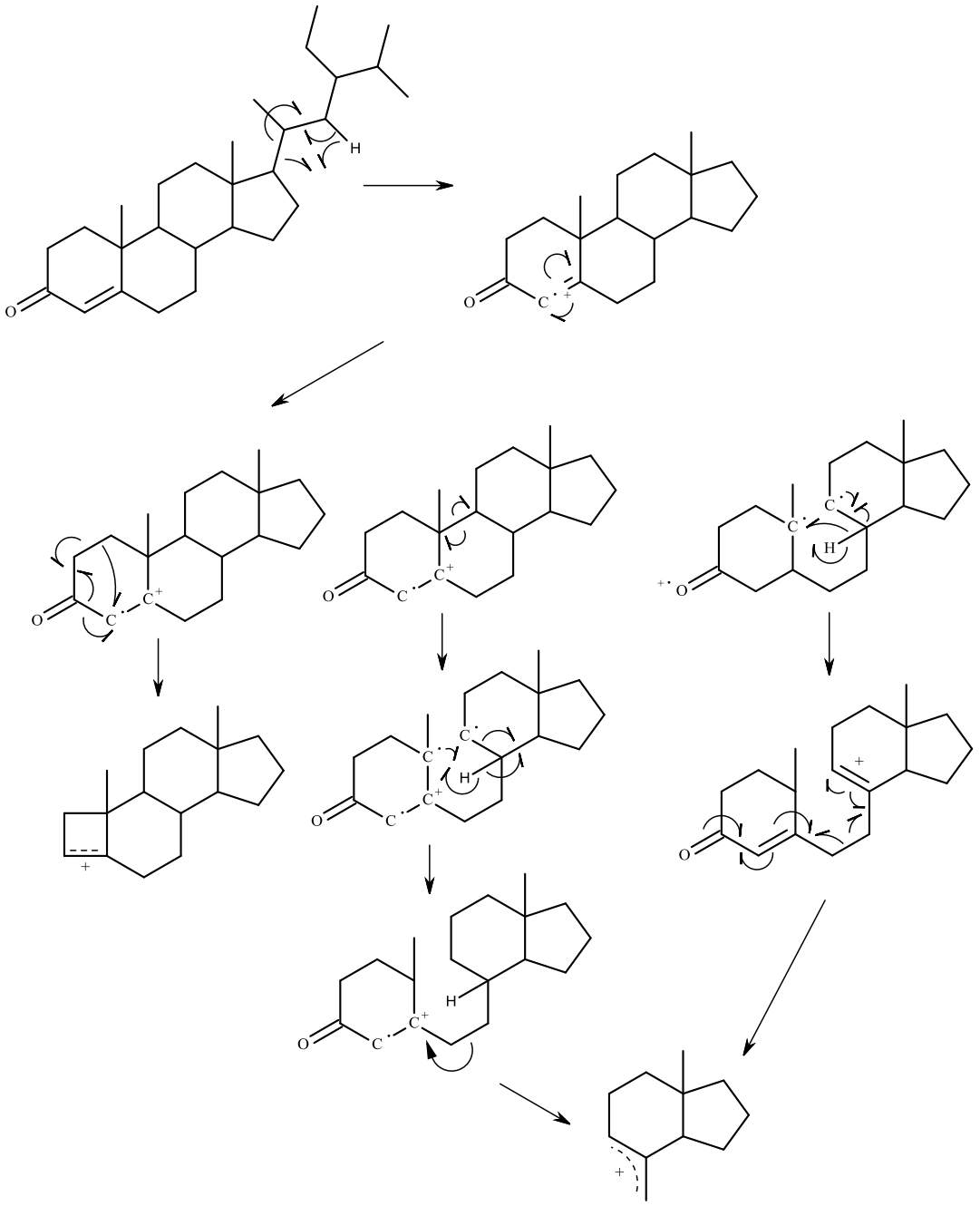


Figure 2.38: Formation of fragment ions m/z = 230 and m/z = 149[197]

Table 2.14: Identification of sterols and sterol derivatives in solvent extracts including EI fragmentation pattern with percentage base peak intensity and calculated KI

Identification	M^+	EI Fragmentation	Calculated KI	Literature KI
Sterols				
Campesterol	400.7	43(100), 55(73), 57(62), 105(55), 81(50), 107(48), 95(48), 91(44), 93 (38), 145(37)	3238	3193[198]
Stigmasterol	412.7	55(100), 83(67), 43(53), 81(51), 69(42), 91(33), 57(33), 93(32), 95(32), 107(28)	3272	3249[199]
β-sitosterol	414.7	43(100), 55(76), 57(62), 81(51), 107(50), 105(47), 93(45), 69(45), 145(40), 91(39)	3334	3408[200]
Sterol 1	-	55(100), 69(61), 43(40), 81(37), 67(32), 79(31), 83(29), 97(25), 109(22) 105(22)	3400	-
Δ^4-Sitosten-3-one	413.7	124(100), 43(70), 55(53), 57(31), 95(30), 229 (29), 105(25), 109(23), 69(23), 147(23)	3476	-
Sterol 2	-	43(100), 55(86), 57(60), 69(49), 81(41), 95(36), 93(34), 107(31), 67(31), 137(30)	3636	-

2.4.7 Beta-diketones

Another interesting class of molecule in the wheat straw extracts are the β-diketones which are commonly identified as a component in plant cuticle wax. β-Diketones have been identified in many other cereal crops such as barley, oat and flax.[88, 92, 100] Hentriacontane-14,16-dione (C_{31}) occurs as one of the major wax components in wheat straw which is in agreement with a previous study carried out by Deswarte.[16] This is a common wax component and had been reported extensively as the key β-diketone in young wheat plant.[91, 97, 98, 99, 105] Triatriacontane-16,18-dione (C_{33}) was also identified in the extracts which is in contradiction to other publications on wheat.[16, 97] However, this had been identified as one of the major β-diketone in *Eucalyptus spp*.[201] It commonly has the 1,3-dicarbonyl group in positions, 12 and 14 or 14 and 16 or 16 and 18 of C_{29}, C_{31} and C_{33} alkyl chains.[68] These odd-numbered β-

diketones are synthesised during the elongation process in making the fatty acids precursors.[93] The β-keto group is protected by metal ions such as copper in various enzymes which can have important applications as metal chelators.[93, 202] These diketones can also be used for making super-hydrophobic coatings.[203] The β-diketones only exist as odd-numbered as a similar decarboxylation step to alkanes also occurs when the molecules are formed.[93] β-Diketones have tautomeric structures and can undergo keto-enol tautomerism as shown in Figure 2.39. The tautomerism occurs by the migration of a hydrogen atom between a carbon atom and the oxygen on an adjacent carbon to form an equilibrium mixture of keto and enol tautomers. As the R groups on the β-diketone are long alkyl chains and the carbon between the two carbonyl groups are unbranched, the equilibrium can be predicted to lie towards the enol tautomer.[204] Due to their tautomeric structure, β-diketones can undergo a derivitisation step such as silylation and be converted into TMS enol ethers to improve chromatography and assist in characterisation. However, this step was not necessary as it was possible to deduce the structures of the β-diketones from direct analysis.

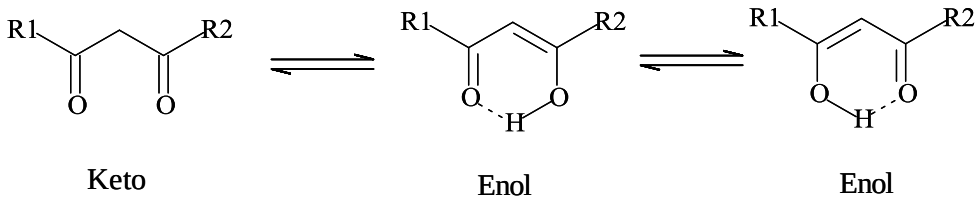


Figure 2.39: Keto-enol tautomerism of β-diketones

Both of the β-diketones were identified using published KI data, EI fragmentation pattern and NIST library 2008. The molecular ion is usually not observed for these compounds but due to its distinct fragmentation pattern, the structure can be deduced easily. When the higher m/z region was magnified, the molecular ion of 492 can be seen at a low intensity as shown in Figure 2.40. With the help of KI values, the number of carbons in the aliphatic alkyl chain can be calculated. It was apparent that there were two CH_2 difference between the two β-diketones as there are about 200 KI unit differences which reflects the two extra carbons in the alkyl chain. Figure 2.40a shows an EI spectrum of hentriacontane-14,16-dione and some distinctive fragment ions which is compared with an EI spectrum from the NIST library (Figure 2.40b). The fragment ion m/z = 100 is a strong characteristic ion for β-diketones. The ion is

formed by a McLafferty rearrangement at the first carbonyl group, cleaving the R group. A series of keto-enol tautomerism can occur after the first McLafferty rearrangement so the second carbonyl is charged. A second McLafferty rearrangement took place as before but on the second carbonyl group and cleaving the second R group which can result in fragment ion m/z = 100 as shown in Figure 2.41. The ion then rearranged and cleaved off a methyl group to give a fragment ion m/z = 85.

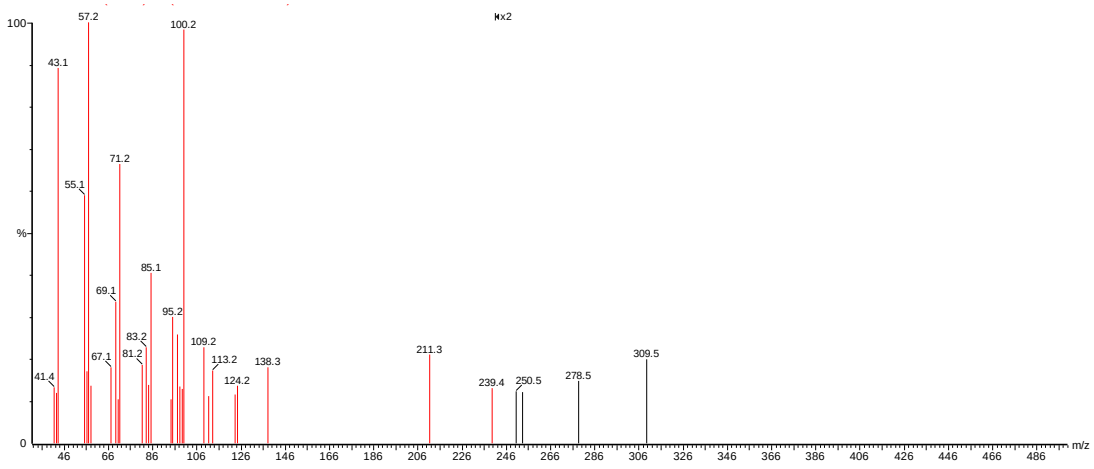


Figure 2.40a: EI spectrum of hentriacontane-14,16-dione (magnified two times from m/z 212– 500) (originally in colour)

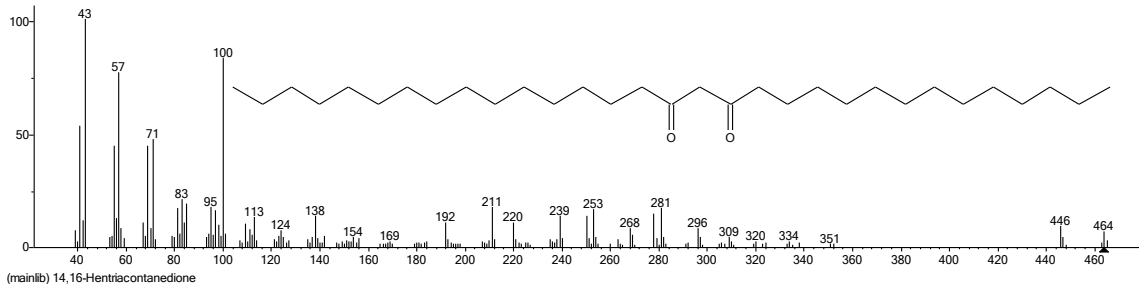


Figure 2.40b: EI spectrum of hentriacontane-14,16-dione from the NIST library

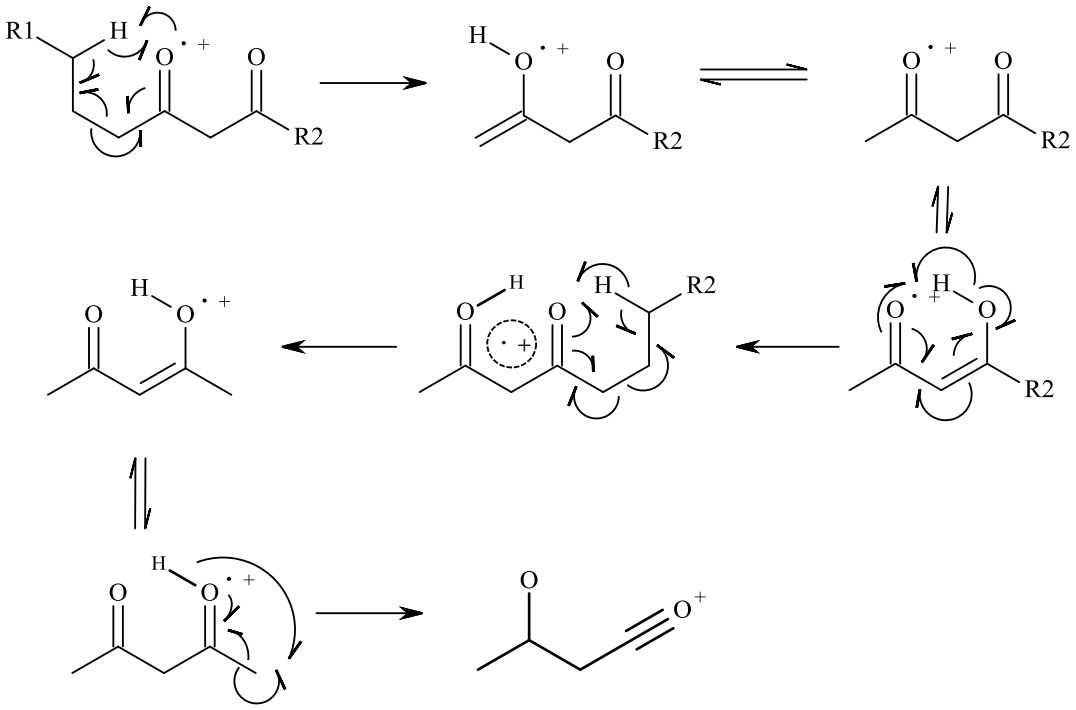
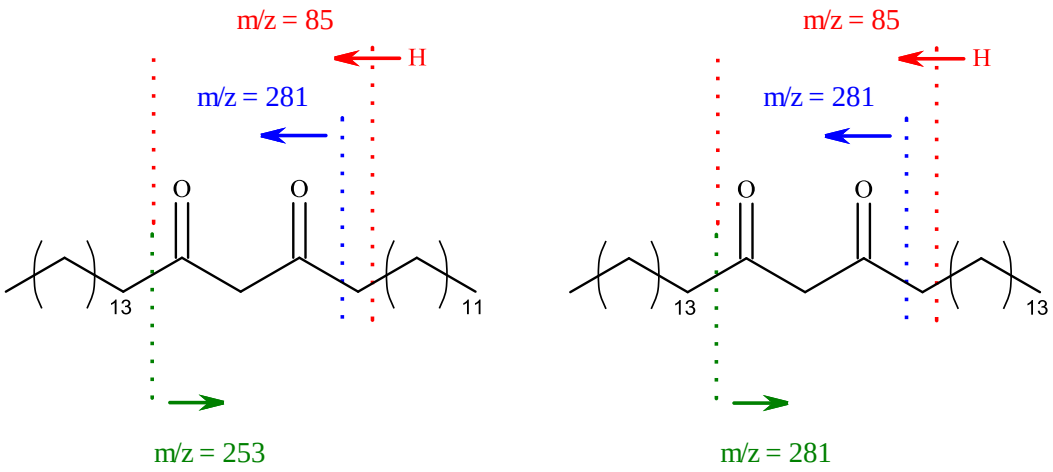


Figure 2.41: Characteristic fragment ions for β-diketones[204]

In order to locate the two carbonyl groups on the alkyl chain, the fragmentation around the functional group is important which is shown in Figure 2.42. Table 2.15 shows the calculated KI values for both β-diketones and shows that the there are two carbons KI difference between the two molecules so the total aliphatic carbon chains can be predicted for 16,18 triatriacontanedione (C_{33}). In 14,16 hentriacontanedione (C_{31}), fragment ions m/z = 253 and m/z = 281 were apparent from the EI mass spectrum whereas for 16,18 triatriacontanedione (C_{33}) a single ion of m/z = 281 was observed.[205]

Table 2.15: Identification of β-diketones in solvent extracts including EI fragmentation pattern with percentage base peak intensity and calculated KI

Identification	M^+	EI Fragmentation	Calculated KI	Literature KI
β-Diketones				
14,16 hentriacontanedione (C_{31})	492.9	100(100), 43(94), 57(82), 71(66), 55(47), 85(41), 69(31), 95(30), 97(28), 83(27)	3377	3375[198]
16,18 triatriacontanedione (C_{33})	520.9	43(100), 57(87), 69(80), 69(80), 71(63), 100(60), 83(50), 95(49), 85(45), 81(44)	3581	-



14,16 hentriacontanedione (C_{31})

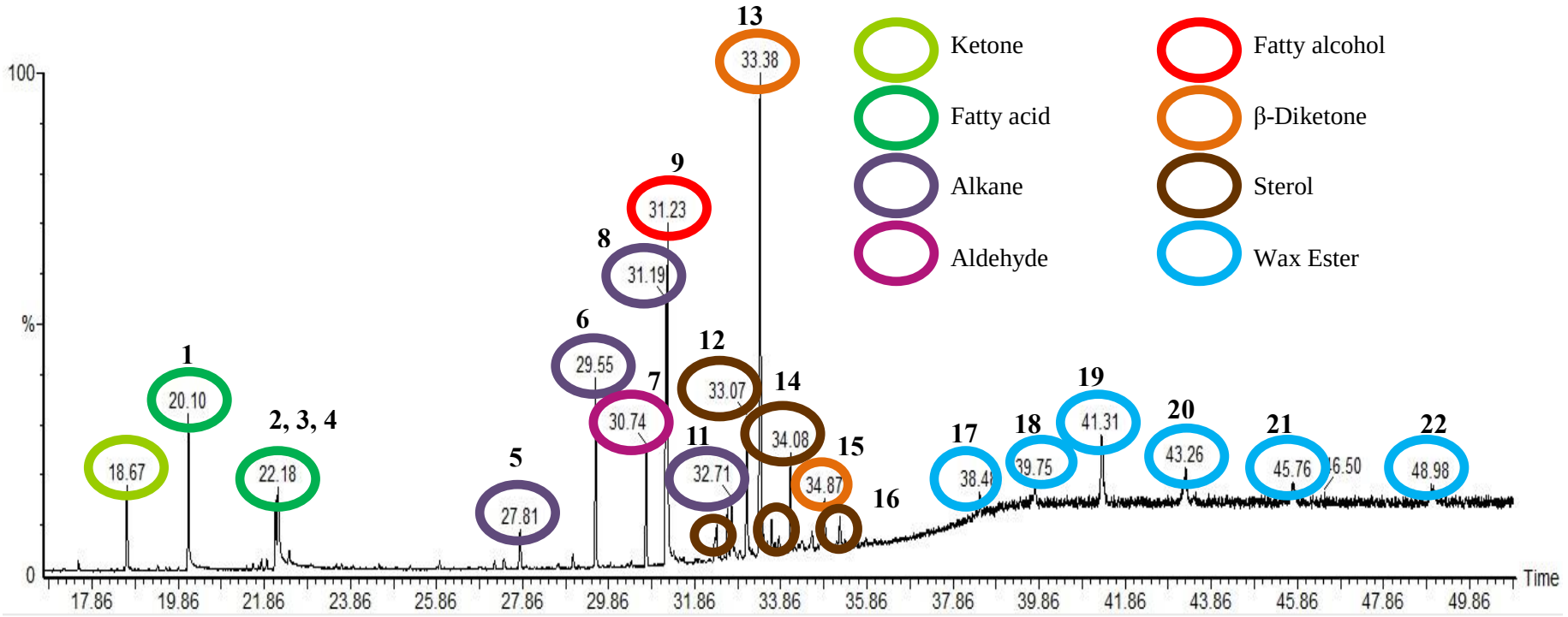
16,18 triatriacontanedione (C_{33})

Figure 2.42: Mass spectral fragmentation of the 14,16 hentriacontanedione (C_{31}) and 16,18 triatriacontanedione (C_{33}) (originally in colour)

Hydroxyl-β-diketones were also common wax component in plant cuticle waxes and had previously been reported to be found in wheat plant, though this had never been reported in wheat straw.[16, 103, 206, 207]

2.4.8 Chemical composition of wheat straw wax summary

The main components on wheat straw cuticle wax can be identified as two main groups: alkyl chain with function group(s) and sterol and its derivatives. The chain lengths of aliphatic wax groups identified followed the pattern from the wax biosynthetic pathway discussed in Section 1.5 where all the components initially stem from a pool of acyl CoAs.[82] The number of carbons of the different compounds is either predominantly all even or all odd-numbered pathway which is expected due to the mechanisms of each wax groups are synthesisd in plant.[82] Figure 2.43 summaries the main components identified in the wheat straw wax.



1	Hexadecanoic acid ($C_{16:0}$)	**6**	Nonacosane (C_{29})	**11**	Triatriacontane (C_{33})	**17**	Tetracosanyl hexadecanoate (C_{40})
2	Octadecadienoic acid ($C_{18:2}$)	**7**	Octacosanal (C_{28})	**12**	Stigmasterol	**18**	Hexacosanyl hexadecanoate (C_{42})
3	Octadecenoic acid ($C_{18:1}$)	**8**	Hentriacontane (C_{31})	**13**	14,16-hentriacontanedione (C_{31})	**19**	Octacosanyl hexadecanoate (C_{44})
4	Octadecanoic acid ($C_{18:0}$)	**9**	Octacosanol (C_{28})	**14**	β-Sitosterol	**20**	Octacosanyl octadecanoate (C_{46})
5	Heptacosane (C_{27})	**10**	Campesterol	**15**	16,18-triatriacontanedione (C_{33})	**21**	Octacosanyl eicosanoate (C_{48})
				16	Δ^4-Sitosten-3-one	**22**	Octacosanyl docosanoate (C_{50})

Figure 2.43: Typical GC chromatogram of solvent wheat straw wax (Viscount 09) extract (originally in colour)

2.5 Quantification of key wax components in wheat straw wax

It can be observed using GC-FID that the various wax extracts from different organic solvent extractions consist of identical wax components so therefore it is important to calculate the relative abundance of the different wax components.

2.5.1 Quantification of key wax components by calibration

The different key wax components have successfully been identified and are then quantified using the method described in Section 6.3.1. The calibration graphs for alkane, free fatty acid, free fatty alcohol, sterol, wax ester, aldehyde and β-diketone have been established using a minimum of eight different standard concentrations along with tetradecane as the internal standard. Figure 2.44 shows a calibration graph for octadecanoic acid as an example. The area and mass ratio were calculated using Equations 2.12 and 2.13 respectively to generate a list of directly proportional area and mass ratio so a line of best fit can be generated. The response factor can be deduced from the gradient of the best fit line. Table 2.16 shows the list of standards along with the calibration range analysed for the quantification of wax components in the extracts. The lowest concentration of the calibration range of the standard is used as the LOQ for that specific wax group.

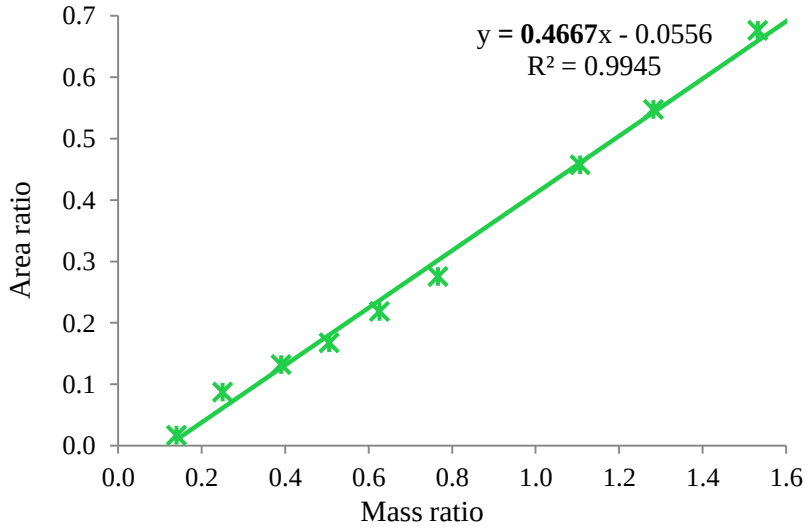


Figure 2.44: Calibration graph for octadecanoic acid (free fatty acid representative) (originally in colour)

$$Area\ ratio = \frac{Area\ of\ standard\ peak}{Area\ of\ internal\ standard\ peak}$$

Equation 2.12: Calculation of area ratio

$$Mass\ ratio = \frac{Mass\ of\ standard\ in\ 1\ mL\ (mg)}{Mass\ of\ internal\ standard\ \ in\ 1\ mL\ (mg)}$$

Equation 2.13: Calculation of mass ratio

Table 2.16: List of standards and calibration ranges with the r^2 and the calculated response factor value

Wax group	Standard	Calibration range ($mgmL^{-1}$)	R^2	Response factor
Fatty acid	Octadecanoic acid	0.25 - 3.0	0.9945	0.4667
Alkane	Hentriacontane	0.1 – 4.0	0.9983	0.4251
Fatty alcohol	Octacosanol	0.25 – 3.0	0.9902	0.3272
Sterol	Stigmasterol	0.25 – 5.0	0.9931	0.3124
Wax ester	Octadecanyl hexadecanoate	0.25 – 5.0	0.9960	0.2721
Aldehyde	Dodecanal	0.25 – 3.0	0.9967	0.7655
β- Diketone	3,5-Heptanedione	0.5 – 5.0	0.9997	0.5464

For wax groups, fatty acid, alkane, fatty alcohol and sterol the chosen standards are wax components that are present in the extracts. As previously discussed, the components identified in each wax group have similar carbon chain lengths and structures so it is valid to use one representative standard to represent the wax group. The largest wax ester standard that can be obtained is octacosanyl hexadecanoate which only consists of 34 carbons but as the carbon length increases, the significance of each extra carbon on the chain length reduces so the response factor of octadecanyl hexadecanoate calibration was used for all the wax esters. Unfortunately, long chain aldehydes and β-diketones standards are not commercially available so the standards obtained were most structurally similar to what were identified. The ECN method was

used to calculate the response factor value when authentic standards were not available.[208] The predicted response factor values were calculated using Equation 2.14 for octacosanal (C_{28}) and 14,16 hentriacontanedione (C_{31}) for the aldehyde and β-diketone group, where IS is tetradecane the internal standard and std is the wax group standard. Table 2.17 shows the molecular weight of the standards and the calculated response value.

$$Response\ factor\ value\ (ECN\ method) = \left(\frac{MW\ of\ IS\ \times number\ of\ C\ in\ std}{MW\ of\ std\ \times number\ of\ C\ in\ IS}\right) \times \left(\frac{MW\ of\ IS}{MW\ of\ std}\right)$$

Equation 2.14: Calculation of response factor value using ECN method[208]

Table 2.17: List of standards with the calculated response factor value from ECN method[208]

Standards	MW	Response factor value
Tetradecane (C_{14}) – Internal	198.390	-
14,16-hentriacontanedione (C_{31})	466.834	0.4516
Octacosanal (C_{28})	410.760	0.50

As indicated by Tables 2.16 and 2.17, there is a large difference between the calculated response value and experimental response value for aldehyde. Since there is a large difference in the chain length between experimental standard aldehyde used and aldehydes identified, the calculated response factor value was used. The two response factor values for the β-diketone were similar but due to the same reason as aldehyde, it is more valid and appropriate to use the calculated response factor value.

2.5.2 Free fatty acids

The five free fatty acids in wheat straw were found to be presented in the extracts ranging from about 1.5 – 11%. Sun *et al.* showed much higher percentage free fatty acids can be extracted with a percentage range of 11 – 34% being reported.[111, 152] By using a solvent mixture system, about 34% of free fatty acids can be extracted using toluene/ethanol/methanol at ratio of 1:1:1.[152] Interestingly, Sun *at al.* also showed that

the extraction times can have a significant change in the relative abundance as reported that extracting using DCM for four hours gave a percentage of about 15% and with 6 hours, there was a reduced percentage to less than 12%.[111] Figure 2.45 shows the percentage of free fatty acids in the solvent extracts (normalised to 100%). DMC was shown to be most selective and diethyl ether as the least selective. The same linear regression with various solvent parameters was carried out as previously but no trends were identified. It has been previously reported that free fatty acids from C_{10} to C_{28} can be extracted from wheat straw. As the extracts were analysed without further fractionation, it will be difficult to identify and quantify any components in low concentrations. A further fractionation step prior to analysis and a better separation of fatty acids in the GC method would improve on the identification and quantification which might enable a correlation between free fatty acids and solvent parameters.

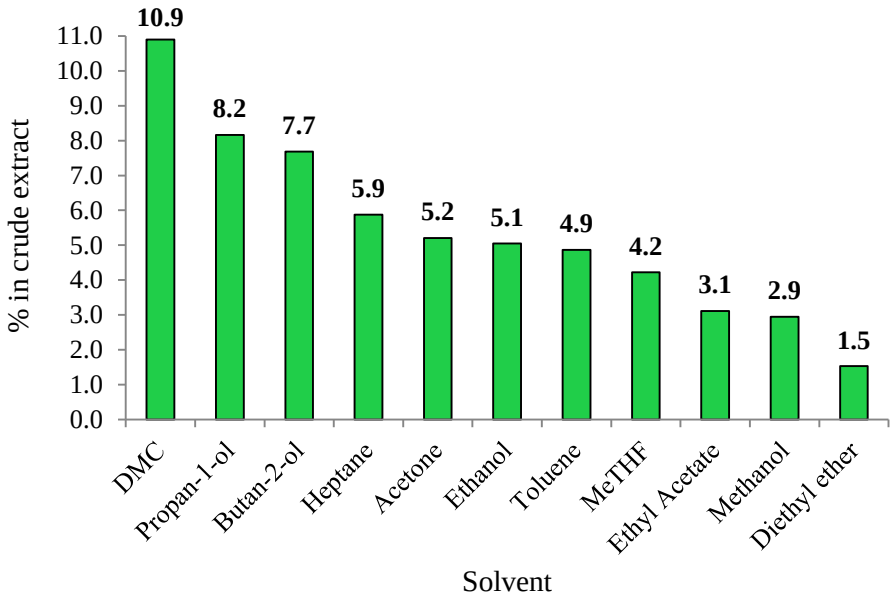


Figure 2.45: Percentage of total fatty acids in solvent crude extracts (originally in colour)

In Figure 2.46, hexadecanoic acid ($C_{16:0}$) is the most abundant free fatty acid found in all solvent extracts which can be presented up to about 5.3% in the propan-1-ol extract. Octadecenoic acid ($C_{18:1}$) is shown to be the second most predominant free fatty acid up to 3.2% in DMC extract. This is in agreement with results reported by Deswarte over 70% of the total free fatty acids present were hexadecanoic acid, octadecenoic acid and octadecanoate acid.[16] Interestingly, no octadecadienoic acid

was reported which is in contradiction to data shown in Figure 2.46.[16] Sun *et al.* reported that the most abundant free fatty acids were tetradecanoic acid and pentadecanoic acid in wheat straw.[111, 152] This is unexpected as odd-numbered free fatty acids are usually found in low concentrations as a C_2 moiety is added during the free fatty acids biosynthetic pathway.[82]

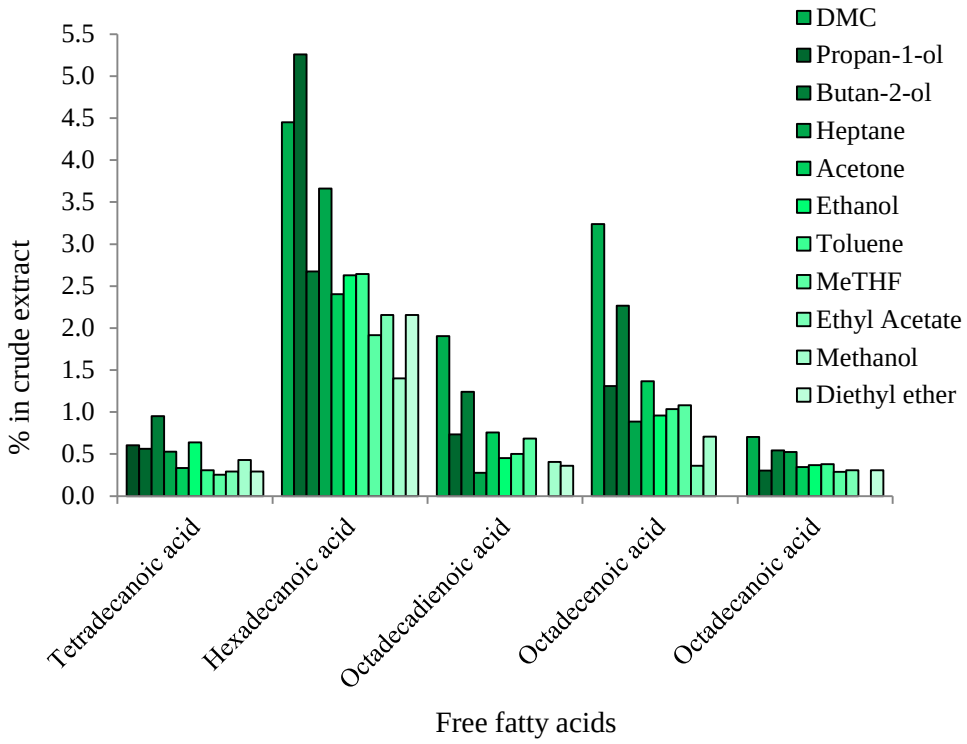


Figure 2.46: Percentage of individual free fatty acids in solvent crude extracts (originally in colour)

2.5.3 Hydrocarbons

The hydrocarbons identified were all exclusively *n*-alkanes with odd-numbered carbon chains between C_{27} to C_{33}. Hentriacontane (C_{31}) sometimes co-elutes with octacosanol (C_{28}) using the GC method described in Section 6.3.1 due to the similarity in the retention index. Figure 2.47 shows the percentage of total alkanes in the extracts but the bars highlighted in patterns are a combination of both hentriacontane (C_{31}) and octacosanol (C_{28}). The alkane fraction in wheat straw can be separated using column chromatography as demonstrated by Deswarte.[16] For the purpose of this work, this was not necessary as further greener extraction and fractionation techniques are

explored. In the solvent extracts, over 4% of *n*-alkanes only can be found in the wheat straw. The percentage of *n*-alkanes appeared to be very similar in many different solvents with varied solvent parameters. This could be an indication that the maximum amount of *n*-alkanes had been extracted.

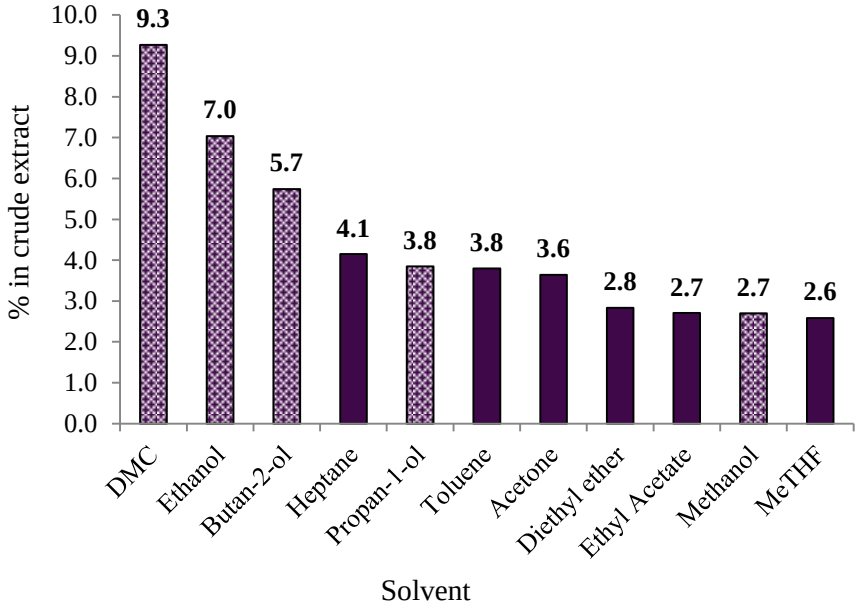


Figure 2.47: Percentage of total *n*-alkanes in solvent crude extracts (originally in colour)

As shown in Figure 2.48, nonacosane (C_{29}) and hentriacontane (C_{31}) were the two most abundant *n*-alkanes found in wheat straw wax which has been previously reported.[16] These valuable *n*-alkanes are not always recovered by solvent extractions; no *n*-alkanes were identified in the wheat straw by Sun *et al*. The level of all the solvent extracts were found to be very similar with the exception of DMC where it appears that a high proportion of all four *n*-alkanes were extracted. It would be interesting to carry out more work using DMC to see how the solvent-solute interactions were formed. No mathematical modelling to correlate solvent properties with the recovery of *n*-alkanes was carried out as there is a co-elution of peaks which means the total *n*-alkanes were not calculated.

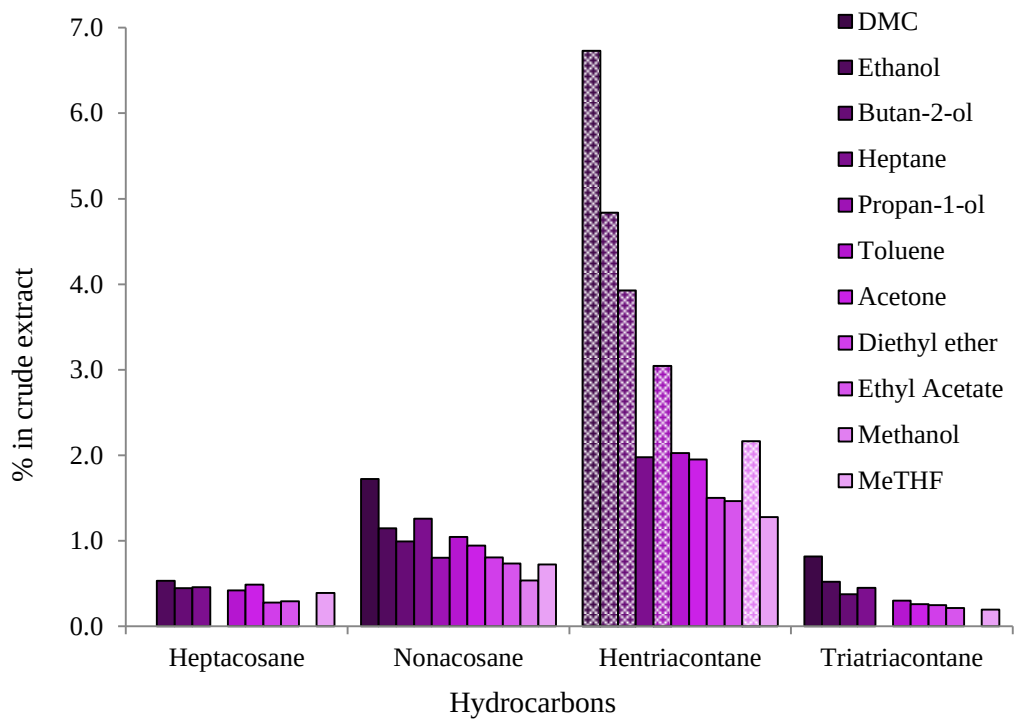


Figure 2.48: Percentage of individual *n*-alkanes in solvent crude extracts (originally in colour)

2.5.4 Free fatty alcohols

Only octacosanol (C_{28}) was identified and as mentioned previously. Octacosanol (C_{28}) co-eluted with hentriacontane (C_{31}) in some cases which made the quantification more difficult. It would be beneficial to separate the fatty alcohol from the other wax components or derivitise it for more accurate quantification but the results presented in Figure 2.49 gives an indication of the quantity of the octacosanol in the extracts. Same as Figure 2.48, the patterned bars indicate the co-elution of two compounds. More than 5% of pure octacosanol can be found in the butan-2-ol extract. Policosanols of C_{24} to C_{32} have been reported in wheat straw after separation by column chromatography with octacosanol being the most abundant fatty alcohol.[16] As policosanol is a very high value product, the content has been well studied. Dunford *et al.* studied the effect of policosanol content in different wheat varieties and under different growing environment.[180, 209] It was shown that policosanols of chain length

C_{20} to C_{36} can be found and the most predominant were tetracosanol (C_{24}), hexacosanol (C_{26}) and octacosanol (C_{28}).

A similar trend can be observed with the fatty alcohol and the *n*-alkanes as DMC, ethanol, propan-1-ol and heptane extract the highest percentage of compounds in the crude extracts. Again, no mathematical modelling to correlate solvent properties with the recovery of fatty alcohol was carried out as there is a co-elution of octacosanol (C_{28}).

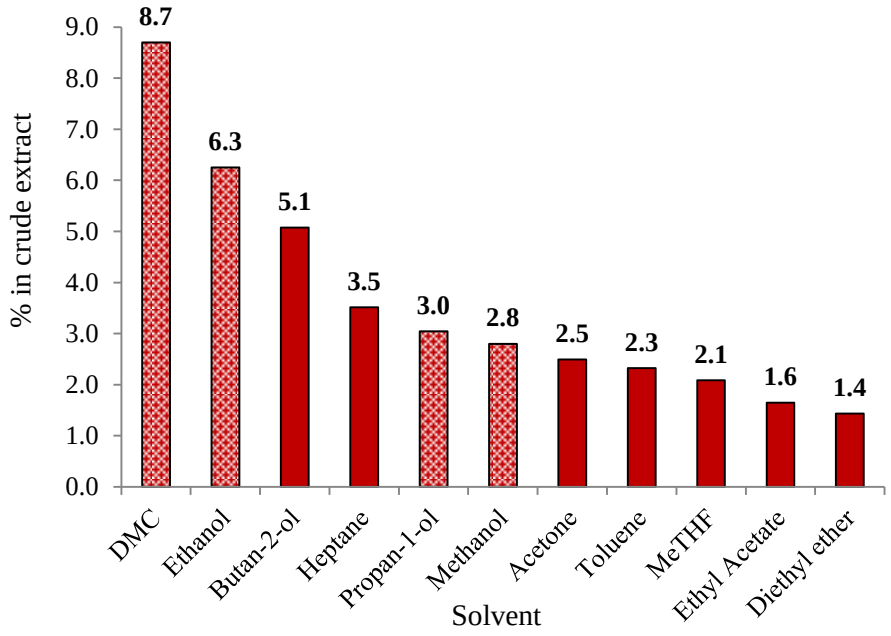


Figure 2.49: Percentage of octacosanol (C_{28}) in solvent crude extracts (originally in colour)

2.5.5 Aldehydes

Octacosanal (C_{28}) was the only aldehyde found in the wax extracts and this aldehyde was a minor component in wheat straw wax. As shown in Figure 2.50, less than 1% of the extracts are octacosanal except in the DMC extract. Surprisingly, DMC showed an exceptional solvent power for octacosanal in which 5.7% of the extract was octacosanal. It would be interesting to further investigate the DMC-octacosanal interactions in details. Sun *et al.* reported that aldehydes are completely absent and Deswarte identified the presence of octacosanal but did not carry out any quantification work so this is the first time in which aldehyde was quantified in the

wheat straw extracts.[16, 87, 152] Due to the very low concentration of octacosanal in the extracts, no mathematical modelling to correlate solvent properties was carried out.

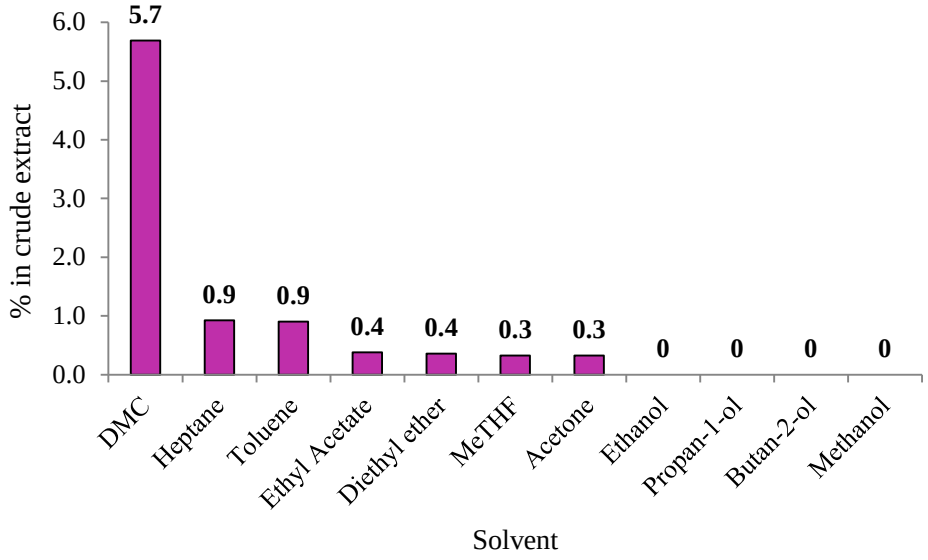


Figure 2.50: Percentage of octacosanal (C_{28}) in solvent crude extracts (originally in colour)

2.5.6 Wax esters

Wax esters are one of the most valuable groups of compounds in the wheat straw extracts. A range of 2.8 – 13% of the extracts was found to be wax esters depending on the organic solvents used as shown in Figure 2.51.

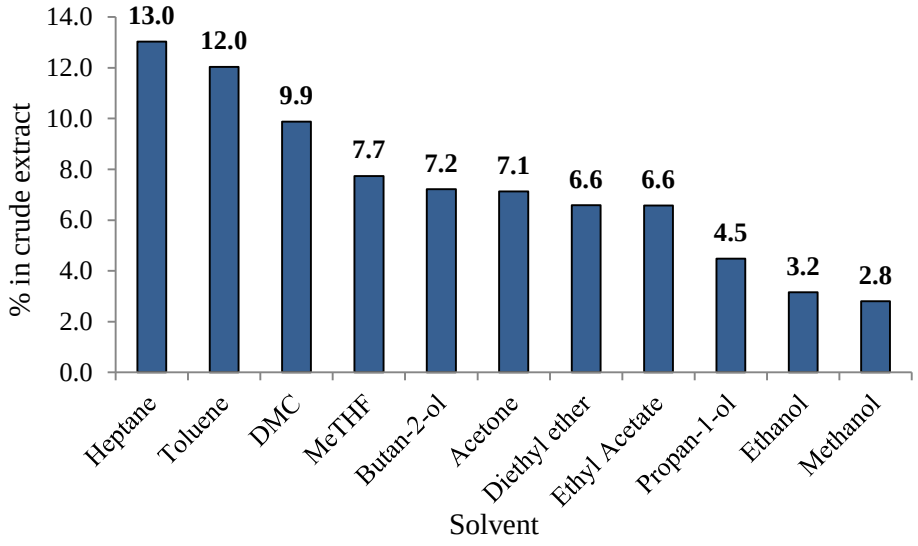


Figure 2.51: Percentage of wax esters in solvent crude extracts (originally in colour)

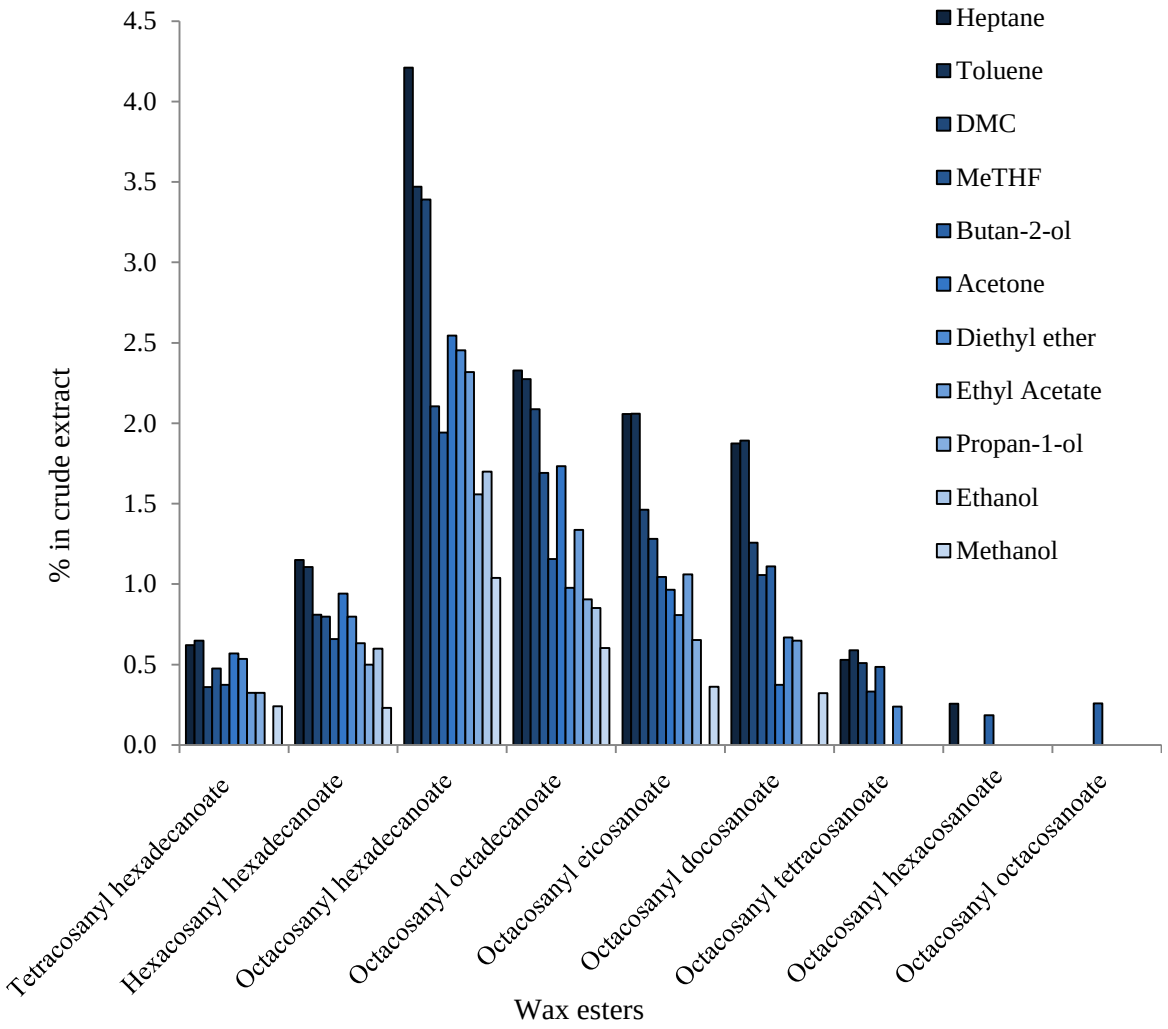


Figure 2.52: Percentage of wax esters in solvent crude extracts (originally in colour)

Figure 2.52 shows the breakdown of the percentage of individual wax ester in the extracts. Octacosanyl hexadecanoate (C_{28}:C_{16}) is the most predominant wax ester and represents up to 4.2% of the whole extract. The distribution of the wax esters were consistent with results reported by Deswarte where octacosanyl hexadecanoate was the most predominant then followed by octacosanyl octadecanoate. It was noted that the two highest percentages of wax esters in the extracts derived from the free fatty acids (hexadecanoic and octadecanoic acid) and fatty alcohol (octacosanol) identified previously. Octacosanyl octadecanoate (C_{28}:C_{18}), octacosanyl eicosanoate (C_{28}:C_{20}) and octasanyl docosanoate (C_{28}:C_{22}) are similar which suggests that high levels of octacosanol (C_{28}) are synthesised by the plant. Hexadecanyl hexadecanoate (C_{16}:C_{16}),

octadecenyl hexadecanoate ($C_{18:1}$:C_{16}) and octadecenyl octadecenoate ($C_{18:1}$:$C_{18:1}$) were the three wax esters previously identified in wheat straw by Sun *et al.* which is in contradiction to the results shown in Figure 2.52. About 2 – 14% of wax esters were identified in the extracts with both petroleum ether and hexane extracting up to 14%.[111, 152] Fractionation of wax esters might be necessary to ensure the shorter wax esters have been recovered and there is no co-elution with other compounds. Deswarte fractionated the extracts and the shortest chain length identified was C_{40}.[16] It would be interesting to quantify the odd-numbered wax esters and compare the distribution, though they represent at such low level, the focus had been based solely on even-numbered wax esters.

LSER have been proposed to correlate the properties of solvents in order to understand the solvent-wax ester interactions. Different physical and chemical properties of the solvents were included into the expression as described in Section 2.5. Again, DMC was the chosen solvent for any validation of the results produced. Strong correlation with two important parameters V_m and Kamlet-Taft parameter α (R^2= 0.903) was developed giving a new normalised LSER equation shown as Equation 2.15 for wax esters presented in wheat straw.

$$\ln\left(\frac{\%\ Wax\ ester}{100 - \%\ Wax\ ester}\right) \times \frac{T_{Expt}}{T_{Ref}} = -3.37 - 0.745\alpha + 0.00798V_m$$

Equation 2.15: New normalised LSER equation with percentage wax ester

The equation was used to predict the percentage of wax esters in DMC. The model has proved to be accurate as the experimental percentage of wax esters was 9.88% and the predicted value from Equation 2.15 was 8.88% which results in only 10% error. Kamlet-Taft α parameter describes the hydrogen bond donating ability of the solvent and is highly significant when extracting wax esters from wheat straw. The residual errors were calculated using Equation 2.7 and the plot is shown in Figure 2.54 with the data evenly distributed both positively and negatively meaning that there is no bias over or under estimation.

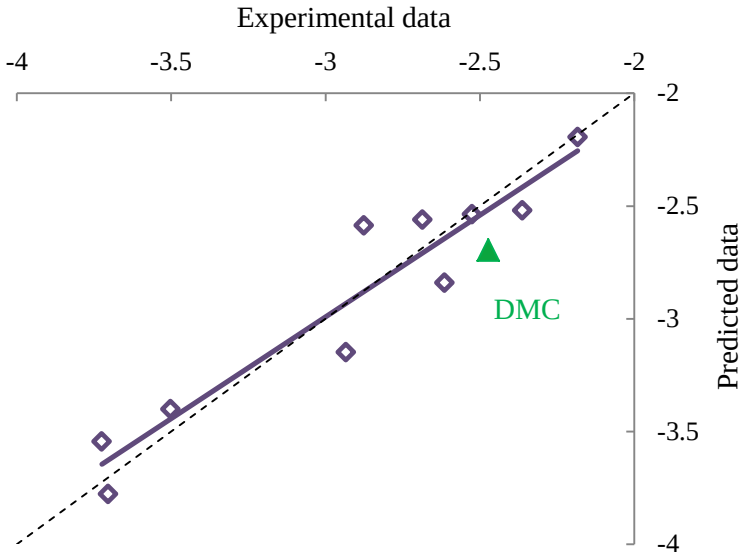


Figure 2.53: A comparison of experimental and predicted percentage wax esters in crude extracts (originally in colour)

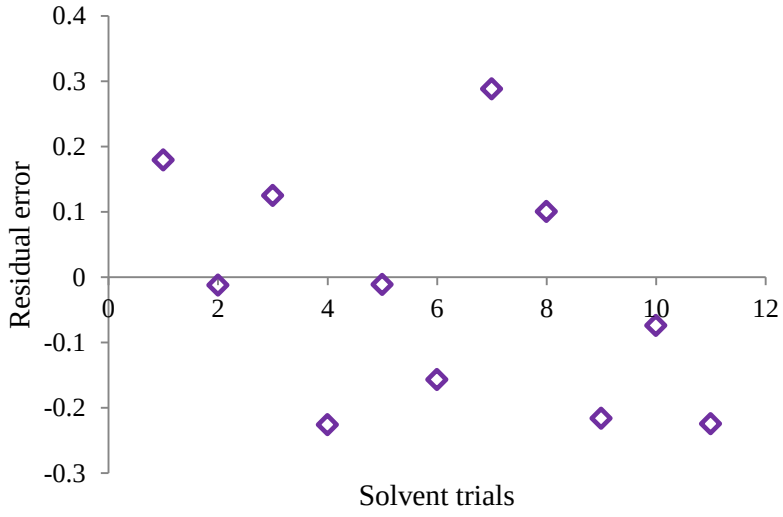


Figure 2.54: Residual error plot for experimental and predicted percentage wax esters in crude extracts (originally in colour)

2.5.7 Sterols

A range of 2.6 – 10.6% of sterols were found in the solvent extracts and interestingly, DMC can extract the highest level of sterols as shown in Figure 2.55. Figure 2.56 shows the breakdown percentage of the individual sterols. Sun *et al.* reported that approximately 3 – 25% of total sterols were found in the extracts with both petroleum

ether and hexane extracts. [111, 152] This is higher than the results shown in Figure 2.56 but no cholesterol, stigmastanol and ergostanol were identified in the extracts which might explain the lower sterol contents. The most abundant sterol in the extracts is identified to be β-sitosterol. This is usually the case in higher plants as β-sitosterol is synthesised first amongst all the other sterols in the biosynthetic pathway.[82] This is in good agreement with data produced by Deswarte as it was noted that more than half of the total sterol content was β-sitosterol.[16] It is important to note that campesterol, stigmasterol, β-sitosterol and Δ^4-sitosten-3-one were the main sterols identified. LSER was also carried out on this important group but no positive correlation was deduced between the sterol levels and the properties of solvent.

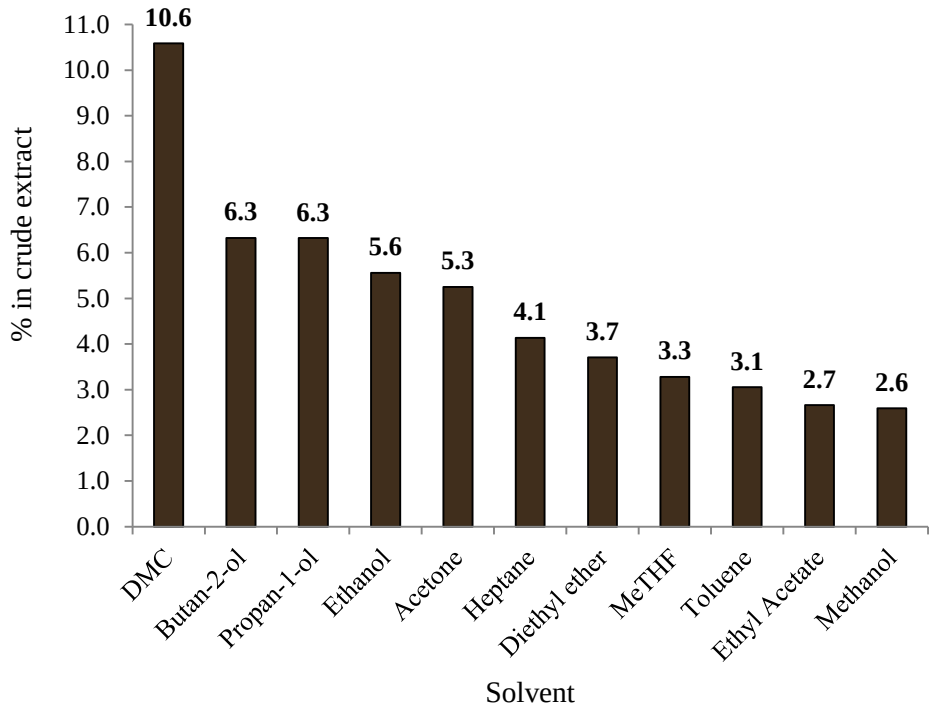


Figure 2.55: Percentage of sterols in solvent crude extracts (originally in colour)

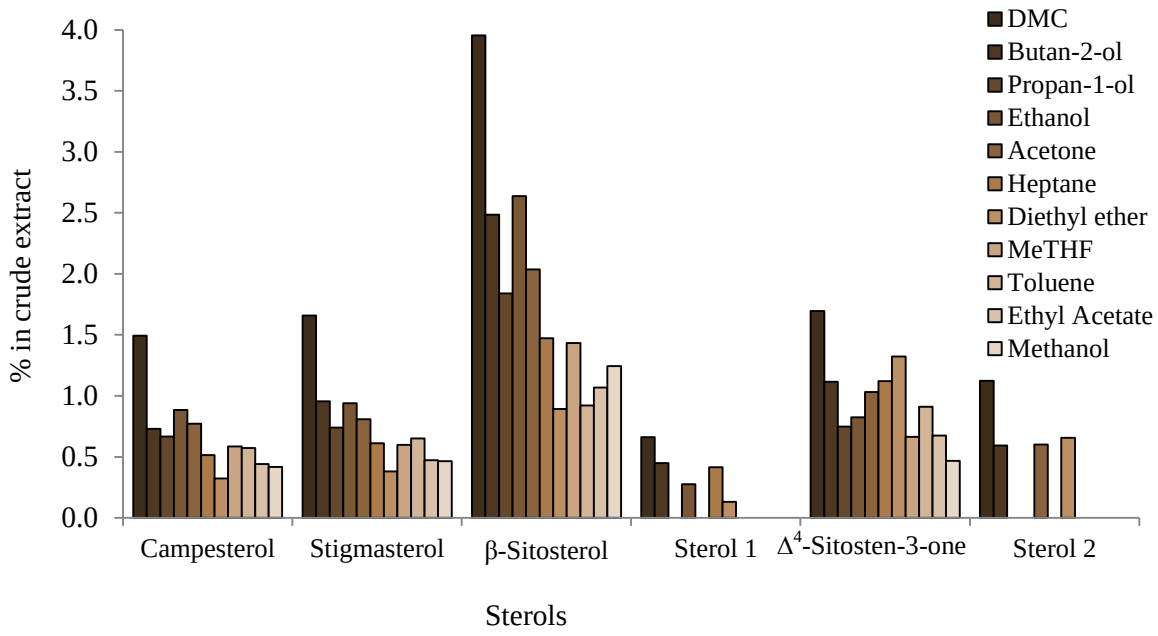


Figure 2.56: Percentage of individual sterols and its derivatives in solvent crude extracts (originally in colour)

2.5.8 β-Diketones

The level of β-diketones detected in the extracts varied depending in the organic solvent and a range of as low as 1.5% and as high as almost 9% was found as shown in Figure 2.57. β-Diketones are common in cereal crops and has been quantified in wheat but this is the first time that β-diketones have been quantified in wheat straw.

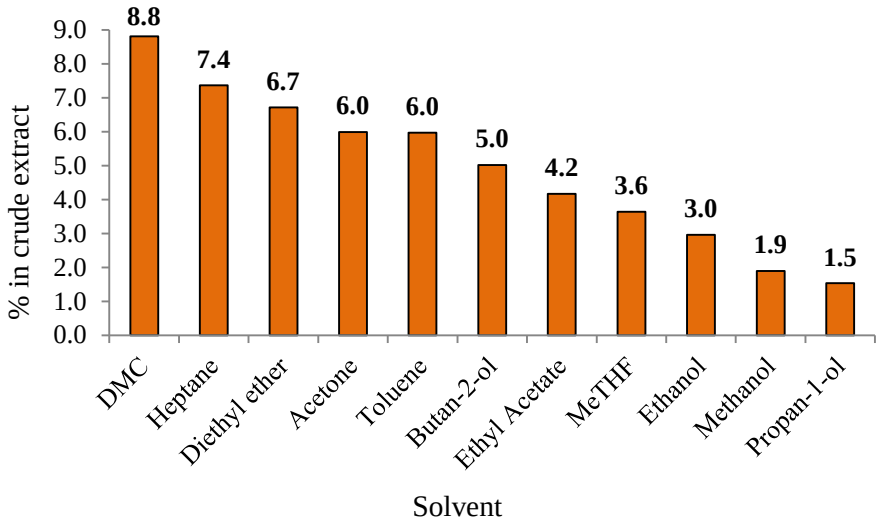


Figure 2.57: Percentage of β-diketones in solvent crude extracts (originally in colour)

Two main β-diketones have been identified in wheat straw (Figure 2.58) which is in agreement with the literatures on wheat and that 14,16- hentriacontanedione is the sole β-diketone.[16, 91, 98, 99] Almost 8% of the DMC extract was only 14,16-hentriacontanedione which makes it a very significant component in the wheat straw wax. 16,18-Triatriacontanedione is only present at very low level and under some solvents, there is a complete absence of the compound. Despite that β-diketones is a common group of compound found in Gramineae, there have been reports that no β-diketones were found in wheat straw.[111, 152] LSER was carried out with the β-diketone groups but no positive correlation was found between solvent properties and percentage of β-diketones extracted.

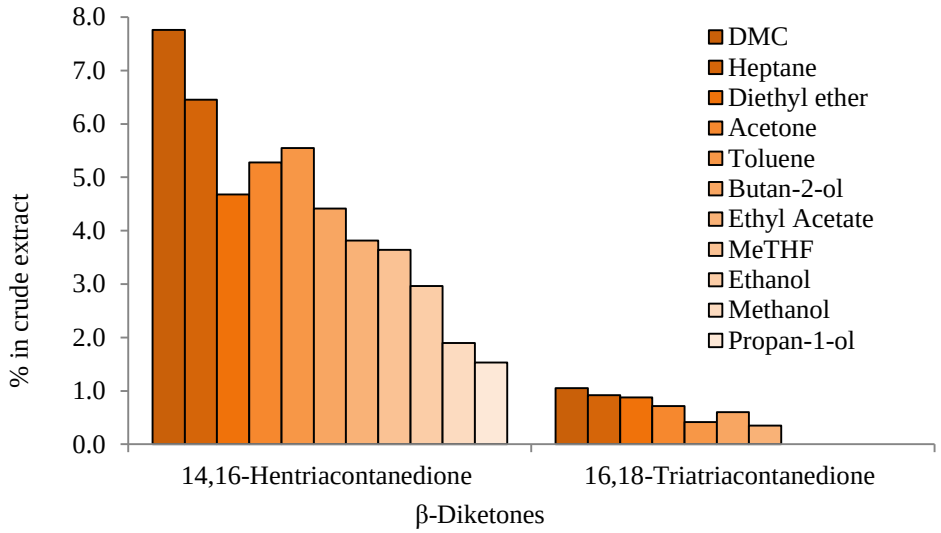


Figure 2.58: Percentage of individual β-diketones in solvent crude extracts (originally in colour)

2.5.9 Summary of wax components quantified in organic solvent extracts

The lipophilic fraction of the wheat straw wax from different organic solvent extracts have similar composition so a quantitative analysis of the different wax groups was carried out. The quantified components were grouped and plotted into a chart against the percentage yield as shown in Figure 2.59. The result showed a deeper insight into the chemical composition of the extracts and the lipophilic components are only a minor portion of the extracts. Only a range of 14 – 57% of the extracts were identified

and quantified which shows that there is still a large proportion of the extracts that need further investigation. DMC has the highest proportion of waxy components to total extractives with the highest percentage of quantified components.

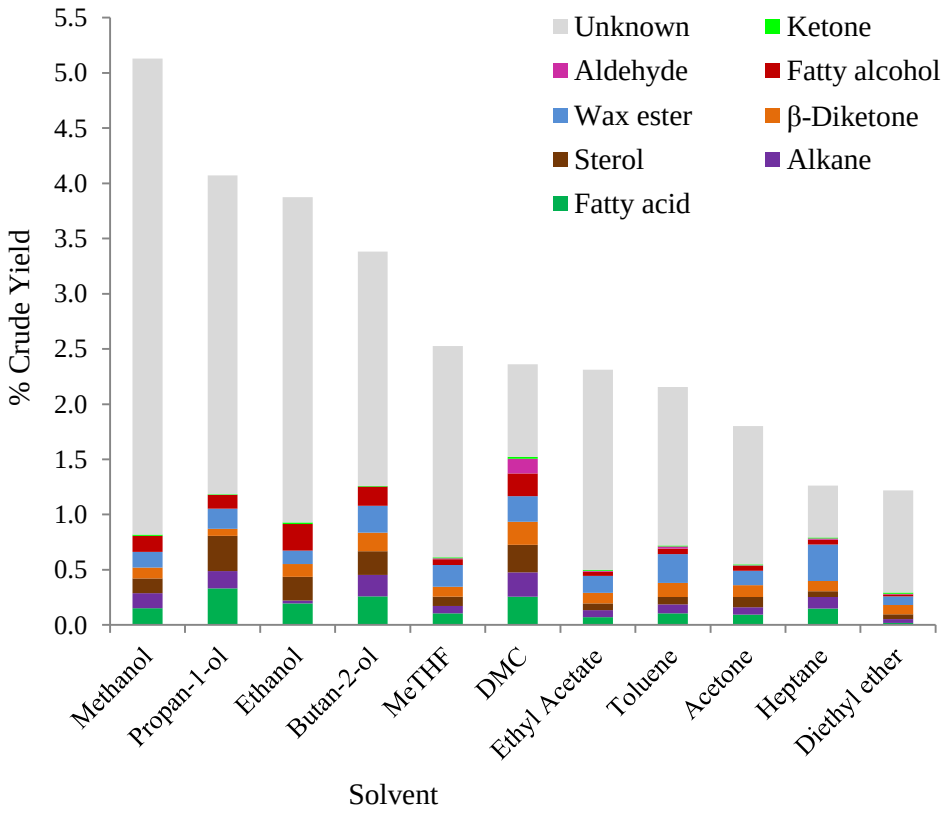


Figure 2.59: The lipophilic fraction of the wheat straw wax in various crude solvent extracts (originally in colour)

There are other minor wax components such as phenolic acids, resin acids and glycerides that have also been previously identified in wheat.[111, 210] Phenolic acids are commonly found in cereal crops especially grains and bran.[211] They are an important group of molecules found to have strong antioxidant activities against free radicals and reactive oxygen species which are found to be one of the main causes of a large number of chronic human diseases such as cancer and cardiovascular disease.[212, 213, 214] Syringic acid, vanillin, vanillic acid, *p*-coumaric acid and *p*-hydroxybenzoic acid have all been previously identified in wheat straw so it would be interesting to investigate the level of phenolic acids in these extracts and its anti-oxidant activity.[215] Resin acids

have also been reported in wheat straw with the most common being azelaic acid, which is frequently used in medical creams and polymers.[215, 216, 217] Glycerides have been reported in wheat straw and are mostly found as triglycerides but can occasionally exist as diglycerides.[111] FAME analysis was carried out using the method described in Section 6.3.4 (Figure 2.60) on the ethanol extract to check for the presence of glycerides in wheat straw. FAME of hexadecanoic, octadecanoic, octadecadienoic and octadecatrienoic acids were identified and can be confirmed that the FAME were the products from glycerides as the wax esters can still be observed. Figure 2.61a shows an EI spectrum of methyl octadecanoate comparing with Figure 2.61b which is an EI spectrum from the NIST library. However, the FAME only indicated the presence of glycerides and not the level of substitution of the glycerol (mono, di or triglycerides). In the ethanol extract, only up to 2.7% of glycerides in the extract can be found which shows that there are still a lot of unknown compounds in the extracts. Previously, dipalmitin, tripalmitin and dipalmitoyl-oleylglycerol were reported to be found in wheat straw.[111] Other compounds that could also be co-extracted are free sugars and pigments which are also not detectable by GC.

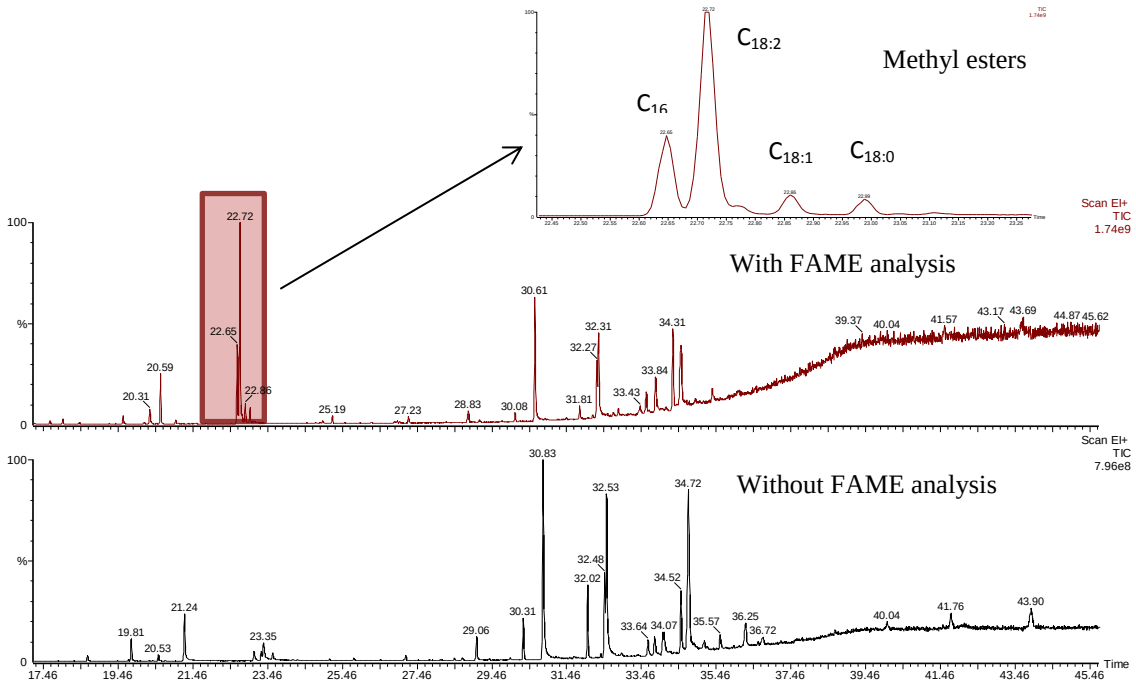


Figure 2.60: FAME analysis of wheat straw wax (originally in colour)

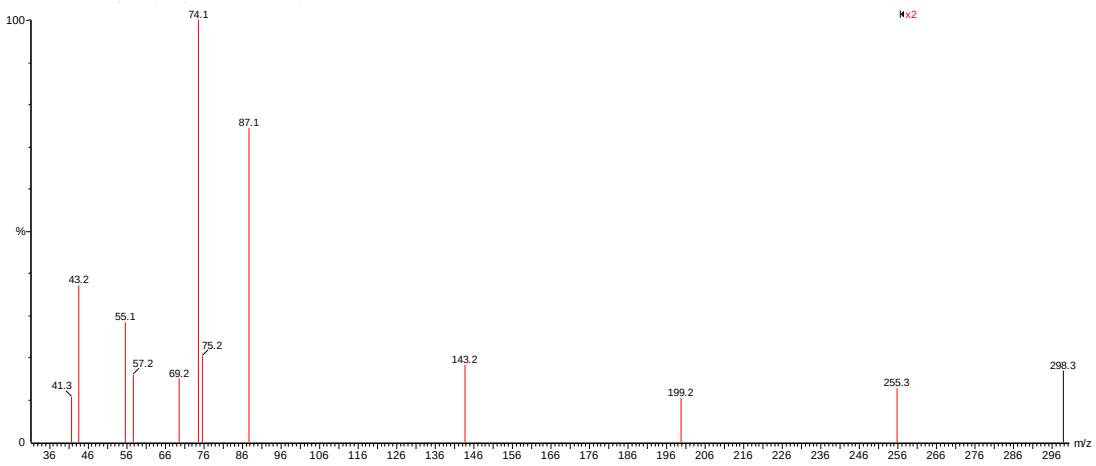


Figure 2.61a: EI spectrum of methyl octadecanoate (magnified two times from m/z 260–300) (originally in colour)

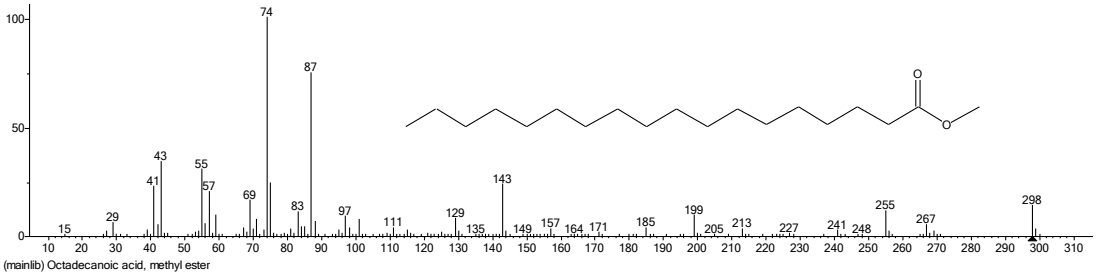


Figure 2.61b: EI spectrum of methyl octadecanoate from the NIST library

In summary, the quantified components were expressed in $\mu g.g^{-1}$ of wheat straw in Table 2.18. This gives a different dimension to the data as it shows the amount of extractives from the biomass. Alkanes, fatty alcohols, wax esters, sterols and β-diketones are the most valuable components amongst the complex extract so it is important to highlight the highest level that can be extracted from the various solvents. As the total amount of wax components in the biomass is unknown, this quantitative work can be used as a rough guideline on the level of compounds are presented for future extractions. This allowed the percentage solvent efficiency to be calculated as shown in Table 2.19 in which the data are all normalised to the highest quantity of the wax group being extracted. The percentage solvent efficiency values expressed in Table 2.19 are calculated using Equation 2.16. Table 2.19 shows that DMC is the most efficient solvent as it can extract above 70% of the wax components.

$$\%\ Solvent\ efficiency = \frac{Concentration\ of\ compounds\ in\ biomass\ (\mu g g^{-1})}{Highest\ concentration\ of\ compounds\ in\ biomass\ (\mu g g^{-1})} \times 100$$

Equation 2.16: Calculation for percentage solvent efficiency

Table 2.18: Quantification of wheat straw wax components in various solvent extracts

Identification	µgg^{-1} of wheat straw (n ≥ 2)										
	Methanol	Propan-1-ol	Ethanol	Butan-2-ol	MeTHF	DMC	Ethyl acetate	Toluene	Acetone	Heptane	Diethyl ether
Tetradecanoic acid	220 ± 23	229 ± 45	248 ± 44	322 ± 31	64 ± 6	142 ± 7	67 ± 14	66 ± 43	60 ± 23	134 ± 34	17 ± 7
Hexdecanoic acid	719 ± 13	2140 ± 11	1019 ± 20	904 ± 12	484 ± 48	1050 ± 12	498 ± 66	570 ± 50	433 ± 19	925 ± 54	129 ± 37
Octadecadienoic acid	209 ± 45	298 ± 90	175 ± 33	420 ± 23	173 ± 19	449 ± 11	*Tr*	108 ± 25	136 ± 37	70 ± 13	22 ± 2
Octadecenoic acid	363 ± 50	533 ± 78	372 ± 51	766 ± 46	274 ± 41	764 ± 23	83 ± 13	223 ± 13	246 ± 12	224 ± 27	*Tr*
Octadecanoic acid	*Tr*	124 ± 12	143 ± 16	184 ± 32	73 ± 28	166 ± 19	71 ± 23	82 ± 10	62 ± 29	133 ± 32	18 ± 7
Total free fatty acids	**1511** ± 131	**3324** ± 236	**1957** ± 164	**2596** ± 144	**1067** ± 142	**2572** ± 72	**719** ± 116	**1049** ± 141	**937** ± 120	**1485** ± 160	**186** ± 53
Heptacosane	*Tr*	*Tr*	206 ± 38	151± 12	98 ± 23	*Tr*	67 ± 22	91 ± 29	88 ± 43	116 ± 31	34 ± 4
Nonacosane	275 ± 33	327 ± 21	445 ± 34	336 ± 32	183 ± 54	407 ± 34	170 ± 59	226 ± 57	170 ± 34	318 ± 19	98 ± 12
Hentriacontane	1111 ± 98[a]	1239 ± 43[a]	1874 ± 112[a]	1327 ± 149	323 ± 18	1588 ± 80[a]	338 ± 47	437 ± 27	351 ± 57	500 ± 26	183± 10
Triatriacontane	*Tr*	*Tr*	202 ± 12	127 ± 40	49 ± 19	193 ± 50	50 ± 11	65 ± 13	47 ± 10	114 ± 32	30 ± 5
Total hydrocarbons	**1386** ± 50	**1567** ± 64	**2726** ± 196	**1940** ± 233	**653** ± 114	**2188** ± 164	**625** ± 139	**818** ± 126	**656** ± 144	**1048** ± 108	**345** ± 31
Octacosanol	1436 ± 42[a]	1239 ± 59[a]	2422 ± 71[a]	1716 ± 52	527 ± 57	2053 ± 30[a]	380 ± 21	501 ± 11	449 ± 43	444 ± 36	174 ± 24
Total fatty alcohol	**1436** ± 42	**1239** ± 59	**2422**± 71	**1716** ± 52	**527** ± 57	**2053** ± 30	**380** ± 21	**501** ± 11	**449** ± 43	**444** ± 36	**174** ± 24
Octacosanal	*Tr*	*Tr*	*Tr*	*Tr*	82 ± 5	1343 ± 59	87 ± 14	195 ± 27	59 ± 11	117 ± 13	43 ± 14
Total aldehyde	***Tr***	***Tr***	***Tr***	***Tr***	**82** ± 5	**1343** ± 59	**87** ± 14	**195** ± 27	**59** ± 11	**117** ± 13	**43** ± 14
Tetracosanyl hexadecanoate	123 ± 27	132 ± 34	*Tr*	127 ± 17	120 ± 20	85 ± 25	75 ± 5	140 ± 23	102 ± 10	157 ± 32	65 ± 10
Hexacosanyl hexadecanoate	119 ± 24	204 ± 11	232 ± 22	223 ± 23	201 ± 52	191 ± 67	146 ± 15	238 ± 12	169 ± 27	291 ± 19	97 ± 7

Table 2.18 (continued)

Octacosanyl hexdecanoate	533 ± 19	634 ± 25	659 ± 22	656 ± 45	532 ± 23	800 ± 14	536 ± 34	748 ± 24	458 ± 17	1064 ± 70	299 ± 22
Octacosanyl octadecanoate	309 ± 25	369 ± 15	330 ± 13	391 ± 16	427 ± 25	493 ± 15	309 ± 11	490 ± 12	312 ± 32	588 ± 11	119 ± 19
Octacosanyl eicosanoate	186 ± 18	265 ± 23	*Tr*	353 ± 25	324 ± 14	345 ± 23	245 ± 26	444 ± 37	174 ± 23	520 ± 23	98 ± 3
Octacosanyl docosanoate	166 ± 37	222 ± 12	*Tr*	375 ± 23	267 ± 19	297 ± 22	150 ± 45	408 ± 28	67 ± 12	473 ± 19	81 ± 15
Octacosanyl tetracosanoate	*Tr*	*Tr*	*Tr*	164 ± 17	84 ± 38	120 ± 5	59 ± 3	127 ± 15	Tr	134 ± 15	29 ± 2
Octacosanyl hexacosanoate	*Tr*	*Tr*	*Tr*	62 ± 2	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	65 ± 15	13 ± 0
Octacosanyl octacosanoate	*Tr*	*Tr*	*Tr*	87 ± 6	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*
Total wax esters	**1435** ± 150	**1825** ± 120	**1221** ± 57	**2439** ± 174	**1955** ± 191	**2331** ± 171	**1520** ± 189	**2595** ± 151	**1283** ± 121	**3291** ± 204	**802** ± 78
Campesterol	214 ± 23	271 ± 17	343 ± 17	246 ± 11	148 ± 58	352 ± 15	102 ± 9	123 ± 15	139 ± 36	65 ± 13	39 ± 2
Stigmasterol	238 ± 11	301 ± 29	364 ± 15	323 ± 5	151 ± 44	392 ± 24	109 ±13	140 ± 26	146 ± 34	77 ± 14	46 ± 5
β-Sitosterol	638 ± 24	749 ± 38	1022 ± 25	840 ± 28	362 ± 12	933 ± 33	247 ± 23	199 ± 36	367 ± 15	186 ± 38	109 ± 13
Sterol 1	*Tr*	*Tr*	106 ± 14	152 ± 16	*Tr*	156 ± 5	*Tr*	*Tr*	*Tr*	52 ± 4	16 ± 5
Δ^4-Sitosten-3-one	240 ± 14	305 ± 11	319 ± 12	377 ± 26	168 ± 22	400 ± 17	156 ± 23	196 ± 29	186 ± 29	142 ± 32	161 ± 23
Sterol 2	*Tr*	*Tr*	*Tr*	201 ± 22	*Tr*	265 ± 14	*Tr*	*Tr*	108 ± 34	*Tr*	80 ± 26
Total sterols	**1330** ± 58	**3192** ± 95	**2154** ± 83	**2138** ± 108	**829** ± 136	**2498** ± 108	**614** ± 68	**659** ± 106	**945** ± 148	**522** ± 101	**451** ± 74
14,16 hentriacontanedione	974 ± 45	624 ± 58	1148 ± 60	1491 ± 13	919 ± 45	1832 ± 87	882 ± 45	1196 ± 34	950 ± 14	814 ± 21	689 ± 14
16,18 triatriacontanedione	*Tr*	*Tr*	*Tr*	204 ± 21	*Tr*	248 ± 6	81 ± 29	90 ± 27	129 ± 23	116 ± 11	129 ± 13
Total β-diketones	**974** ± 45	**624** ± 58	**1148** ± 60	**1695** ± 34	**919** ± 45	**2080** ± 93	**963** ± 74	**1287** ± 61	**1079** ± 37	**930** ± 32	**818** ± 27
Total identified	**8072** ± 476	**11770** ± 632	**11627** ± 631	**12524** ± 745	**6032** ± 690	**15066** ± 697	**4910** ± 621	**7104** ± 623	**5408** ± 624	**7837** ± 654	**2821** ± 301

[a] = co-elution and values shown are the combined quantities, *Tr* = Trace levels < LOQ, LOQ shown in Section 2.5.1

Table 2.19: Normalised percentage solvent efficiency for different wax group

Solvent/ wax group	$\mu g g^{-1}$ of wheat straw	% Solvent efficiency										
		Methanol	Propan-1-ol	Ethanol	Butan-2-ol	MeTHF	DMC	Ethyl acetate	Toluene	Acetone	Heptane	Diethyl ether
Propan-1-ol/ **Free fatty acids**	3324	45	*100*	59	78	32	77	22	32	28	45	6
Ethanol/ **Hydrocarbons**	2726	51	57	*100*	71	24	80	23	30	24	38	13
Ethanol/ **Free fatty alcohols**	2422	59	51	*100*	71	22	85	16	21	19	18	7
DMC/ **Aldehydes**	1343	0	0	0	0	6	*100*	6	15	4	9	3
Heptane/ **Wax esters**	3291	44	55	37	74	59	71	46	79	39	*100*	24
Propan-1-ol/ **Sterols**	3192	42	*100*	67	67	26	78	19	21	30	16	14
DMC/ **β-Diketones**	2080	47	30	55	81	44	*100*	46	62	52	45	39

2.6 Conclusion and future work

Wheat straw (*Triticum aestivum*) has been highlighted as a low cost, high volume agricultural waste that needed added value applications. From the straw bio-refinery concept, an opportunity to extract of wheat straw for high value chemicals arises. In this chapter, the use of eleven different organic solvents (heptane, ethanol, diethyl ether, 2-methyl tetrahydrofuran, acetone, toluene, ethyl acetate, propanol, butan-2-ol, dimethyl carbonate and methanol) on wheat straw wax extraction was investigated. Linear solvation energy relationships were successfully developed to describe the relationship between properties of solvent and extraction crude and lipophilic yields. It was found that there is a strong correlation of both the percentage of crude and lipophilic yields with Kamlet-Taft parameter β (hydrogen bond accepting ability and molar volume. Two new normalised LSER equations were established for the prediction of percentage crude and lipophilic yields. This aids the understanding between the solvent-solute interactions which can be extremely useful during solvent selection. Ethyl acetate showed the best selectivity for wax components, closely followed by heptane.

A qualitative analysis by HTGC-MS with the assistance of KI and NIST Library 2008 demonstrated that organic solvents had almost no effect on the chemical composition, which means that the important point to note is that various different solvents extract different quantities of each wax components as there is a large variation with the yields. The lipophilic fraction of the wheat straw wax composed mainly of free fatty acids, free fatty alcohols, aldehydes, wax esters, alkanes, β-diketones and sterols. The main components were identified successfully but fractionation of the extracts would enable compounds of lower concentrations to be identified. Phytosterols and policosanols have been highlighted as potential sources for dietary supplements or food additives that can reduce blood cholesterol levels. Alkanes have been shown to have insect repellent properties which are interesting as an additive in insecticides. Wax esters, fatty alcohols, sterols and alkanes are components commonly identified in commercial waxes for many applications in the personal care and cosmetics industry. Wax esters and alkanes are highly hydrophobic which have the potential to be used in wax polishes and coatings. Beta-diketones are an interesting group of molecules that can be used as metal chelators. The above components identified in wheat straw wax have tremendous commercial potential as a UK-sourced alternative or an addition to the continuous growing existing wax market. In order to push this to a commercial

success, physical properties of this new product must be determined to understand the actual market potential and how it can be compared with the existing commercial waxes.

A quantitative analysis of the different wax groups is paramount due to similarities in chemical composition. A comprehensive quantitative analysis was carried out for all the various solvent extracts. LSER was also carried out on some key wax groups but unfortunately no correlation was established between any of the wax groups except the wax esters. This is the first time that a relationship with properties of solvents and specific groups of compounds was successfully established. The newly developed LSER equation specifically for wax esters within a complex mixture is important as it showed that there is a potential in defining the relationship of organic solvents and wax groups. This will prove to be extremely useful in solvent selection for higher selective extractions. It is difficult to understand the solvent-solute interactions as natural products are typically very complex. Fractionation of wax groups and fine tuning of GC methods to the specific groups would give a more accurate quantification analysis which may assist the establishment of any further mathematical modelling.

As with all extraction from a plant material, the total extractives in the biomass originally are unknown but from the solvent study, a deeper understanding of the "potential" quantities of each key wax components was achieved. With this new information, the efficiency of the solvent for the extraction of specific wax groups can be reviewed. It was concluded that DMC is the solvent with the highest efficiency for all the wax groups as it can extract over 70% of the "potential" quantities presented on the wheat straw.

As wheat straw has shown such great potential biomass for wax products, it would be interesting to investigate other possible biomass for natural wax sources. This will give opportunities for other biomasses as well as locating the "best" biomass for extraction by comparison of extraction yields, chemical compositions and yields for specific wax groups. Sourcing wax products from a number of renewable sources can be advantageous when wax blending needs to be applied to achieve various physical properties for different applications. For these reasons, different potential biomasses would be screened for waxes, which is discussed in Chapter 3.

It has been noted that this high value wax product can be produced from a natural, readily available renewable resource but due to tighter legislations and consumers' demands, there is a big drive towards "greener" products. The levels of residual solvent in natural extracts as well as solvent emissions into the atmosphere are of more concern so it has become increasingly restricted over the past few years. This is a great opportunity for both environmental reasons and new marketing opportunity to establish a 100% natural wax product. Alternative environmentally friendly extraction techniques such as supercritical carbon dioxide would not only leave the wax product completely solvent- free but is also a by-product from industrial processes. According to the principles of green chemistry, existing renewable resources should be utilised where it can, so this is an area of research of high interest in this process. Due to all these reasons, this alternative extraction technique is investigated in Chapter 4. With this preliminary organic solvent extraction work, the green extraction technique can be compared so conclusions can be made on whether the use of carbon dioxide is feasible.

Chapter 3

Supercritical carbon dioxide extraction of wheat straw waxes

A.J. Hunt, E.H.K. Sin, R. Marriott and J.H. Clark, *ChemSusChem.* , 2010, **3**, 306-322

V.L. Budarin, P.S. Shuttleworth, J.R. Dodson, A.J. Hunt, B. Lanigan, R. Marriott, K.J. Milkowski, A.J. Wilson, S.W. Breeden, J. Fan, E.H.K. Sin and J.H. Clark, *Energy Environ. Sci.*, 2011, **4**, 471-479

Oral presentation given at 1st NORthern Sustainable Chemistry meeting (NORSC), York, UK, October 2010

Interview for BBC World Services "Can Chemistry Save the World", March 2011

Oral presentation given at 7th International Conference on Renewable Resources and Biorefineries (RRB7), Bruges, Belgium, June 2011

Poster given at 11th International Conference on Carbon Dioxide Utilisation (ICCDU XI), Dijon, France, June 2011

Book chapter titled "Supercritical CO_2 as an environmentally benign medium for biorefinery" for book called "The role of green chemistry in biomass processing and conversion" (authored by R. Marriott and E.H.K. Sin, edited by Haibo Xie and Nick Gathergood) in John Wiley & Sons. Inc.

3. SUPERCRITICAL CARBON DIOXIDE EXTRACTION OF WHEAT STRAW WAX

3.1 Introduction

Components in straw wax have been successfully identified and quantified by organic solvent extraction and preliminary results showe that some of the wax compounds has real commercial potential to replace some existing waxes or as a green alternative wax for many applications such as flavourings, cosmetics, food supplements, coatings etc.[181, 218, 219, 220] Many of these applications are strictly regulated, for example legislation is already in place restricting extraction solvent usage and residues in cosmetics, flavour and fragrances.[221, 222, 223, 224, 225, 226] Lipids are traditionally extracted using an organic solvent such as hexane but with growing environmental concerns over organic solvents, extensive research has been taking place into using greener alternative solvents.[1, 157] Hexane has been recognised as a hazardous air pollutant by the US EPA and the EPA Toxic Release Inventory (TRI) indicated that more than 20,000 tonnes of hexane are released into the atmosphere from extraction.[129, 227] As well as the warnings issued by various government bodies, hexane is also reported to be neurotoxic and can potentially greatly affect the central nervous system.[228, 229] One option is to use CO_2 as a greener replacement solvent for hexane.[230, 231]

With the amount of CO_2 generated and emitted into the atmosphere continuing to rise, it is apparent that a major effort is needed across the world in order to reduce atmospheric CO_2 levels including carbon dioxide capture and storage.[232] Reductions in energy use and an increase in the use of carbon neutral biofuels are already in place in order to tackle global warming.[233, 234] However, despite the negative association of CO_2, this can be a valuable renewable resource for use in many industrial processes.[235] Carbon dioxide is usually obtained as a recovered by-product from industrial processes such as fermentation, ammonia production and the generation of hydrogen.[235] The production of bioethanol in particular generates significant quantities of CO_2 and utilisation of this should be considered in preference to capture and storage in oceans or terrestrial sinks.[232] This is especially the case when CO_2 is used as a reactant as it would be omitted and converted to other products. CO_2 generated as a

by-product is relatively pure and can be easily dried, re-compressed and stored before being used to extract valuable chemicals or used as a reaction solvent.[236]

Due to tighter legislation on solvent usage, the use of supercritical CO_2 is slowly being implemented to replace many industrial applications due to its many advantages compared with organic liquid solvents.[221, 222, 223, 224, 225, 237] It is also very well-known for its environmental benefits e.g.: it does not contribute to smog, cause ozone depletion, create acute eco-toxicity and most importantly it generates no liquid wastes.[238, 239, 240, 241] It also fulfils many health and safety issues such as being non-carcinogenic, non- toxic, non-mutagenic and non-flammable. Carbon dioxide is commonly used as a SCF due to its low critical point of 304 K and 7.3 MPa. [238, 239, 240, 241] The properties of SCFs have been compared with both gases and liquids; they are often described as the intermediate between these two states as shown in Table 3.1. Due to these physical properties, SCF is a defined state that is neither a gas nor liquid. Above the critical point, a substance is at a higher temperature and pressure range than a gas and liquid in equilibrium so giving this special state as shown in Figure 3.1. [238, 239, 240, 241]

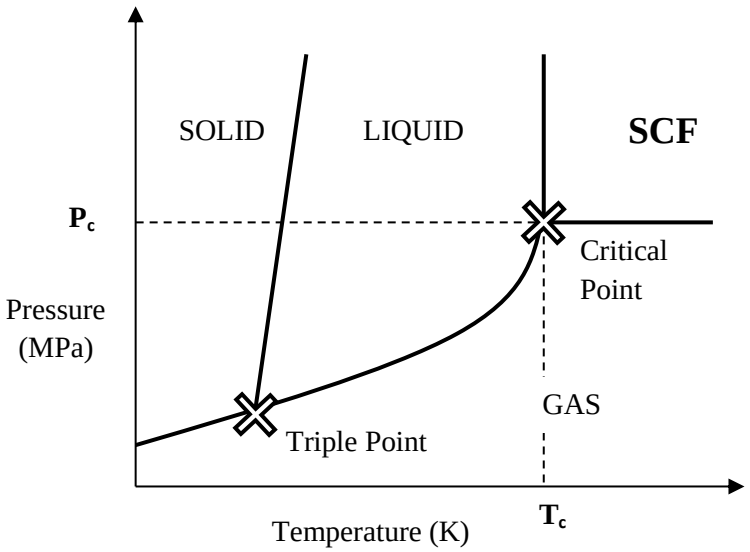


Figure 3.1: Phase Diagram for supercritical fluid

Table 3.1: Physical properties of gaseous, supercritical and liquid CO_2

State	Density (gL^{-1})	Viscosity (Pa.s)	Diffusivity (m^2s^{-1})
Gaseous	1	10^{-5}	10^{-4}
SCF	100 - 900	10^{-4}	10^{-6}
Liquid	1000	10^{-3}	10^{-8} - 10^{-9}

As well as its low critical point, it is also non-flammable, non-toxic and renewable which makes supercritical CO_2 the most common SCF used. The critical point for each substance has its own specific critical pressure (P_c) and temperature (T_c). [238, 239, 240, 241] Supercritical fluids have many unique and unusual properties which make them of interest to many chemists and industries. [238, 239, 240, 241] The most crucial properties of a SCF are its density, viscosity, diffusivity and heat capacity. The physical properties of supercritical CO_2 and liquid CO_2 are highlighted in Table 3.2 where they are compared with common organic solvents. High densities generally means enhanced solubilisation of compounds and low viscosities mean greater penetration into solids and easier flow with minimal friction. [238, 239, 240, 241] The properties can be manipulated by temperature and pressure and it has been reported that $scCO_2$ is a good solvent for the extraction of lipids and gives a very high recovery. [238, 239, 240, 241] Under $scCO_2$ conditions, the temperature of extraction tends to be lower than of organic solvent extraction which minimises thermal destruction of the raw materials. As the extraction is carried out under pressure, the system can be depressurised after completion, leaving both the raw materials and extracts completely solvent-free.

Table 3.2: Physical properties of CO_2 compared with organic solvents

Solvent	Density (gL^{-1})	Viscosity (Pa.s)	Heat capacity ($kJkg^{-1}K^{-1}$)	Reichardt's polarity scale E_T^N
DCM	1326	4.06×10^{-4}	1.19	0.309
Hexane	655	2.95×10^{-4}	2.27	0.009
Water	1000	8.94×10^{-4}	4.18	1
Ethanol	789	1.074×10^{-3}	2.44	0.654
Ethyl acetate	894	4.31×10^{-4}	1.9	0.228
$scCO_2$	956 (at 313 K and 40 MPa)	1.06×10^{-4} (at 313 K)	0.846 (at 313 K)	0.09 (variable)
lCO_2	1000 (298 K and 40 MPa)	1.2×10^{-4} (at 298 K)	3.14 (at 283 K)	0.09 (variable)

Processes using CO_2 do not add to CO_2 emissions and CO_2 in the atmosphere but they create an opportunity to reuse the by-product CO_2 from other industrial processes. According to the principles of green chemistry, the use of CO_2 as the extraction solvent for wheat straw wax has double benefits as not only can the extraction add value to the low cost, high volume agricultural waste, it also utilises an industrial by-product so avoiding unnecessary extra production of organic solvents.

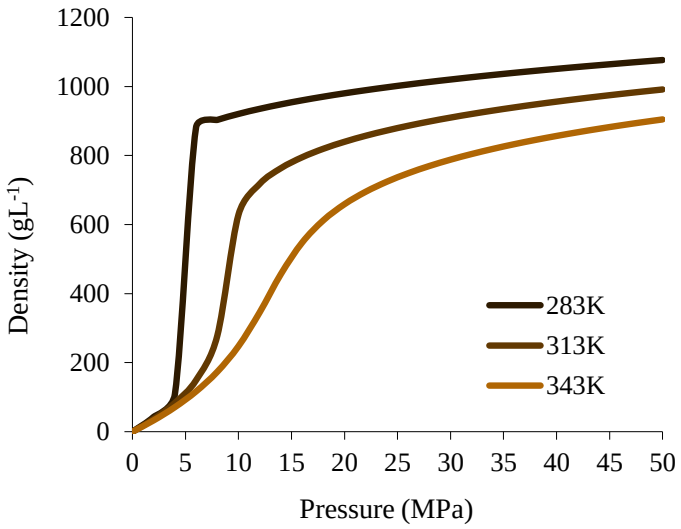


Figure 3.2: Density of CO_2 with change in temperature and pressure (originally in colour)

The solvent properties of $scCO_2$ is "tuneable" and this can be achieved by the adjustment of temperature and pressure as shown in Figure 3.2.[241] These two parameters directly influence the density which affects the solvating power of CO_2. Carbon dioxide is a very non-polar molecule so as expected $scCO_2$ is a very non-polar hydrophobic solvent which means the selectivity for non-polar molecules is high. Small or non-polar molecules such as alkanes are highly soluble whereas large or polar molecules like fatty acids are less soluble. The molecules that would be soluble in $scCO_2$ can be predicted as mentioned previously since the polarity is comparable with hexane. Due to its low polarity, a co-solvent such as ethanol can be added to the extraction in order to increase the overall polarity of the extraction solvent to extract more polar molecules. Fractionation of different molecules within the extract can be achieved by adjusting the pressure and temperature of the separators so the molecules of interest can selectively precipitate. Using $scCO_2$ as an extraction solvent, extraction

and fractionation can be combined in one step. Previously, supercritical CO_2 had been demonstrated as an environmentally friendly alternative solvent for the extraction of these valuable wheat straw waxes.[18] The extraction trials investigated both liquid and supercritical CO_2 as a solvent as well as the addition of a co-solvent. Due to the success of the laboratory trials, the extraction of wheat straw wax using supercritical CO_2 was carried out in pilot scale in collaboration with Botanix. However, optimisation of the extraction conditions was not fully completed so a lot of the parameters used were recommendations from the company.

In this chapter, the use of CO_2 as an environmentally friendly alternative to organic solvents is investigated thoroughly. The same winter wheat straw (Viscount 09) was used for the CO_2 experiments so it can be compared with results from Chapter 2. The effects of different pressure and temperature on extraction yields as well as chemical composition would be studied. A quantitative analysis was carried out to understand the relationship between solute solubility in CO_2. Finally, optimisation of wheat straw wax extraction was carried using Chrastil model to identify the "ideal" extraction conditions for various wax groups.

3.2 Carbon dioxide extraction of wheat straw

3.2.1 Raw materials, pre-treatment and moisture content

Wheat straw (Viscount 09) was used for all the CO_2 extractions and was milled to reduce particle size using the procedure described in Section 6.2.2 prior to extraction. The milled wheat straw was stored in paper sacks in a cool dry place until the extraction was carried out. Prior to the each extraction, the moisture content was determined using the method described in Section 6.2.1 to ensure it was below 10%. The moisture level must be 10% or less because CO_2 can co-extract residual water in the biomass. The effect of moisture level and the co-extraction of water is discussed in Section 5.2.1 in Chapter 5.

3.2.2 Comparison of organic solvents and carbon dioxide extraction yields

Carbon dioxide is a relatively non- polar solvent and quite often its polarity is compared with hexane. Heller *et al.* compared the solubility parameters of CO_2 with *n*-alkanes in the 1980s.[230] In the 1990s, Ikushima *et al.* carried out $scCO_2$ polarity measurement using IR spectroscopy and concluded that supercritical CO_2 has a very similar solvent polarity to hexane and heptane.[231] Extensive research had taken place to compare and understand the differences between hexane and $scCO_2$ extraction of lipids. Traditionally, lipids are extracted using hexane but $scCO_2$ has been adopted as a more environmentally friendly alternative extraction method. One of the biggest worldwide industrial $scCO_2$ plants for the extraction of sesame oil was built in South Korea.[242] An extractor volume of 2 x 3800 L and a pressure of up to 55 MPa is used for this edible oil production.[242]

In this study, extraction of wheat straw wax by liquid, supercritical CO_2 and supercritical CO_2 with co-solvent were compared with traditional organic solvents. The extractions were carried out using the procedure described in Section 6.2.5. The extraction times were selected based on previous investigation with wheat straw wax carried out by Deswarte.[16] Extraction of $scCO_2$ is quite often being compared with hexane or heptane extraction. Table 3.4 shows a comparison of extraction yields between $scCO_2$ and heptane.

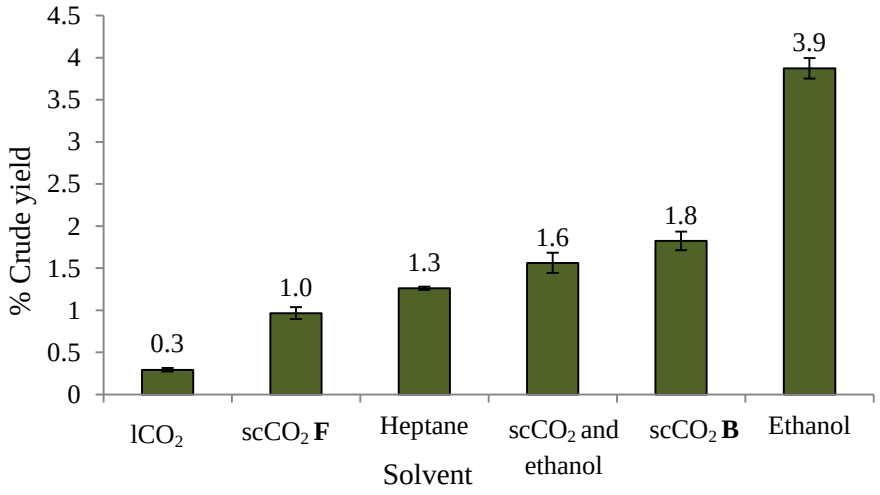


Figure 3.3: Comparison of percentage crude yields of organic solvents and carbon dioxide (originally in colour)

Table 3.3: CO_2 extraction conditions outlined in Figure 3.3

	Temperature (K)	Pressure (MPa)	Co-solvent
lCO_2	278	6.5	-
$scCO_2$ **F**	323	35	-
$scCO_2$ **B**	373	40	-
$scCO_2$ + ethanol	323	35	10%

As shown in Figure 3.3, it can be observed that the extraction yields varied, depending on temperature and pressure and the addition of ethanol co-solvent with carbon dioxide. The CO_2 conditions for the different extractions presented in Figure 3.3 are shown in Table 3.3. The yield for heptane is in between the two $scCO_2$ conditions and $scCO_2$ with ethanol which is expected as $scCO_2$ is more selective for non-polar molecules compared to heptane but with the addition of a polar co-solvent, the polarity increases and it is capable of extracting more polar molecules. The addition of co-solvents with CO_2 means the total overall polarity would alter and this is dependent on the percentage of co-solvent added and the polarity of the organic solvent. Due to the co-extraction of more polar molecules, the increase in the relative proportion of polar to non-polar molecules means a reduction in selectivity.[243] Previously, it was shown from the extraction of wheat straw wax that any co-solvents added to the system would provide enhanced extraction efficiency and the extraction yield is directly related to the polarity of the co-solvent. In this case, ethanol is chosen as the co-solvent and has E_T^N of 0.654, it can be expected that a co-solvent with higher E_T^N such as methanol (E_T^N= 0.762) would give rise to a higher extraction yield.[16] In this experiment, heptane was used as a more environmentally friendly alternative to hexane due to lower toxicity.[157] Table 3.4 shows some literature that suggested hexane and $scCO_2$ extraction yields are highly comparable in lipid extraction but it is common to find that hexane yields tend to be higher than $scCO_2$ yields unless a higher pressure is used.[244] In a separate experiment where the conditions were at 373 K and 40 MPa, a 1.8% extraction yield was observed for wheat straw wax. The yield for liquid CO_2 is a lot different to heptane as the properties of the solvent completely changes when CO_2 is used in liquid phase. Due to the higher viscosity, the penetration through the biomass is more difficult which can greatly reduce the compounds being extracted due to poorer penetration and reduced solubility.[245] Less work has been carried out using liquid CO_2 compared to supercritical CO_2 even though it has a relatively high density

and therefore high solvent power although in some cases, liquid CO_2 can lead to extraction of unwanted contaminants compared to $scCO_2$.[246] The compositional difference between lCO_2 and $scCO_2$ extracts will be discussed later in the Chapter.

Table 3.4: Comparison of supercritical CO_2 and hexane lipids extraction yields

Extract	$scCO_2$			Hexane
	Pressure (MPa)	Temperature (K)	Yield (%)	Yield (%)
Soy bean oil[227]	30	313	16.4	19.9
Sunflower seed oil[245]	32 - 35	313 - 323	36.0	38.4
Rape seed oil [245]	35	313	39.3	40.1
Soybean oil[245]	34.5	323	20.0	19.9
Rice bran wax/oil[247]	28	343	7.1	12.0
Flax dust wax[112]	55.2	333	7.4	4.0
Wheat straw wax[16]	30	313	0.63	1.0
Triticale straw wax[90]	25	343	0.82	0.8

3.2.3 Effect of temperature and pressure in supercritical carbon dioxide crude extraction yields by first order polynomial modelling

As discussed previously, the temperature and pressure in $scCO_2$ determines the density of CO_2 which is one of the most crucial physical properties in extraction. Milled wheat straw was extracted using a range of temperatures and pressures in an experimental 2 x 2 plot as shown in Figure 3.4. The main advantage of carrying out the experimental design is that two variable parameters can be investigated simultaneously unlike the traditional method of only changing one variable parameter at any single time.[248] The plot was designed to give a working range for temperature and pressure. The temperature was set at 305 K and 373 K and the pressure was set at 7.5 MPa and 40 MPa. Traditionally, there is a limitation with the working pressure as older commercial plants cannot usually operate above 30 MPa. Due to advances in engineering, some of the bigger and newer plants built nowadays can operate up to 55 MPa.[236, 249] A total of four experimental points at the minimum and maximum operating temperature and pressure were obtained with the exact conditions shown as points **A**, **B**, **C** and **D** in Figure 3.4. An extra point at the centre of the working ranges was also carried out (labelled **E**: 338K and 25 MPa) to clarify any relationships

identified and minimise the risk of missing a non-linear relationship in the middle of the working range.[250]

The significance of temperature and pressure were modelled using the dimensionless factor coordinate system.[16, 250] A similar optimisation on wheat straw wax has been modelled before and a successful first order polynomial function was developed to describe the relationship of extract crude yield to temperature and pressure.[16] The work described by Deswarte is further developed in this study. Bigger temperature and pressure ranges were incorporated into the study as technologies have advanced. The same qualitative analysis was carried out as in Chapter 2 and the conclusions can be drawn that the CO_2 extraction conditions have very little effect on chemical compositions. This is expected as using CO_2 under different conditions will only moderately modify the polarity of the solvent and in the solvent study described in Chapter 2, a larger polarity range was studied and there were still little compositional difference between the extracts. Due to this reason, an identical quantitative analysis on the different wax groups was undertaken to further understand the relationship of temperature and pressure on specific groups of compounds. No work has been carried out before on correlation of specific wax groups in cereal straw wax due to the complexity of the natural wax extracts.

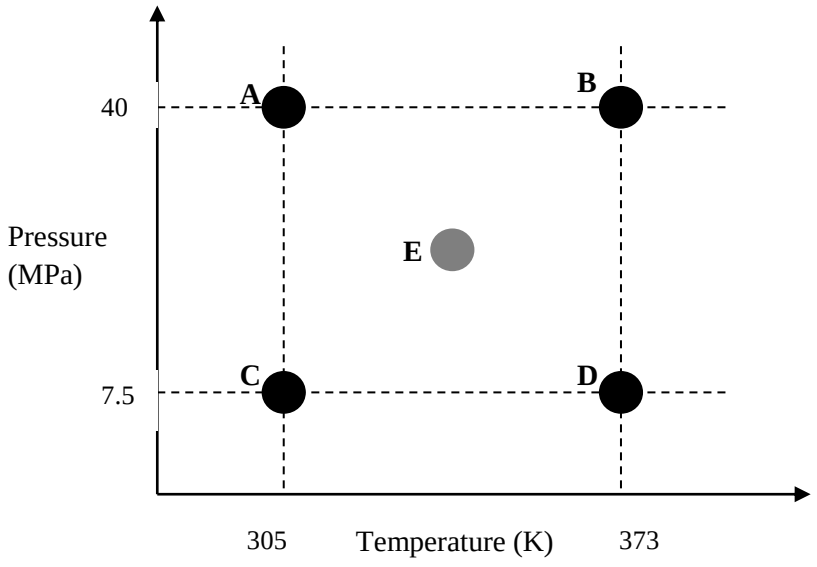


Figure 3.4: Experimental design for extraction conditions optimisation

A dimensionless factor coordinate system has the convention of "-1" for the low level and "+1" for the high level of each parameter. The coordinate for the centre point was "0" as it coincided with the origin of the system. The newly coded coordinate values are shown in Tables 3.5 and 3.6. As the centre point **E** was 338 K and 25 MPa which is not exactly central, in the calculations the coordinate "0" used were exact calculated values to increase accuracy. The values were adjusted and resulted in $x_1 = 0.0097$ and $x_2 = 0.107$.

Table 3.5: Experimental design with the coded coordinate values

Factor	Experimental variable	Coded coordinate values		
		-1	0	+1
X_1	Temperature (K)	306	338	373
X_2	Pressure (MPa)	7.5	25	40

Table 3.6: Experimental design for optimisation

Experiment point	Coded coordinate values		Experimental conditions	
	X_1	X_2	Temperature (K)	Pressure (MPa)
A	-1	+1	305	40
B	+1	+1	373	40
C	-1	-1	305	7.5
D	+1	-1	373	7.5
E	0	0	338	25

A total of five experiments were carried out for the optimisation and the relationship between percentage crude yield and temperature and pressure were deduced using multiple linear regression. Equation 3.1 shows the first order polynomial function used for the multiple linear regression where Y was the total percentage extraction yield, b_1 and b_2 account for the main effects of the coded coordinate values of x_1 (temperature) and x_2 (pressure), b_{12} represents the second order interaction term and b_0 is the response at "zero" level which corresponds to the yield at experiment point **E**.

$$Y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2$$

Equation 3.1: First order polynomial function

A standard four hours extraction was carried out and the total yields were calculated using Equation 3.1. A standard extraction time was chosen. Previous work carried out by Deswarte showed that more than 90% of the compounds were extracted out of the wheat straw within four hours.[16, 18] There is no need to extend extraction further because of the significant increase in the operating cost for the low increase in yield. An operating flow rate of 40 $g.min^{-1}$ was used based on previous extractions demonstrated by Deswarte which means that a total of 9.6 kg of CO_2 was used for each four hour extraction.[16] A similar factorial design study carried out by Meireles showed that the effect of flow rate was not significant which means the important factor is the total amount of CO_2 used in the extraction.[251] The total percentage crude yield for the five experiments is shown in Figure 3.5.

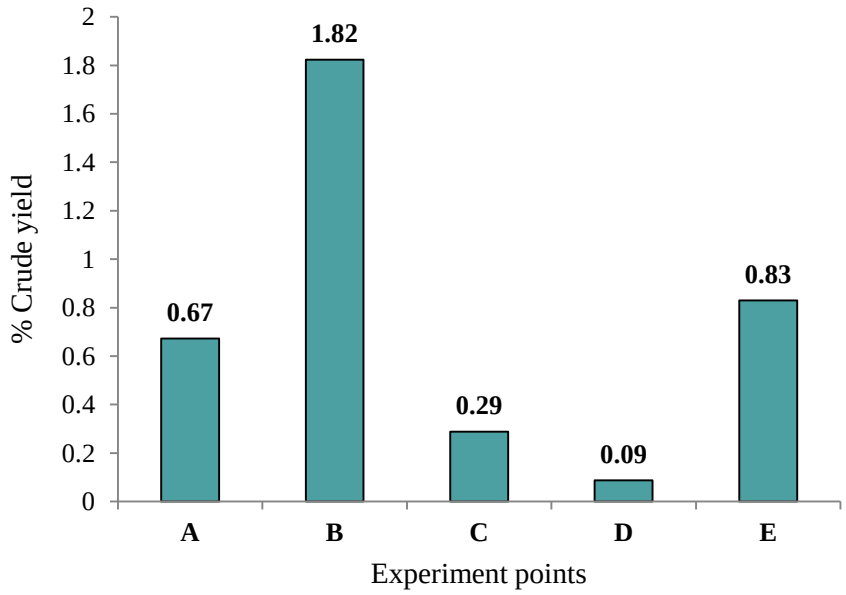


Figure 3.5: Percentage crude yields for $scCO_2$ extraction at different pressure and temperature (originally in colour)

Multiple linear regression was carried out using the percentage yield data presented in Figure 3.5 using Equations 3.2, 3.3, 3.4 and 3.5 and as a result, a new first order function modelling the $scCO_2$ extraction of wheat straw waxes was deduced, which is presented as Equation 3.6.

$$b_0 = \frac{1}{4}(y_1 + y_2 + y_3 + y_4)$$

$$b_1 = \frac{1}{4}(-y_1 + y_2 - y_3 + y_4)$$

$$b_2 = \frac{1}{4}(-y_1 - y_2 + y_3 + y_4)$$

$$b_{12} = \frac{1}{4}(y_1 - y_2 - y_3 + y_4)$$

Equations 3.2, 3.3, 3.4 and 3.5 (top to bottom): Calculation for coefficients of the first order polynomial function

$$Y = 0.72 + 0.24x_1 + 0.53x_2 + 0.34x_1x_2$$

Equation 3.6: First order function modelling the $scCO_2$ extraction of wheat straw waxes

Equation 3.6 shows the coefficients for both temperature and pressure which give further details on the influence of the individual parameters and the combination of the two parameters that determine the density of CO_2 and hence solvent strength.[241] It is important to note that temperature, pressure and density are not the only factors that can have an effect on extraction yield. Other factors such as viscosity, diffusivity and polarity of the $scCO_2$ have also been reported to have an important role in terms of extractions.[252] In general, the coefficient can give a rough idea on the influence of temperature and pressure on the yields. Here, the coefficient for x_2 (pressure) is double the value for x_1 (temperature) which indicates the pressure of the extraction is more influential in giving higher crude yields. From the coefficient, it is apparent that the density of the CO_2 is important. An inverse effect of the temperature with constant pressure can be seen. The increase in temperature leads to a decrease in CO_2 density under identical pressure conditions which can cause a drop in the solubility of the wax compounds. This is the case when the system is under low pressure as shown by experimental points **C** and **D**. The density dropped from 311.5 gL^{-1} to 130.4 gL^{-1} and this was reflected in the massive decrease in yields from 0.29% to 0.09%. As a result of reduced solubility, the yields should also decrease from experimental points **A** and **B** but the opposite occurred as the system did not behave as predicted. The increase in temperature from 306 K to 373 K at 40 MPa means the density would drop from

978.7 gL^{-1} to 756.7 gL^{-1}. This indicates that there are other factors that come into play when describing the influence of temperature and pressure. The increased temperature would provide sufficient energy to break the crystal lattice of the wax compounds which can lead to enhanced solubility. It can be concluded that the effects of temperature on wax extraction yield are controlled by two opposing factors. At high temperatures, the vapour pressures for the wax compounds are high and the wax on the straw surfaces are molten meaning that this factor overrides the negative influence of reduced density. Research carried out by Lucas also suggests that increased solubility of lipids at high temperatures was more important than the density giving a higher yield.[247] In contrast, the effect of density dominated at low temperatures where the wax remained crystalline on the straw surfaces. The percentage crude yield for extraction point **E** in the middle of the experimental design was calculated using Equation 3.6 and compared with the experimental value. The predicted value was calculated to be 0.73% and the average actual experimental value was 0.83% giving a 0.1% difference with a 13% error. The model shows relatively good prediction for percentage crude yield in wheat straw wax extraction which is in agreement with previous first order polynomial modelling work carried out by Deswarte.[16]

3.2.4 Effect of temperature and pressure in supercritical carbon dioxide extraction yields of different wax groups

The five extractions described in Section 4.3.4 were characterised following the procedure described in Section 6.3.2. Identical wax compounds were identified in the $scCO_2$ extracts compared to the solvent extracts so it is necessary to carry out a quantitative analysis. In this section, the effect of temperature and pressure on individual wax groups is discussed and the details of the breakdown of the individual compounds within each wax groups are described later in the chapter. The calibration and methods used are identical to Chapter 2 and Table 3.7 shows the percentage of the different wax groups within the extracts.

The physical properties such as colour and texture of the $scCO_2$ extracts ranged from soft pale yellow to harder light green at different extraction conditions, already suggesting that there are compositional differences between the extracts. The purpose of using experimental design was to optimise the extraction conditions for groups of

compounds and single compounds. This proved successful and this is the first time, correlation of wax groups has been carried out using such experimental design.[250, 253]

Table 3.7: Percentage of different wax groups extracted from wheat straw

Experimental point	Percentage of wax group in wheat straw					
	Fatty acid	Nonacosane	Aldehyde	Wax ester	Sterol	β-Diketone
A	0.079	0.019	0.009	0.179	0.083	0.125
B	0.103	0.028	0.0108	0.320	0.113	0.170
C	0.0079	0.02	0.0033	0.004	0.029	0.099
D	0.013	0.001	0.0006	0.015	0.0004	0.0004
E	0.097	0.023	0.017	0.133	0.097	0.125

In order to understand the relationship between the relative abundance of various wax groups with temperature and pressure, the same multiple linear regression was carried out for each individual wax group using Equation 3.6. In these cases, Y represents the normalised percentage of different wax groups in the crude extracts. Table 3.8 shows the coefficients from the multiple linear regression method. Hydrocarbons and fatty alcohols were omitted from the modelling as there were co-elutions between hentriacontane (C_{31}) and octacosanol (C_{28}). Nonacosane (C_{29}) is one of the most predominant *n*-alkanes in wheat straw wax so this was used to represent the hydrocarbon wax group in the first order polynomial modelling. As octacosanol (C_{28}) is the only fatty alcohol which was measurable because of co-elution, the fatty alcohol was not investigated.

Table 3.8: Coefficients for different wax groups

Wax group	Coefficients			
	b_0	b_1	b_2	b_{12}
Fatty acid	0.0254	0.00465	-0.000476	-0.0134
Nonacosane	0.0170	-0.00260	0.00643	0.00705
Aldehyde	0.00267	-0.000826	0.0000205	-0.000162
Wax ester	0.0456	0.00520	0.0181	-0.0182
Sterol	0.0211	-0.0115	0.00570	0.00255
β-Diketone	0.0453	-0.0312	-0.00504	0.01780

Unlike the coefficients derived for percentage crude yield, all four coefficients are positive meaning that there is positive influence on the described parameters. A mixture of both positive and negative coefficients can be seen on Table 3.8 which means that the temperature and pressure can affect the yields of certain wax groups both positively and negatively. However, it is important to evaluate the validity of the newly developed first order polynomial equations for each wax group by comparing the predicted yield for a specific wax group with the actual experimental yield for experiment point **E**. Table 3.9 compares the calculated predicted and actual percentage yield for each wax group. The percentage errors indicate the difference between the predicted and actual experimental percentage yields.

Table 3.9: Prediction of percentage yield for different wax groups

Wax group	% Yield			% Error
	Predicted	Actual	Difference	
Fatty acid	0.025	0.034	0.0082	24.3
Nonacosane	0.017	0.023	0.0060	26.1
Aldehyde	0.003	0.017	0.014	84.0
Wax ester	0.046	0.046	0.001	1.1
Sterol	0.021	0.034	0.013	37.4
β-Diketone	0.045	0.043	-0.002	4.1

The percentage yield difference between the two values was also calculated along with the percentage error. It can be observed that there are good matches between the wax esters and β-diketones as the differences between the predicted and actual percentage yield is very low which means there is a linear relationship with temperature and pressure. The proposed equations for wax ester and β-diketone are shown as Equations 3.7 and 3.8 respectively.

$$Y\ (Wax\ esters) = 0.0455 + \ 0.00520x_1 + \ 0.0181x_2 - 0.0182x_1x_2$$
$$Y\ (\beta - diketones) = 0.0453 - 0.0312x_1 - 0.00504x_2 + \ 0.0178x_1x_2$$

Equations 3.7 and 3.8 (top to bottom): First order function modelling the wax esters and β-diketones extraction in wheat straw waxes

The coefficients for the wax esters and β-diketones are plotted in Figure 3.6 to show the important parameters in achieving high yield. For wax esters, the coefficients suggested that both pressure and temperature of CO_2 have a positive influence on high extract yields. However, the coefficient b_{12} showed a negative value which means that the density has little influence on the yield of wax esters and this data is supported by no increase in values from experimental points **B** and **D** and a large rise in yield from experimental points **C** and **D**. At low temperature, the pressure appears to have a huge positive impact (experimental points **A** and **C**) but at high temperature, the increased pressure has zero influence (experimental points **B** and **D**). The results show that a combination of low temperature and high pressure can achieve the highest wax ester yield. The results shown are not expected as the solubility of wax esters increase when the compounds are molten under higher vapour pressure as discussed previously.[247] The data is also in disagreement with work carried out by Jones *et al.* on the extraction of wool wax from $scCO_2$.[254] The study showed that wool wax containing 88% of esters with more than 95% of esters with molecular weight of above 600 Da behaved in the opposite manner as at critical temperature, the pressure has no influence on the yield.[254] These findings were also supported by $scCO_2$ extraction of wool wax by Bayona *et al*.[255] Interestingly, Cygnarowicz-Provost *et al.* extracted wool grease using $scCO_2$ in a pressure range between 20 – 52 MPa using three different temperatures and showed that above 45 MPa at high temperatures, the amount of wool grease extracted dropped giving 45 MPa as an optimum pressure.[256] From the data, a non-linear relationship between pressures can be established for high temperature in wax esters extraction in wheat straw. It would be interesting to conduct a pressure study on high temperature for wax esters.

For β-diketones, the results were very interesting as both temperature and pressure showed a negative impact on the extraction yield alone but in combination, the density appeared to have a large effect on the extraction yield. This means that the highest percentage yield for β-diketones can be achieved by using low temperature and high pressure which result in high CO_2 density. Even though temperature and pressure can alter a lot of other physical properties such as viscosity and diffusivity, a number of studies have suggested that the density of CO_2 is one of the most crucial parameter in terms of extraction.[257, 258, 259]

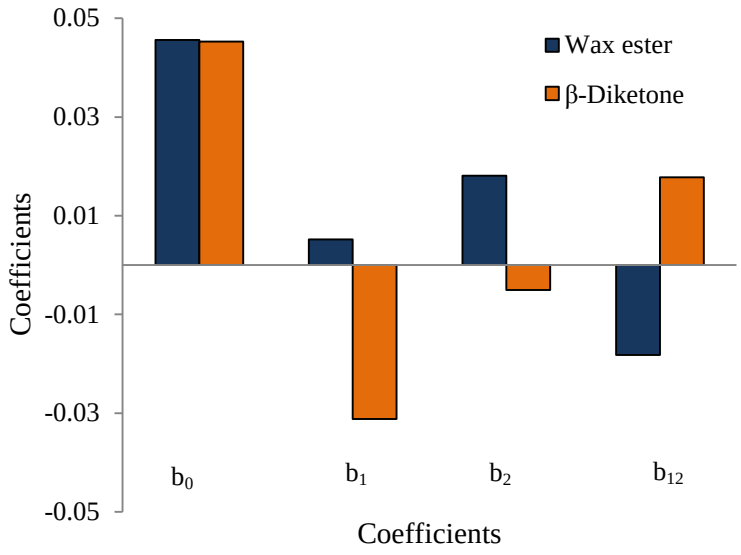


Figure 3.6: Coefficients in first order modelling for wax ester and β-diketone (originally in colour)

The prediction for the fatty acids and sterols yields was relatively poor which indicates that there is no linear relationship between temperature and pressure. However, in order to confirm that this is true, fractionation of the individual wax groups and using specific methods of analysis would improve the accuracy of the quantification analysis. The aldehydes in the wheat straw wax showed very interesting results as the predicted and actual values for experimental point **E** are extremely different and have a large percentage error of 84%. Due to the high percentage error, no linear relationship between temperature and pressure can be demonstrated which indicates that there may be "hot spots" in the experimental design where at a specific temperature and pressure, the yield is high. A similar result was seen with the extraction of bagasse wax using $scCO_2$ by a student Gabriel Guihard in the Green Chemistry group in the University of York, where a "hot spot" for high yields was observed.[260] A 2^2 experimental design of 308 - 338 K and 10 – 40 MPa was carried out and especially high crude yield was found at 333K and 35 MPa.[260] The chemical composition study for bagasse wax shown later in the thesis (Chapter 4) highlighted that the main wax group in the extracts are aldehydes. In wheat straw hexane extract, the aldehydes levels are shown to be up to 800 $\mu g g^{-1}$ (about 16% of the total extract). A high level of long chain aldehydes in sugarcane was previously observed by Wada *et al*. This may explain the abnormal "hot spot" located in the experimental design as

aldehydes in waxes do not behave as predicted. It would be interesting to carry out further extractions to try and understand the relationship of aldehyde extraction with $scCO_2$ extraction parameters.

3.2.5 Optimisation of wheat straw wax extraction using supercritical carbon dioxide by Chrastil modelling

The effect of temperature and pressure on the wheat straw wax yield has been previously analysed using first order polynomial function in order to establish any relationship between temperature and pressure. The established first order functions showed the positive and negative influence of temperature and pressure on the extractions which are useful information when designing optimised $scCO_2$ conditions for maximum yields. Although the change in temperature and pressure of the system ultimately reflects the change in density, as highlighted before other parameters such as diffusivity and viscosity may also be altered. The yield of extracts is strongly dependent on the solubility of the different compounds in $scCO_2$ at specific temperatures and pressures. Effectively, the wax compounds are assumed to be in equilibrium between the straw and $scCO_2$ so the supercritical solubility behaviour can be calculated. In the past, many density-based supercritical models have been explored to describe this behaviour (Chrastil, Del Valle and Aguilera, Adachi and Lu, Méndez-Santiago and Teja, Bartle).[261, 262, 263, 264] The most well-known density-based empirical supercritical model was developed by Chrastil in 1982 and it is one of the first models proposed which directly relates the solubility of solute and the density of CO_2.[261] Many of the others are derived from the Chrastil model but modified to improve their suitability for different circumstances. Del Valle and Aguilera highlighted the limitations of the model such as high solute solubility and limited temperature range in their study of extraction of vegetable oil and created an "improved" equation to account for the variations of heat of vaporisation with temperature.[262] Adachi and Lu modified the Chrastil equation for more density dependable functions within the model.[265] Here, the original density-based Chrastil equation was used to model the behaviour of wheat straw wax in $scCO_2$ from nine different extraction conditions in order to understand the behaviour of solvent and solute interactions. The Chrastil model describes the association of solvent and solute molecules in equilibrium resulting in the formation of a solvated complex. The

Chrastil equation is shown as Equation 3.9, where *c* is the mass of the extract (g) divided by the volume of CO_2 (g), k is the average number of solvent molecules in a solvated complex, *a* is a function of the enthalpy of solvation and enthalpy of vapourisation, *b* is a function of *k*, *T* is the temperature (K) and *d* is the density of carbon dioxide ($g.L^{-1}$).[261]

$$\ln(c) = kln(d) + \frac{a}{T} + b$$

Equation 3.9: Chrastil equation

Table 3.10 shows the nine different extraction conditions used for the Chrastil modelling and the average mass of extracts recovered. The extractions are labelled as experimental points **A** – **I** (with **A** – **E** corresponding to the previous first order polynomial function). As a standard extraction time and flow rate were used in all the extractions a total of 9.6 kg of CO_2 was being used for each extraction. The total crude yields for the nine experiment points are also listed in Table 3.10 and displayed as a contour graph as Figure 3.7.

The content of the wax in the straw is low and only comprised 0.5 – 1.0% of the dry weight so in order to achieve 1% crude yield, the extraction data showed that a minimum temperature of 320 K and pressure of 25 MPa (density = 848.7 $g.L^{-1}$) is required for the extraction.[49] If a 1% crude yield can be achieved in the straw extraction then a potential of 7.5 – 25 million tons of valuable waxes can be obtained each year worldwide.[49] By modelling the $scCO_2$ extraction, this can ensure that a certain crude yield can be achieved by applying specific temperature and pressure. A full economic assessment must be carried out to establish the most financially viable extraction conditions for the highest percentage crude yield. From Figure 3.7, it can be observed that it is a combination of high pressure and temperature that enhanced the percentage crude yield and these results are in agreement with other $scCO_2$ straw wax extractions.[90] A temperature of 305 K and a pressure of 40 MPa results in a CO_2 density of 982.4 $g.L^{-1}$ and only gives a low yield of 0.67% confirming that the density of CO_2 is important. There are other factors that dictate the solubility of wax constitutes in CO_2. Both temperature and pressure dictate the density and dielectric constant of the CO_2 which influence the solvating power, thereby increasing the

solubility of solutes in the extracting fluid.[266] Cuticular wax is semi-crystalline and enough thermal energy is required in order to overcome the lattice energy and increase the solubility of wax components.[48]

Table 3.10: Extraction conditions and percentage crude yield for Chrastil modelling

Experimental point	Temp (K)	Presure (MPa)	Mass (g)	Density (gL^{-1})	Crude yield (%)
A (n = 3)	305	40	0.87	981.9	0.67 ± 0.15
B (n = 3)	373	40	2.37	756.7	1.82 ± 0.22
C (n = 3)	305	7.5	0.37	365.9	0.29 ± 0.08
D (n = 3)	373	7.5	0.05	130.4	0.09 ± 0.02
E (n = 3)	338	25	1.25	761.9	0.83 ± 0.11
F (n = 3)	323	35	1.26	899.2	0.97 ± 0.14
G (n = 3)	373	25	1.27	588.5	0.98 ± 0.09
H (n = 3)	373	10	0.24	188.6	0.18 ± 0.02
I (n = 3)	323	7.5	0.2	193.9	0.15 ± 0.04

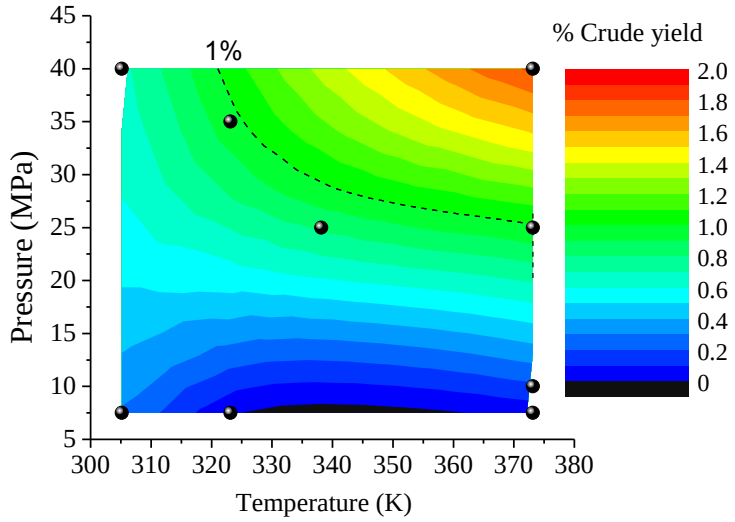


Figure 3.7: Percentage crude yield of wheat straw waxes from $scCO_2$ extraction (originally in colour)

According to the Chrastil model, ln(*d*) versus ln(*c*) at constant temperature should give a straight line if the system obeys the theory.[261] This model was applied to the $scCO_2$ extraction of the total crude yield and it was found that the extraction of wheat straw wax obeys the model well. The coefficient can be improved when other functions are incorporated into the Chrastil equation. Multiple linear regression was

applied to the ln(d) and ln(c) crude yield data to obtain the coefficients k, a and b. The crude yield of wheat straw waxes extracted using $scCO_2$ can be predicted using equation 3.10 with $r^2 = 0.96$.

$$\ln(c) = 1.41 \ln(d) - \frac{1256.37}{T} - 14.6$$

Equation 3.10: Chrastil equation modelling the $scCO_2$ extraction of wheat straw waxes

Figure 3.8 shows a linear relationship between experimental ln(c) and predicted ln(c) between (305 K and 7.5 MPa) to (373 K and 40 MPa) which indicated the extraction can be modelled using Equation 3.10 derived from the Chrastil model. From Figure 3.7, it can be concluded that high temperature and pressure would favour the extraction of waxes from wheat straw. This finding is in agreement with previous first order polynomial modelling of temperature and pressure.

However, as part of the straw bio-refinery, it is crucial that the straw remained in a usable condition for exploitation such as co-firing for energy or microwave pyrolysis for bio-oil so the high temperature and pressure conditions must not alter the physical condition of the straw residues.[19] This is where the use of CO_2 as a solvent is superior to traditional organic solvents as the straw residues are left completely solvent-free, unlike solvent extractions where an extra drying step may be required after extraction.

The limitation of the model (temperature 305 - 373K and pressure 7.5 - 40 MPa) might mean that the optimum wax extraction conditions may not have been reached. Most large commercial plants built in the 1980s and 1990s will not operate above 35 MPa due to equipment design so the maximum pressure would be limited. However, modern plants have been designed to operate at up to 55 MPa so the extraction times can be significantly reduced. Operating at a high pressure means $scCO_2$ extraction is an energy intensive and costly process, so a full economic assessment must be carried out to calculate the extra costs against extra wax yield. The total wax content present on the straw surfaces is unknown at this stage so it is difficult to conclude that the straw has been completely de-waxed and extraction has gone to completion. Further extending the models extraction limits would be of interest from an academic point,

but would add limited commercial value due to the added cost of production scale extractions at higher pressures.

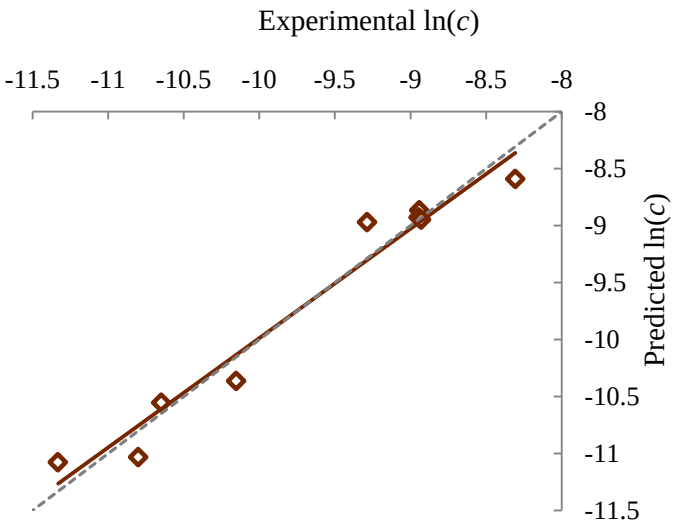


Figure 3.8: Experimental ln(*c*) against predicted ln(*c*) for percentage crude yield (originally in colour)

For the first time, the Chrastil equation has been applied to just a single group of molecules within a complex mixture of molecules. As in previous first order polynomial modelling, hydrocarbons and fatty alcohols were omitted due to co-elution of hentriacontane (C_{31}) and octacosanol (C_{28}). Nonacosane (C_{29}) was used to represent hydrocarbon wax group and the fatty alcohol wax group was not investigated. The same multiple linear regression was applied with the mass of wax group extracted in ln(*c*) with ln(*d*) to calculate new *k*, *a* and *b* for the development of a new model that is specific to the different wax groups. Table 3.11 shows the values for coefficients *k*, *a* and *b* for the selected wax groups.

Table 3.11: Chrastil modelling for different wax groups

Wax group	Coefficients			r^2
	k	*a*	*b*	
Fatty acid	1.32	-2090	-13.87	0.68
Nonacosane	1.98	-3661	-15.63	0.65
Aldehyde	-0.34	-5726	6.34	0.11
Wax ester	2.60	-2441	-20.76	0.90
Sterol	2.44	1518	-31.87	0.73
β-Diketone	2.44	2155	-33.30	0.74

Some wax groups have showed good correlation with density and temperature in the Chrastil model. There are a number of reasons for the correlations so further investigation is required to optimise the individual wax group. An obvious reason is that no equilibrium was achieved in the residence time in the extractor, as the experiments were carried out using a fixed flow rate under a flow-through method at different temperature and pressure. An improvement to this would be to stop the CO_2 flow once the extractor is filled but the disadvantage of this method is that as the wax groups become soluble in the $scCO_2$, the properties of the CO_2 such as polarity would change and also become more saturated as more molecules are dissolved. Friedrich *et al.* demonstrated in a $scCO_2$ extraction study using soybean flakes that the extraction efficiency is not uniform through the CO_2 extractor column. The soybeans were removed and divided into three fractions, the three fractions showed a large difference in colour and oil content showing that the CO_2 becomes more saturated as it penetrates through the materials.[227] By stopping the flow, the extraction efficiency will be more uniform which is a benefit over the existing method. Another method is to alter the flow rate of the system and remain as a flow-through method so that the residence time for each extraction conditions are the same and the effect of extraction yield with various residence time can be investigated. However, achieving equilibrium for all the different wax groups at a specific residence time is impossible but nonetheless; it would be interesting to in investigate this extraction parameter.

Only the wax ester group correlates well with the Chrastil model which indicated that the extraction of wax esters has reached equilibrium in the extraction conditions carried out and is also dependent on CO_2 density and temperature. The other groups may not have a correlation as the extraction cannot be modelled by density and

temperature alone. Other important parameters such as polarity and viscosity of the CO_2 may influence the extraction performance. From this preliminary optimisation trial, it can be concluded that the individual wax groups (except wax esters) do not correlate well with the established Chrastil equation under the testing conditions. However, other CO_2 parameters can be considered and incorporated in order to establish a new equation for modelling the extraction of certain wax groups. It is also important to carry out extraction over shorter times. As the influence of extraction times has not been investigated for each individual group, it is very difficult to ensure that saturation has not already occurred during the four hour extraction as currently four hour extraction data only gives a "snapshot" of yield results. This may be the reason for the lack of correlation against some of the wax groups. As the wax ester group obeys the Chrastil model in the extraction conditions under investigation, a new equation can be established for the wax esters in wheat straw wax as displayed in equation 3.11 with r^2 = 0.90. Figure 3.9 shows a good match of experimental and predicted ln(c) for percentage wax ester.

$$\ln(c) = 2.60\ln(d) - \frac{2440.52}{T} - 20.8$$

Equation 3.11: Chrastil equation modelling the $scCO_2$ extraction of wax esters in the wheat straw waxes

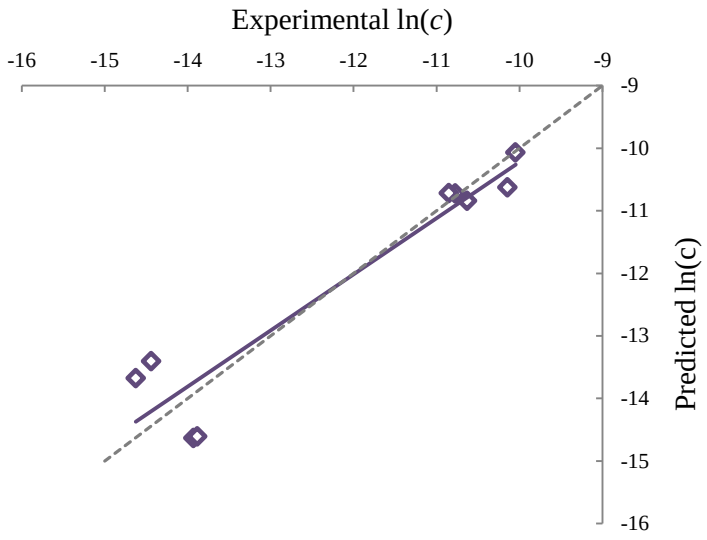


Figure 3.9: Experimental ln(c) against predicted ln(c) for percentage wax esters (originally in colour)

As shown in Figure 3.9, there were two distinct clusters of points in which low ln(*c*) indicated high wax esters yield and high ln(*c*) showed low wax esters yield. The trend cannot be explained by density of CO_2 alone. It can be noted that extractions carried out using 25 MPa or higher belonged to the higher wax esters yield. There are two opposing factors that are important for wax esters extraction. The elevated extraction temperature leads to high T_m long chain wax esters melting on straw surfaces so therefore increasing the solubility in CO_2. However, the increase of temperature would result in the decrease in CO_2 density leading to reduced solvating power of solvent so therefore it is vital to understand and optimise the influence of extraction conditions through modelling.

It can be concluded that using the existing extraction conditions, the percentage crude yield and wax ester yield can be predicted using the Chrastil model. More work is needed to investigate extraction time and establishing equilibrium of wax molecules between CO_2 and straw. New density-based models may need to be developed in order to describe the extraction of wheat straw wax completely.

3.3 Identification and quantification of key wax components in wheat straw wax

The different key wax components have been successfully identified with the full identification process described in Chapter 2. The compounds found in the $scCO_2$ extracts were identical to the wax components identified in the solvent extracts. The use of $scCO_2$ at different temperatures and pressures has been used to replace traditional organic solvents so it is not surprising that the wax compounds identified are identical. The calibrations used for the quantification of the $scCO_2$ extracts are detailed in Section 2.5.1. The percentages are all normalised to 100% for the percentage of wax groups within the extracts. Liquid CO_2 and $scCO_2$ with co-solvent ethanol are labelled with different colours (dark grey and light grey) in order to distinguish between the extracts. Co-elution of compounds often occurs when analysing crude samples directly and without fractionation. In the wheat straw wax, hentriacontane (C_{31}) and octacosanol (C_{28}) eluted with the same retention times so the quantified percentage of both compounds is indicated as patterned bars in the Figures. The use of CO_2 as a solvent at different temperatures and pressures has often been compared with different traditional organic solvents. Since it is the first time that any quantification of wax components has been carried out in wheat straw waxes extracted using CO_2 so no

comparison with previous literature were made thus the results are compared with data previously reported from solvent extracts in Chapter 2.

3.3.1 Free fatty acids

A total of five free fatty acids were identified in the CO_2 extracts and these are tetradecanoic acid ($C_{14:0}$), hexadecanoic acid ($C_{16:0}$), octadecadienoic acid ($C_{18:2}$), octadecenoic acid ($C_{18:1}$) and octadecanoic acid ($C_{18:0}$). The fatty acids presented in the CO_2 extracts are all even-numbered and are one of the shortest aliphatic carbon chain wax compounds identified in the wheat straw wax. Due to their relatively short aliphatic carbon chain, it can be expected the compounds are highly soluble in CO_2. This was reflected by the quantities of the total free fatty acids presented which varied depending on extraction conditions and this is displayed as Figure 3.10. Percentages ranged of 5.6 – 29.5% of free fatty acids were reported in the $scCO_2$ extracts and liquid CO_2 and $scCO_2$ with ethanol consist of a low percentage of 7.2% and 3.6% respectively.

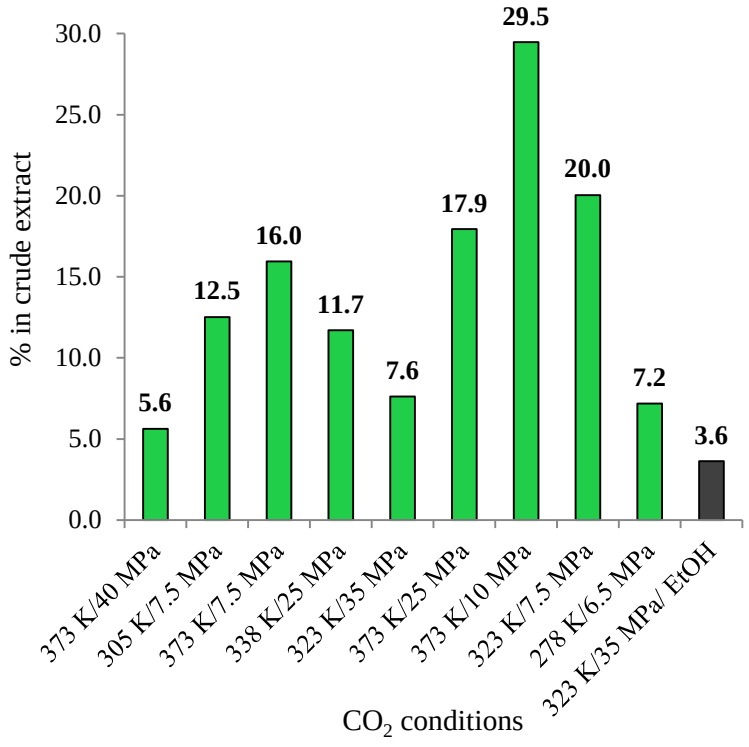


Figure 3.10: Percentage of total free fatty acids in CO_2 crude extracts (originally in colour)

From Figure 3.11, hexadecanoic acid ($C_{16:0}$) is shown to be the most abundant free fatty acids in the CO_2 extracts which is the same as identified in the solvent extracts. About 14.5% of hexadecanoic acid ($C_{16:0}$) in 373 K and 10 MPa $scCO_2$ extract and the yield data suggested that low CO_2 density tend to lead to relatively high free fatty acid extracts due to other wax components identified in the extracts not being soluble at such low CO_2 density. It is difficult to predict the extent of solubility of free fatty acids in $scCO_2$ under different conditions from wheat straw but significant studies have been carried out to establish the solubility of free fatty acids in $scCO_2$. Other factors will affect the solubility behaviour of free fatty acids in wheat straw wax extraction as properties such as polarity of the CO_2 will alter as wax molecules become soluble in the solvent and other issues such as diffusivity of CO_2 which would impact on the penetration of CO_2 through the straw to solubilise the compounds. In a multi-component system, the solubility of compounds may be impeded or enhanced when other compounds become soluble in the CO_2 which can then effectively act as a "co-solvent" and the extent of this effect is highly dependent on the other wax compounds, its quantities and the location of various wax compounds in the straw.[267]

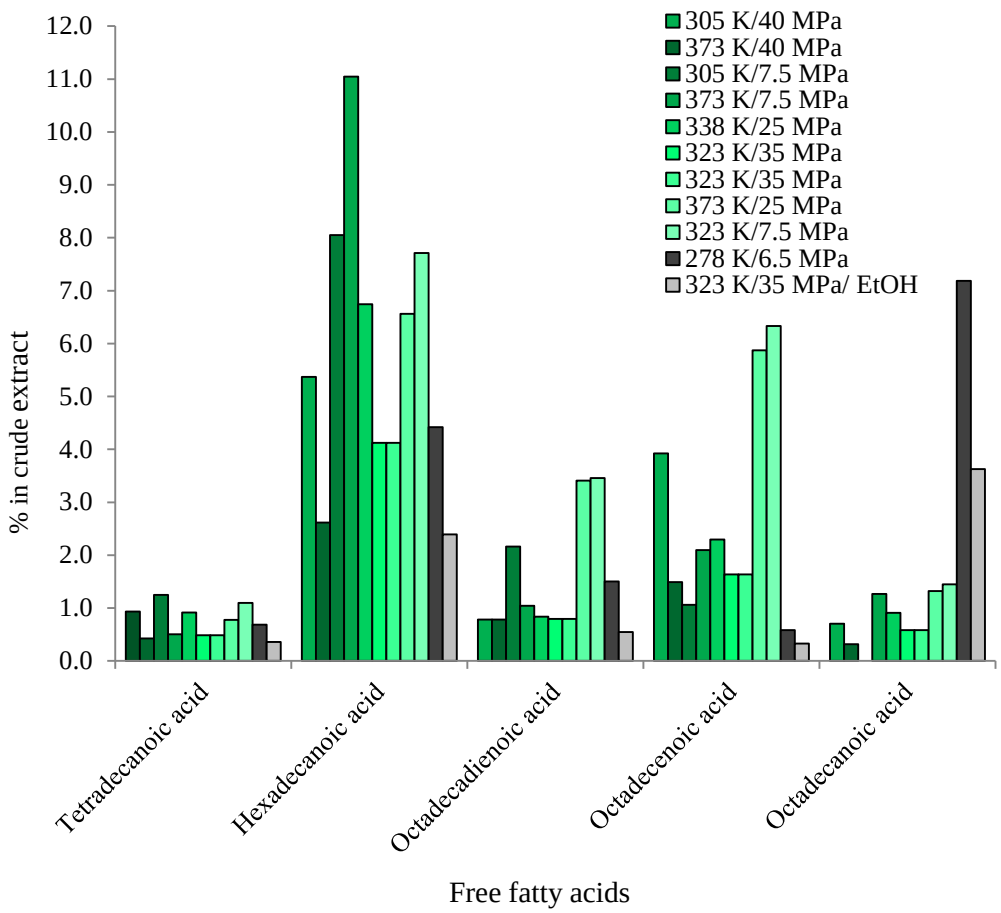


Figure 3.11: Percentage of individual free fatty acids in CO_2 extracts (originally in colour)

Table 3.12 shows a list of published solubility data for free fatty acids in CO_2. The table shows a range of conditions where fatty acids are known to be soluble in CO_2. The published solubility data for the various free fatty acids in CO_2 are similar so therefore it is suggested that the distribution of fatty acids displayed in Figure 3.11 is predominantly based on the abundance in the wheat straw. Iwai *et al.* investigated the influence of solubility of fatty acids and showed that the use of ethanol as a co-solvent can enhance the solubility in CO_2.[268] However, the use of ethanol as a co-solvent can also improve the solubility of other compounds which will lead to less selective extraction and more downstream processing. The balance of the use of neat CO_2 and various co-solvents must be further investigated in order to conclude the feasibility of the use of co-solvents.

Table 3.12: Published solubility data for fatty acids in CO_2[269]

Fatty acids	Temperature range (K)	Pressure range (MPa)	Density range (gL^{-1})	Solubility (kg/kg of CO_2)
Tetradecanoic acid ($C_{14:0}$)	308 - 333	14 - 41.9	563 - 980	0.5×10^2 - 63×10^2
Hexadecanoic acid ($C_{16:0}$)	308 - 328	13.8 - 41.4	620 - 976	1.9×10^3 - 76×10^3
Octadecadienoic acid ($C_{18:2}$)	313 - 333	13.8 - 27.6	563 - 899	0.11×10^2 - 2.7×10^2
Octadecenoic acid ($C_{18:1}$)	313 - 333	13.8 -27.6	563 - 899	0.048×10^2 - 2.3×10^2
Octadecanoic acid ($C_{18:0}$)	308 - 328	13.8 - 41.2	620 - 976	0.28×10^3 - 33×10^3

3.3.2 Hydrocarbons

As identified previously, the hydrocarbons in the solvent extracts were all exclusively *n*-alkanes with odd-numbers due to the wax biosynthetic mechanism.[82] The four main *n*-alkanes identified were heptacosane (C_{27}), nonacosane (C_{29}), hentriacontane (C_{31}) and triatriacontane (C_{33}) which were also identified in the solvent extracts. Figure 3.12 shows the percentage of total alkanes in the extracts and the co-elution has been highlighted. Further work of fractionation is needed to be carried out for alkane fractions. In Figure 3.13, the breakdown of the individual alkanes is displayed and nonacosane (C_{29}) and hentriacontane (C_{31}) were found to be the most abundant *n*-alkanes which is also consistent with the solvent data in Chapter 2.

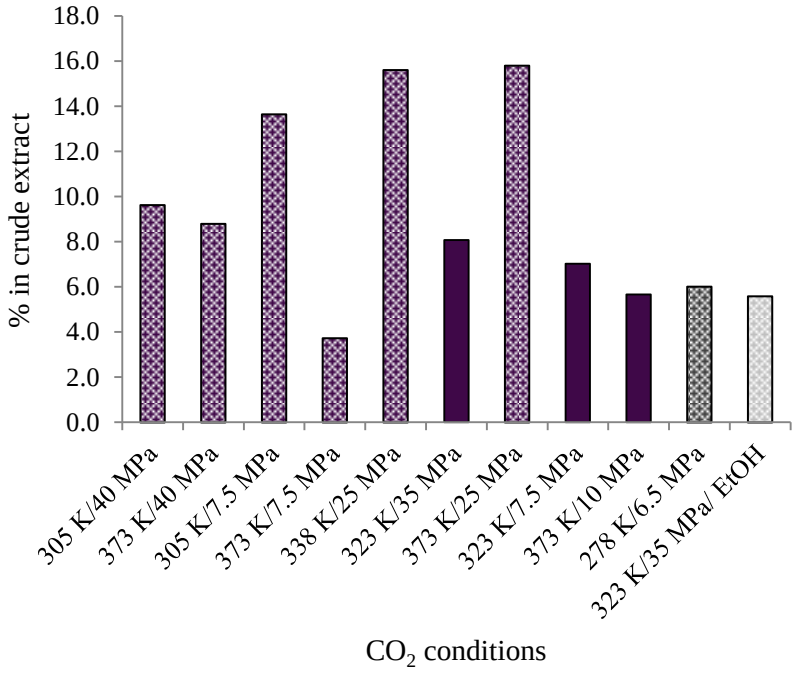


Figure 3.12: Percentage of total *n*-alkanes in CO_2 crude extracts (originally in colour)

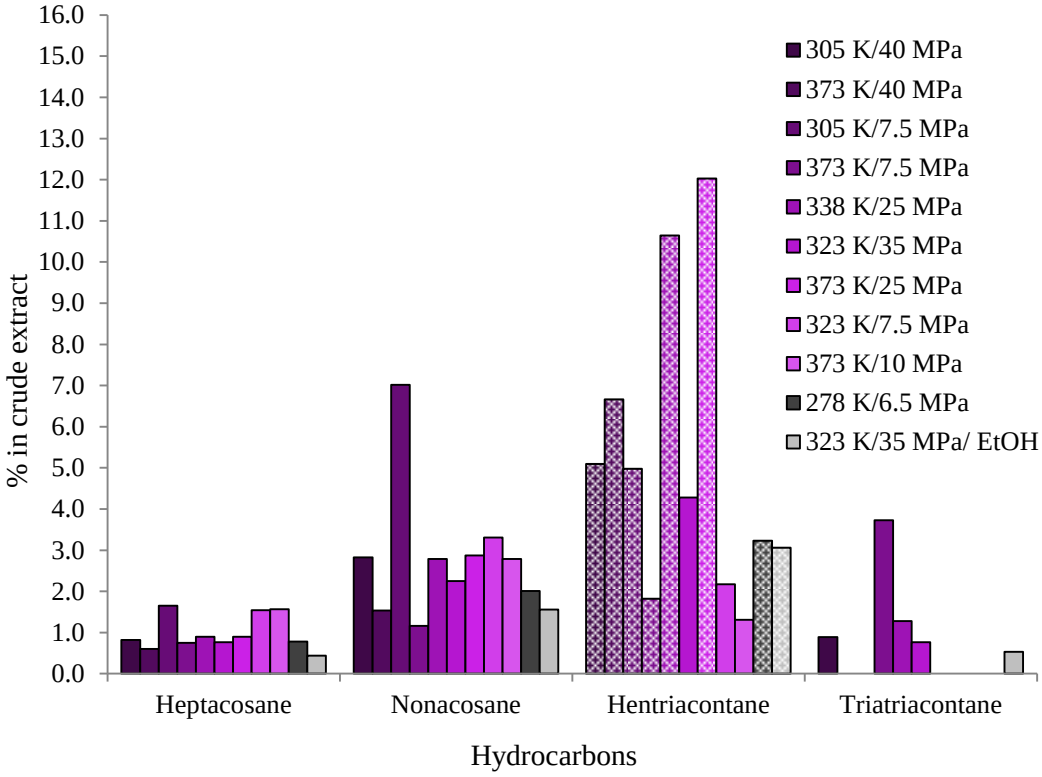


Figure 3.13: Percentage of individual *n*-alkanes in CO_2 extracts (originally in colour)

Table 3.13 shows a list of published solubility data for the *n*-alkanes with chain lengths between C_{26} to C_{32} in CO_2. From this data, its suggested that the aliphatic carbon chain lengths do not have a large influence on the solubility in CO_2. In a multi-component system of long chain molecules, diffusivity of the alkanes in CO_2 is an important factor. Wakao *et al.* demonstrated with FAME that as the chain length increases, diffusivity decreases so the larger alkanes present such as triatriacontane (C_{33}) would be less diffusive compared to heptacosane (C_{27}) making it more difficult to extract out of the straw.[270] It was been shown that as the number of carbons in the aliphatic chain increases, the miscibility with CO_2 decreases which means solubility also decreases.[271] It was demonstrated that branching in alkanes would aid the solubility.[271]

Table 3.13: Published solubility data for *n*-alkanes in CO_2[272]

Hydrocarbons	Temperature range (K)	Pressure range (MPa)	Density range (gL^{-1})	Solubility (kg/kg of CO_2)
Hexacosane (C_{26})	343	15.8 - 26.4	541 - 754	0.4 - 3.1
Octacosane (C_{28})	343	16.2 - 24	557 - 725	0.25 - 1.42
Dotriacontane (C_{32})	343	18.5 - 31	626 - 797	0.23 - 1.38

3.3.3 Fatty alcohols

Octacosanol (C_{28}) was the sole fatty alcohol found in the CO_2 extracts and again this was also demonstrated by the solvent extracts. Figure 3.14 shows the percentage of octacosanol presented in the extracts and the patterned bars highlighted the combined yield of hentriacontane (C_{31}) and octacosanol (C_{28}). It can be noted that generally the yield of octacosanol is higher in CO_2 extracts than the solvent extracts and this may be due to the higher selectivity by using CO_2. No published octacosanol (C_{28}) solubility data was found but fatty alcohols are readily soluble in a wide range of CO_2 conditions.

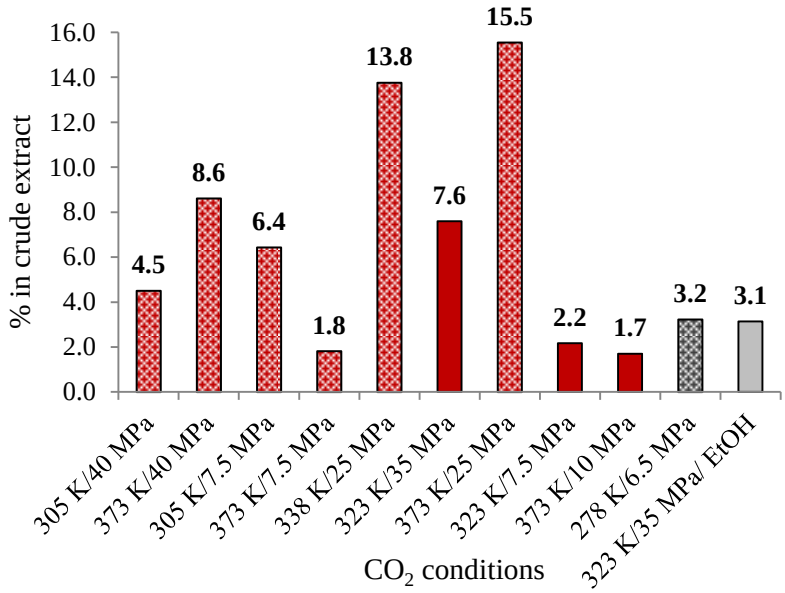


Figure 3.14: Percentage of total octacosanol (C_{28}) in CO_2 crude extracts (originally in colour)

3.3.4 Aldehydes

Octacosanal (C_{28}) was the only aldehyde identified in the CO_2 extracts and is present at a very low level. As shown in Figure 3.15, the maximum quantity found in the CO_2 extract is 2.2% and at low CO_2 density, quite often the octacosanal (C_{28}) is not extracted.

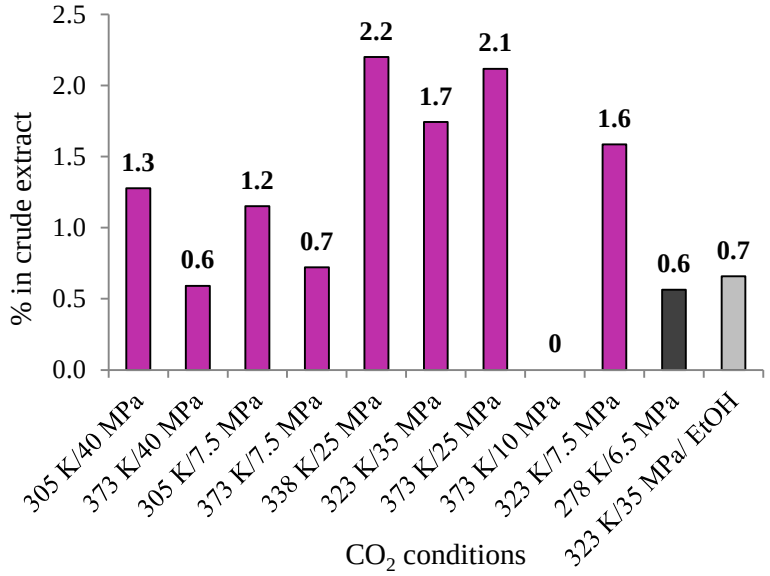


Figure 3.15: Percentage of total octacosanal (C_{28}) in CO_2 crude extracts (originally in colour)

3.3.5 Wax esters

Aliphatic wax esters are the main wax group presented in the CO_2 extracts at levels up to 30.1% of the extracts. The percentage yields of wax esters found in the CO_2 extracts are displayed in Figure 3.16. Wax esters of $C_{40} - C_{56}$ chain length were found in the CO_2 extracts as shown in Figure 3.17. As discussed previously the wax esters were predominantly octacosanol (C_{28}) and hexadecanoic acid (C_{16}) and these are some of the most abundant wax compounds in their free form. Octacosanyl hexadecanoate (C_{28}:C_{16}) and octacosanyl octadecanoate (C_{28}:C_{18}) were the main aliphatic wax esters identified. No published solubility data was found for long chain wax esters but Dandge *et al.* published data on methyl and ethyl esters and found that the presence of the ester functional group can increase the solubility of the equivalent chain length compound.[271] The solubility would be lower in the wax esters present in wheat straw as the aliphatic carbon chain lengths are much higher. The $scCO_2$ with co-solvent ethanol showed a very high percentage of wax esters in the extract and it has been demonstrated that the use of co-solvent can enhance of the solubility of esters.[273] The use of ethanol as an addition to supercritical CO_2 does not have any economic advantages over the use of neat $scCO_2$ as high temperatures and pressures are both still required for the increased yield.

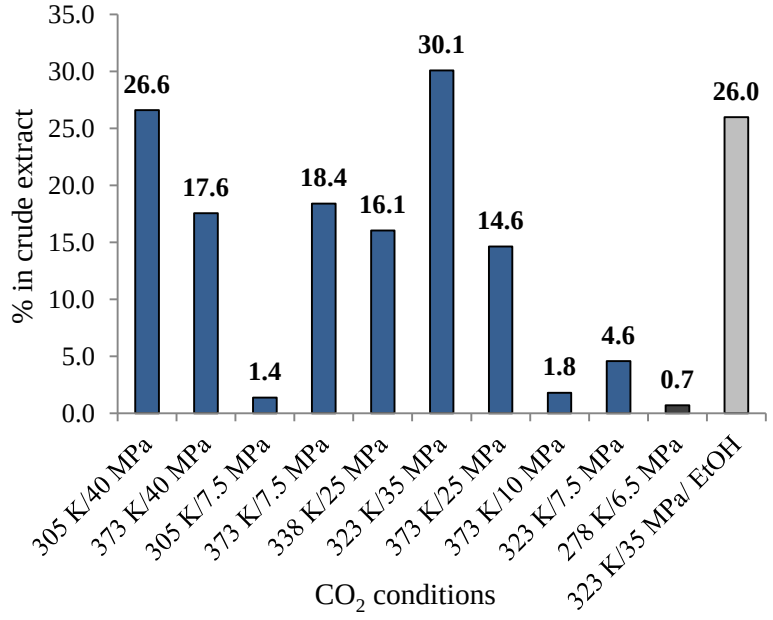


Figure 3.16: Percentage of wax esters in CO_2 crude extracts (originally in colour)

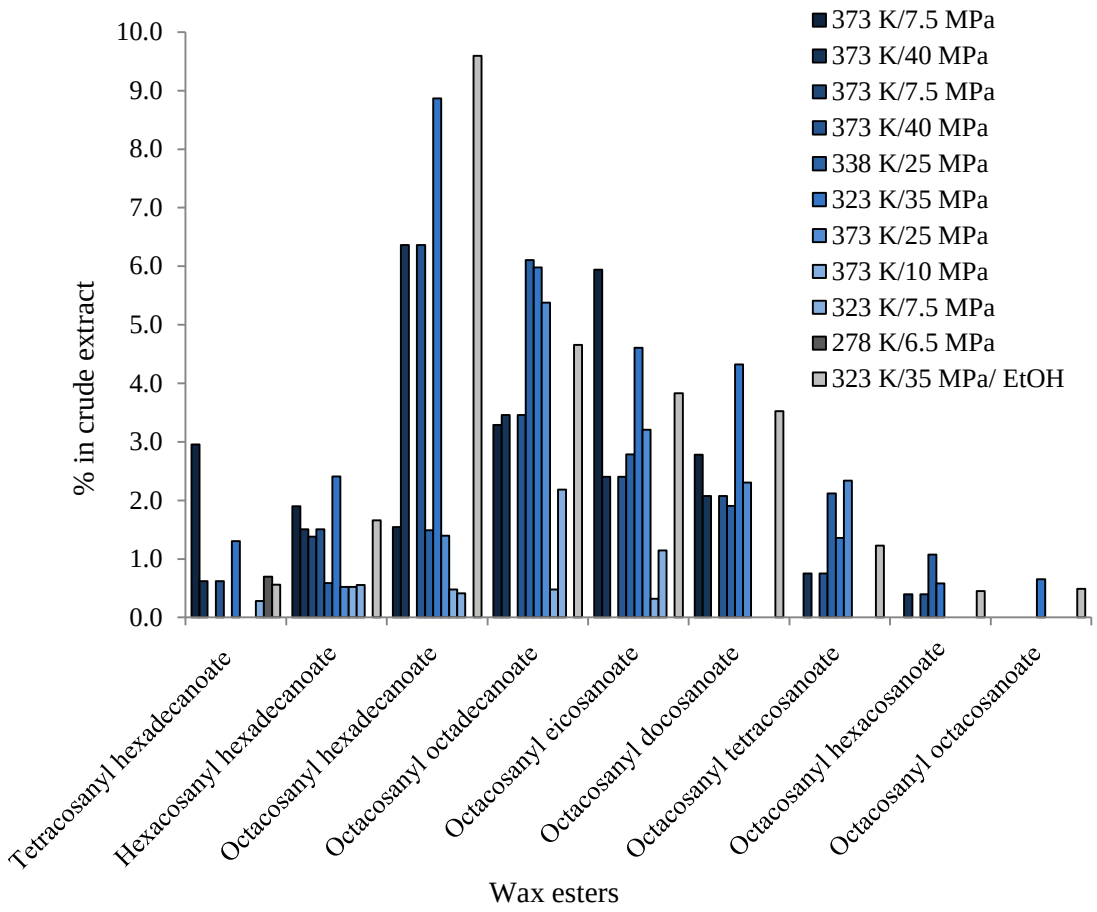


Figure 3.17: Percentage of individual wax esters in CO_2 extracts (originally in colour)

3.3.6 Sterols

Sterols and their derivatives were found at between 0.5 – 27.4% in the CO_2 extracts as shown in Figure 3.18. The 3 main plant sterols, campesterol, stigmasterol and β-sitosterol were identified again in the CO_2 extracts with β-sitosterol being the most abundant which is in agreement with the solvent extracts. The relative abundance for the individual molecule and the percentage presented in the crude extracts is shown in Figure 3.19. The second most abundant sterol derivative in the extracts was the oxidised form of β-sitosterol: Δ^4-sitosten-3-one, which was also found to be a molecule of high abundance in the solvent extracts.

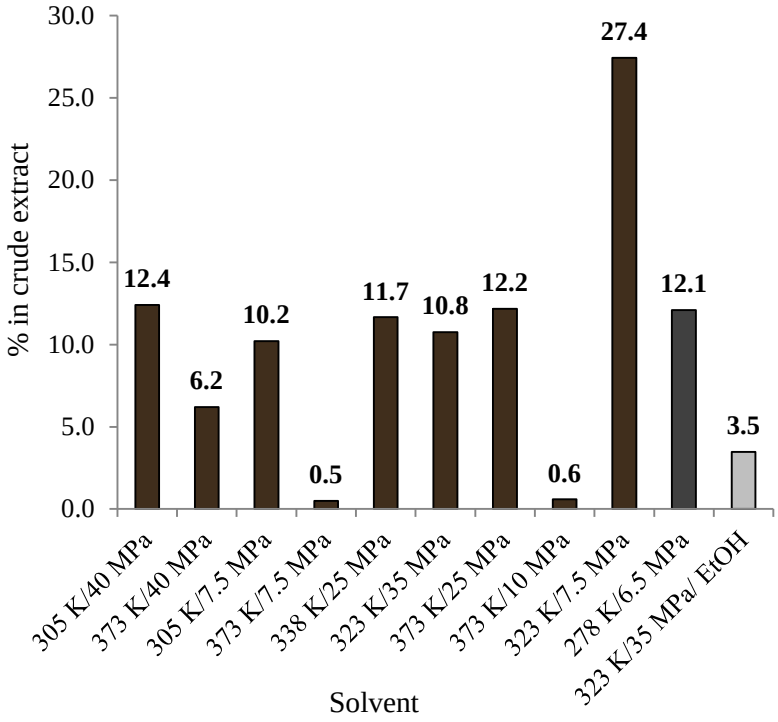


Figure 3.18: Percentage of sterols and its derivatives in CO_2 crude extracts

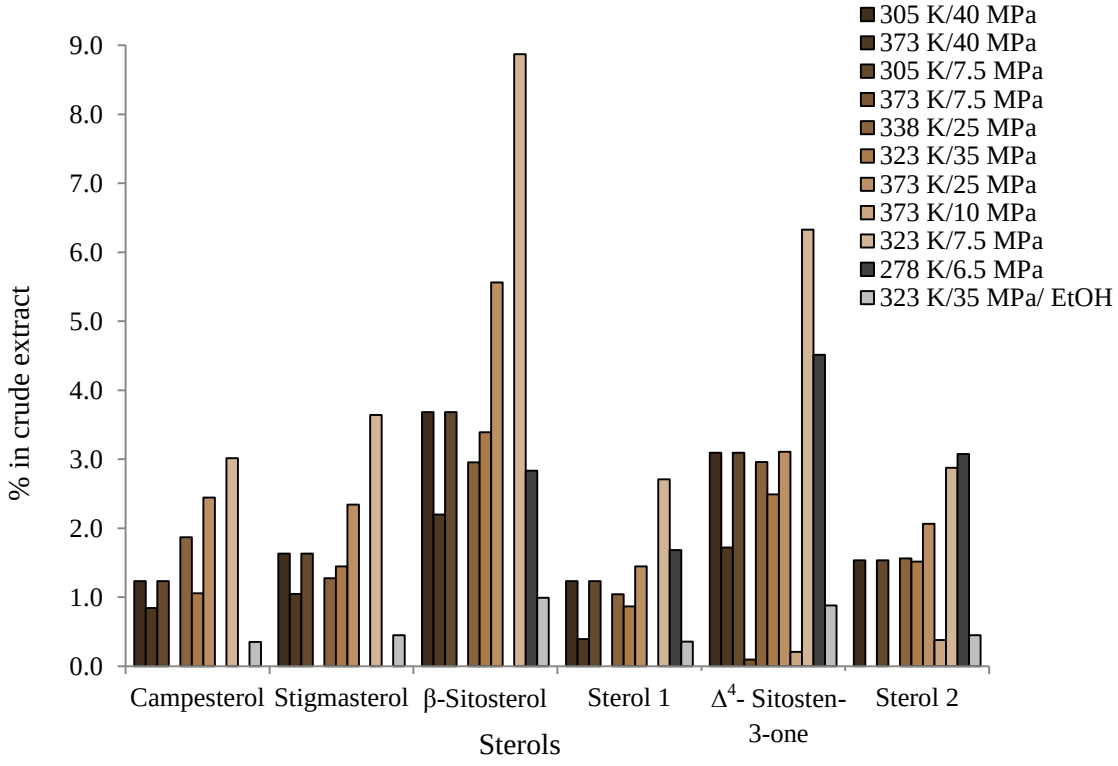


Figure 3.19: Percentage of individual sterols and its derivatives in CO_2 extracts

3.3.7 β-Diketones

In agreement with the solvent extracts, only two β-diketones were found in the CO_2 extracts. This group of compound is presented at a very high level in the CO_2 extracts as up to 34.5% of β-diketones can be found in a single wax extract as shown in Figure 3.20. As shown in Figure 3.21, 14,16-hentriacontanedione is the most abundant β-diketone identified and the highest β-diketone containing extract has up to 34.5% of just 14,16-hentriacontane as the other β-diketone 16,18-triatriacontanedione was not found. No published solubility of β-diketones in CO_2 was found but β-diketones from wheat straw showed great solubility in liquid CO_2, supercritical CO_2 and supercritical CO_2 with co-solvent ethanol.

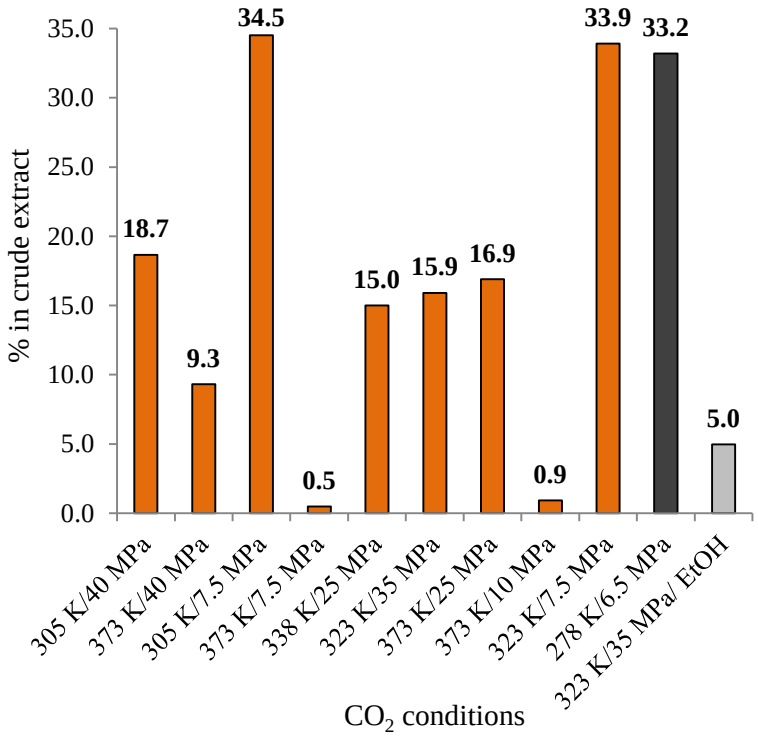


Figure 3.20: Percentage of β-diketones in CO_2 crude extracts

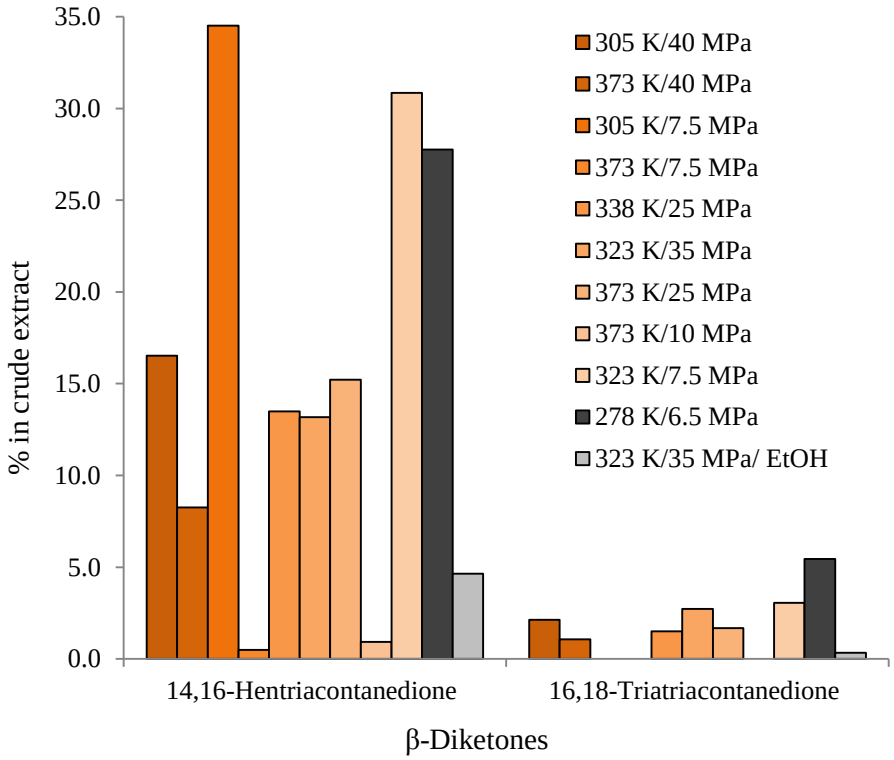


Figure 3.21: Percentage of individual β-diketones in CO_2 extracts

3.3.8 Summary of wax components quantified in CO_2 extracts

The lipophilic fraction of the wheat straw wax from different CO_2 extracts were successfully identified and quantified. The wax compounds identified were identical to the solvent extracts so it can be concluded that the difference between both organic solvent and CO_2 extracts obtained under different conditions is the relative abundance of the wax compounds. The quantified components were grouped and plotted against the percentage yield as shown in Figure 3.22 and the data is also displayed in Table 3.14. Unlike the solvent extracts where up to 84% of the extracts were not identified using GC-FID, only 43% of unidentified compounds were present in CO_2 extracts. This suggests that using CO_2 as a solvent is more selective for wax extracts. By simply manipulating the temperature and pressure of the CO_2 conditions, it is possible to change the solubility of wax compounds to give a distinct mixture of wax compounds which was apparent through colour, texture and odour. The colour of the straw waxes ranged from light yellow to a darker brown-yellow. The liquid CO_2

extract was the lightest and the supercritical CO_2 extract was the darkest in colour and this can reflect the selectivity of wax compounds in Figure 3.22.

Liquid CO_2 is selective for smaller molecular weight compounds as the solubility of wax compounds in liquid CO_2 was lower compared to CO_2 under supercritical conditions and this was reflected by the lack of wax esters in the extract. For this reason, it can be concluded that the use of liquid CO_2 is not ideal for the extraction of wheat straw waxes but could be used as a pre-treatment process to extract some of the wax compounds selectively and then increasethe temperature and pressure of the CO_2 above its critical point to extract the wax esters remaining on the straw. This would result in one fraction containing the majority of wax groups (free fatty acids, hydrocarbons, fatty alcohols, aldehydes, sterols and β-diketones) and another high wax esters-containing fraction. A 10% co-solvent ethanol was also explored and it showed the solubility of compounds increased due to the increased polarity. This can be seen from the rise in extraction yield from 1% to 1.6% but the percentage of identified lipophilic compounds remain the same so it can be concluded that the use of 10% ethanol as a co-solvent has no advantage over the use of neat CO_2 under the same conditions. However, the use of ethanol has been shown to increase the solubility of wax esters by giving the highest percentage of wax esters presented in the extract.

The identified wax compounds were shown as µg per g of wheat straw in Table 3.15 and some important trends have been noted. In hydrocarbons, at medium pressure (338K/373K and 25 MPa), the elevated temperature does not appear to have any beneficial effect on the yield. When the temperature was increased from 305 K to 373 K at 40 MPa, the level of alkanes almost tripled. This highlighted the importance of increased pressure on alkane extraction. For sterols, the data from Table 1 shows increased pressure from 25 to 40 MPa does not have a significant effect on sterol yield which indicated that increasing the CO_2 density to above 600 $g.L^{-1}$ would not benefit sterol extraction. Up to 30% of long chain aliphatic wax esters are extracted at high temperature (373 K). The data showed that a higher proportion of wax esters have been extracted at higher temperature which could be due to the high melting points. The melting point (T_m) of wax esters are much higher than other compounds found in the extracts due to the extended chain length. Saturated wax ester tetracosanyl tetracosanate ($C_{24:0} - C_{24:0}$) has a T_m of > 348 K and an increase of 1 – 2 °C per extra

carbon on the molecules.[274] The main factor influencing the T_m was shown to be the chain length of the wax esters.[274] The smallest wax ester identified was tetracosanyl hexacosanoate ($C_{24:0} - C_{26:0}$) and this means that the fraction is not molten until the extraction temperature is at least 348 K. At high (373 K) temperature, it is believed that the wax esters melt on the surface of the straw which can enhance the solubility of these long chain wax esters (> C_{40}) in $scCO_2$. With β-diketones, it is important to note that about 1000 $\mu g.g^{-1}$ of 14,16 hentriacontanedione in straw can be extracted at critical temperature and pressure and the level remained similar at increased temperature of up to 373 K and increased pressure of 35 MPa. This indicates that the solubility of β-diketone in $scCO_2$ is very high. The data showed that this valuable molecule can potentially be isolated at the critical point of CO_2 (at about 35% purity), then increasing the temperature and pressure for the extraction of the other wax components.

As well as the identified wax components, there are other molecules that were expected to be present in the extracts which were discussed in Chapter 2. Since the polarity of CO_2 is similar to hexane, the extractives can be predicted to be similar.[231] Other minor wax components such as phenolic acids, resin acids and glycerides were identified in wheat.[111, 210] The presence of glycerides was assessed using FAME analysis as per the method described in Section 6.3. The highest CO_2 density extract (373 K/ 40 MPa) and the extract carried out using co-solvent ethanol under the same conditions, both contain the highest percentage of unknown compounds. In the extracts obtained using $scCO_2$ condition (373 K/ 40 MPa) and $scCO_2$ with ethanol, the level of glycerides was quantified using the method described in Section 6.3.1giving up to 5.5% and 14.5% respectively. The calibration for the fatty acids can be found in Section 2.5.1. Further investigations need to be carried out to fully characterise these whole extracts.

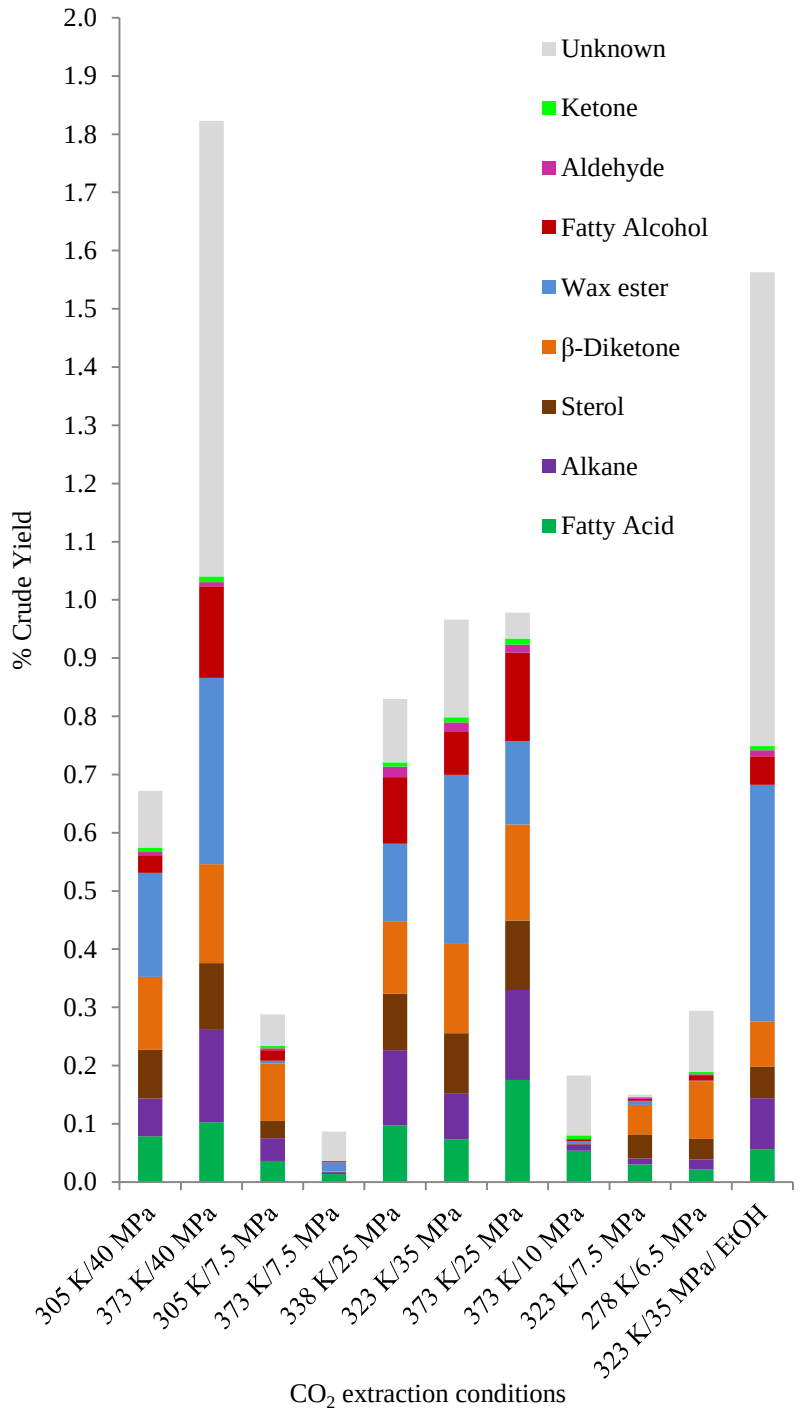


Figure 3.22: The lipophilic fraction of the wheat straw wax in various crude CO_2 extracts

Table 3.14: Quantification of wheat straw wax components in various solvent extracts

Identification	µg/g of biomass (n = 3)										
	373 K/ 40 MPa	373 K/ 25 MPa	323 K/ 35 MPa	338 K/ 25 MPa	305 K/ 40 MPa	305 K/ 7.5 MPa	373 K/ 10 MPa	323 K/ 7.5 MPa	373 K/ 7.5 MPa	278 K/ 6.5 MPa	323 K/ 35 MPa/ EtOH
Tetradecanoic acid	78 ± 12	76 ± 11	47 ± 4	76 ± 12	63 ± 1	36 ± 5	48 ± 5	11 ± 5	4 ± 1	20 ± 4	56 ± 13
Hexadecanoic acid	476 ± 13	642 ± 34	398 ± 34	560 ± 29	361 ± 24	232 ± 3	267 ± 16	77 ± 14	96 ± 14	130 ± 25	374 ± 22
Octadecadienoic acid	142 ± 16	333 ± 23	77 ± 19	69 ± 12	53 ± 14	62 ± 5	79 ± 13	35 ± 4	9 ± 3	44 ± 1	85 ± 10
Octadecenoic acid	271 ± 23	574 ± 12	158 ± 12	191 ± 10	264 ± 3	*Tr*	121 ± 4	63 ± 7	18 ± 2	17 ± 3	51 ± 12
Octadecanoic acid	58 ± 9	129 ± 45	56 ± 3	76 ± 11	47 ± 5	31 ± 7	24 ± 8	14 ± 2	11 ± 3	*Tr*	*Tr*
Total free fatty acids	**1026** ± 73	**527** ± 125	**736** ± 72	**344** ± 74	**787** ± 47	**361** ± 20	**539** ± 46	**589** ± 32	**139** ± 23	**211** ± 33	**567** ± 57
Heptacosane	109 ± 10	88 ± 10	74 ± 8	75 ± 11	55 ± 5	15 ± 1	29 ± 1	15 ± 5	7 ± 2	23 ± 7	68 ± 13
Nonacosane	280 ± 18	281 ± 24	218 ± 13	231 ± 14	190 ± 7	33 ± 3	51 ± 5	33 ± 14	10 ± 2	59 ± 9	244 ± 27
Hentriacontane	1214[a] ± 45	1176[a] ± 37	414 ± 45	883[a] ± 35	342 ± 15	22[a] ± 5	24[a] ± 2	22 ± 4	16[a] ± 3	95[a] ± 17	478 ± 19
Triatriacontane	*Tr*	*Tr*	74 ± 4	106 ± 5	60 ± 3	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	83 ± 7
Total hydrocarbons	**1603** ± 73	**465** ± 71	**779** ± 70	**459** ± 65	**647** ± 30	**207** ± 9	**104** ± 8	**207** ± 23	**33** ± 7	**177** ± 33	**873** ± 66
Campesterol	153 ± 13	239 ± 11	102 ± 10	155 ± 6	83 ± 5	30 ± 7	*Tr*	2 ± 1	*Tr*	0	55 ± 14
Stigmasterol	191 ± 16	229 ± 4	140 ± 11	106 ± 7	110 ± 15	36 ± 1	*Tr*	10 ± 3	*Tr*	0	70 ± 9
β-Sitosterol	401 ± 34	544 ± 30	327 ± 15	245 ± 16	247 ± 4	89 ± 5	*Tr*	29 ± 6	4 ± 1	83 ± 4	155 ± 18
Sterol 1	72 ± 7	141 ± 8	83 ± 15	87 ± 10	83 ± 6	27 ± 6	*Tr*	29 ± 7	*Tr*	50 ± 3	56 ± 6
Δ^4-Sitosten-3-one	313 ± 8	304 ± 19	241 ± 12	246 ± 6	208 ± 13	*Tr*	4 ± 1	35 ± 4	*Tr*	133 ± 15	137 ± 10
Sterol 2	*Tr*	202 ± 10	146 ± 34	130 ± 7	103 ± 8	29 ± 5	7 ± 1	35 ± 4	*Tr*	90 ± 16	70 ± 9
Total sterols	**1130** ± 78	**1191** ± 82	**1040** ± 97	**968** ± 52	**834** ± 51	**274** ± 24	**11** ± 2	**37** ± 25	**4** ± 1	**356** ± 38	**543** ± 66
14,16-Hentriacontanedione	1505 ± 56	1231 ± 56	1052 ± 56	1120 ± 56	1111 ± 19	994 ± 18	17 ± 4	309 ± 23	4 ± 1	816 ± 28	725 ± 34
16,18- Triatriacontanedione	195 ± 16	135 ± 4	217 ± 16	125 ± 22	144 ± 14	*Tr*	*Tr*	31 ± 1	*Tr*	160 ± 17	52 ± 12

Table 3.14 (continued)

Total β-diketones	**1700** ± 72	**1366** ± 60	**1270** ± 72	**1245** ± 78	**1254** ± 33	**994** ± 18	**17** ± 4	**339** ± 34	**4** ± 1	**976** ± 45	**777** ± 46
Tetracosanyl hexadecanoate	113 ± 11	51 ± 4	126 ± 16	*Tr*	79 ± 5	*Tr*	*Tr*	3 ± 1	26 ± 5	20 ± 4	88 ± 11
Hexacosanyl hexadecanoate	274 ± 15	137 ± 8	233 ± 18	49 ± 10	194 ± 14	40 ± 3	10 ± 4	6 ± 2	17 ± 5	*Tr*	260 ± 14
Octacosanyl hexdecanoate	1160 ± 45	526 ± 19	857 ± 39	123 ± 8	863 ± 6	*Tr*	9 ± 4	4 ± 2	13 ± 4	*Tr*	1500 ± 56
Octacosanyl octadecanoate	630 ± 25	314 ± 19	577 ± 27	507 ± 24	358 ± 35	*Tr*	9 ± 3	22 ± 4	29 ± 7	*Tr*	728 ± 35
Octacosanyl eicosanoate	438 ± 33	225 ± 15	445 ± 26	231 ± 17	156 ± 3	*Tr*	6 ± 1	11 ± 1	52 ± 18	*Tr*	599 ± 33
Octacosanyl docosanoate	378 ± 31	229 ± 20	417 ± 24	158 ± 13	83 ± 5	*Tr*	*Tr*	*Tr*	24 ± 4	*Tr*	551 ± 17
Octacosanyl tetracosanoate	137 ± 13	*Tr*	131 ± 13	176 ± 18	53 ± 8	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	192 ± 12
Octacosanyl hexacosanoate	72 ± 11	*Tr*	*Tr*	89 ± 5	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	70 ± 13
Octacosanyl octacosanoate	*Tr*	*Tr*	63 ± 19	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	76 ± 15
Total wax esters	**3203** ± 184	**430** ± 85	**2906** ± 182	**472** ± 95	**1787** ± 76	**40** ± 3	**33** ± 12	**46** ± 10	**160** ± 43	**20** ± 4	**4062** ± 206
Octacosanol	1570[a] ± 78	1521[a] ± 56	733 ± 26	1142[a] ± 37	303 ± 24	185[a] ± 17	31[a] ± 5	22 ± 3	16[a] ± 3	95 ± 10	491 ± 34
Total fatty alcohol	**1570** ±78	**1521** ± 56	**733** ± 26	**1142** ± 37	**303** ± 24	**185** ± 17	**31** ± 5	**22** ± 3	**16** ± 3	**95** ± 10	**491** ± 34
Octacosanal	108 ± 6	207 ± 33	168 ± 15	182 ± 17	86 ± 11	33 ± 4	*Tr*	16 ± 3	6 ± 1	17 ± 1	67 ± 13
Total aldehyde	**108** ± 6	**207** ± 33	**168** ± 15	**182** ± 17	**86** ± 11	**33** ± 4	***Tr***	**16** ± 3	**6** ± 1	**17** ± 1	**67** ± 13
Total identified	**8770** ± 564	**4186** ±	**6898** ± 534	**3670** ± 418	**5395** ± 272	**1908** ± 95	**704** ± 77	**1322** ± 130	**565** ± 79	**1566** ± 164	**6411** ± 488

[a] = co-elution and values shown are the combined quantities, *Tr* = Trace levels < LOQ, LOQ shown in Section 2.5.1

3.4 Conclusion and future work

Following the solvent extraction study in Chapter 2, the use of CO_2 as a green alternative solvent instead of traditional organic solvents for the extraction of low cost, high volume agricultural waste wheat straw (*Triticum aestivum*) has been studied. Qualitative analysis by GC-MS and quantitative analysis by GC-FID were proved to be successful. The analysis can be improved by fractionation of the individual wax groups so that a tailored analytical method could be applied to improve separation and precision. Liquid CO_2, neat supercritical CO_2 and supercritical CO_2 with ethanol were examined. The chemical composition of the CO_2 extracts were found to be identical to the solvent extracts which is expected so the key wax molecules were quantified. Liquid CO_2 extraction give a very low selective wax yield which consist of free fatty acids, hydrocarbons, fatty alcohols, aldehydes, sterols and β-diketones but no wax esters. It can be concluded that liquid CO_2 can be used as part of a pre-treatment on wheat straw to selectively extract high wax esters extract by using $scCO_2$. The addition of co-solvent ethanol with $scCO_2$ lead to complete extraction but failed to extract wax molecules relatively which means the extraction overall is unselective. Supercritical CO_2 extracts showed both high extraction yields with optimised temperature and pressure and selectivity towards wax components so therefore the effect of temperature and pressure was investigated.

Supercritical CO_2 conditions namely temperature and pressure were investigated and optimised using 2^2 factorial design. Temperature and pressure both have direct influence on CO_2 density which was found to be the main parameter that drives the extractions. However, it is important to note that other parameters such as viscosity, diffusivity and polarity are also affected by the modification of temperature and pressure. A first order polynomial equation was applied to both the percentage crude extraction yield and the individual wax groups. The percentage crude yield, wax ester and β-diketone yield correlate well and no correlation was found with free fatty acid, hydrocarbon, aldehyde and sterol yield. This suggested that wax esters and β-diketones have a linear behaviour with CO_2 temperature and pressure. Strangely, the percentage crude yield showed astrong correlation even though not all the wax groups behave linearly. Further work is needed to understand the behaviour of the wax molecules in CO_2 by carrying out solubility tests. Extraction times and flow rate have not been investigated so the experiments can be extended to 2^3 or 2^4 factorial design in order to establish any relationship between extraction times and flow rate.

The most well-established density-based model, the Chrastil model, for supercritical CO_2 was explored for the first time with the extraction of wheat straw wax. This model was used by assuming that equilibrium extraction of wax in a flow system has been achieved and that the $scCO_2$ would form solvated solute complexes with the wax molecules. In the case of applying this model to this extraction, it proved difficult as this is a multi-component system. Again, the model was applied to both the percentage crude extraction yield and the individual wax groups. The model will only have a correlation if there is a CO_2 density relationship with the extraction yields. As the extractions were carried out on a flow system at a fixed extraction time for all nine different $scCO_2$ conditions, it is difficult to gather if the system was in equilibrium. Further work must be carried out at different extraction times and flow rates in order to gain more understanding of the different extractions as the current data only provide a "snapshot" of the investigation. The experiments may need to switch from flow-through to fixed volume at specific temperature and pressure but new problems could arise as the CO_2 properties, such as polarity, may change as molecules are dissolved or saturation of the solvent can occur due to, the vast mixtures of different molecules. As the raw materials is a natural product containing semi-crystallised layered cuticular wax, the different wax molecules would align differently on different layers of the straw surfaces so therefore it may prove difficult to model. Diffusivity of CO_2 through the straw is very important and further investigation on the physical pre-treatment will be needed to optimise the best method for pre-treatment and particle size for CO_2 extraction. However, it is important to be aware that the extracted straw residues is a valuable product and not a waste as it can be used for further processing such as microwave pyrolysis which was discussed in the as the concept of straw bio-refinery in Chapter 1. The Chrastil model was successfully applied to percentage crude yield and wax ester yield based on the extraction conditions, which suggested that equilibrium was achieved during its residence time. Again, strangely, a strong correlation was found with percentage crude yield as the majority of the wax groups do not have any correlations. Even though there is a lack of correlation with the majority of the wax groups, this study had shown the potential to use a density-based model to establish optimised conditions for a specific blend of chemical composition of wax. New improved models have been developed and adapted over the years to suit the extraction process so for future work, this will need to be investigated and draw some correlation between CO_2 conditions and solubility of wax compounds.

Solubility data for the key wax compounds were gathered. The majority are based on binary systems which give only limited information on the behaviour of the wax molecules in the $scCO_2$ phase at different conditions. In a multi-component system such as in the case of extraction of wheat straw waxes, the solubility of the wax compounds would alter. It would be of great interest to investigate the variation in behaviour of wax molecules in both situations. This would provide important solubility information that has not only benefit in extraction but also would assist in process development in using CO_2 for fractionation.

The promising preliminary results show that supercritical CO_2 can be used as a green alternative to traditional organic solvents from the chemical compositional analysis. Wax compounds have shown a vast range of potential applications as discussed in Chapter 2. There is a real interest both academically and industrially to bring the product and process to commercialisation following the success of the use of CO_2 as a green solvent. The effect of temperature and pressure for CO_2 has been studied and conditions have been optimised but further work is needed on the investigation of extraction time and flow rate. It can be concluded that the next stage is pilot scale trials. With the initial optimisation data and success with pilot scale by Deswarte, carried out previously, it would be interesting to increase scale to production scale to examine any scaling up effect.[16] By scaling up, the feasibility of using supercritical CO_2 for straw wax extraction would be assessed and the physical properties of the resulting wax products can be tested and any further downstream processing can be investigated.

Chapter 4

Straw wax screen and production scale supercritical carbon dioxide extraction of cereal straw waxes

Oral presentation given at 1st NORthern Sustainable Chemistry meeting (NORSC), York, UK, October 2010

Oral presentation given at 7th International Conference on Renewable Resources and Biorefineries (RRB7), Bruges, Belgium, June 2011

4. STRAW WAX SCREEN AND PRODUCTION SCALE SUPERCRITICAL CARBON DIOXIDE EXTRACTION OF CEREAL STRAW WAXES

4.1 Introduction

The extraction of wheat straw wax has been demonstrated successfully both by organic solvents and a greener alternative, supercritical CO_2. After assessing the potential of both, it can be concluded that supercritical CO_2 under optimised conditions is the most selective and environmentally friendly method for straw wax extraction so scale up trials were carried out using supercritical CO_2. In this chapter, a number of other low cost straws were screened using the selected organic solvents ethanol and hexane to give an insight into the wax content as well as interesting new molecules that could potentially have a high commercial value. The ethanol and hexane extracts were also quantified by GC-FID to compare the relative abundance of the key important wax groups. It was highlighted previously that the crude wax yield and the chemical composition from hexane extractions are highly comparable with supercritical CO_2 so the data collected from the hexane extractions were used for the selection of raw materials for the production scale supercritical CO_2 extraction. Solvent extraction was used as in preliminary trials instead of $scCO_2$ extraction due to shorter set up time. Wheat, barley and oat were selected from the seven different biomass screens for the scale up trials which were carried out in collaboration with Sundown Products Limited and Evonik Industries. Three tonnes of cereal straw one 1 tonne of wheat, barley and oat straw wax each) was extracted using optimised $scCO_2$ conditions and the scale up effect is discussed. The chemical compositions of the waxes were analysed qualitatively and quantitatively. Physical properties such as melting point were analysed in order to gain a greater understanding of the potential for commercialisation and selected for relevant product formulations. The main advantage of using $scCO_2$ as the extraction solvent is its low environmental impact. As the extraction of waxes requires high pressure it is an expensive technology which can raise many concerns industrially. For this reason, an economic assessment was carried out for the extraction of wax from cereal straw as part of the consideration of using $scCO_2$ extraction.

4.2 Raw materials selection

4.2.1 Raw materials

Cuticular waxes exist on all the plant surfaces. In this section, different biomass wastes are explored as a new source of wax by screening using hexane and ethanol extraction. The focus on the biomass screen is solely based on straw as this part of the plant makes up more than 50% of the dry weight of the crop.[27] Straw 'waxes' from other plant families have also been examined with flax (*Linum usitatissimum*) straw first being first extracted in 1931.[275] However, little work has been carried out on other agricultural straws. The main agricultural crops wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), oat (*Avena sativa*) and oilseed rape (*Brassica napus*), were considered as potential sources of straw as together, the four crops make up about 99% of the total cereal crops grown in the UK.[26] Bagasse was considered as a source of biomass for wax as this is the biggest waste after pressing for its juice in the sugar cane industry. Sugarcane wax has long been of interest and highlighted as a potential new wax, with many attempts to study its chemical composition as far back as 1935 but, yet to be commercialised.[61, 276, 277] Currently, the waste bagasse (*Saccharum officinarum*) is being utilised as a source of bio-ethanol however, if the bagasse can firstly be extracted using $scCO_2$ to create wax products prior to bio-ethanol production then it creates a new idea of the bio-refinery concept.[278] Miscanthus (*Miscanthus giganteus*) is another bio-energy crop and therefore of interest for its potential as a novel source of wax by adding value to the biomass prior to fuel production.[279] Another potential biomass is sunflower *(Helianthus annuus)* and this is of increasing growing volume due to the rise in bio-diesel production.[280] The resulting sunflower straw currently does not have any high value applications so this was a great opportunity to add value to sunflower straw by extraction of high value chemicals. Almost no work had been carried out on the extraction of miscanthus and sunflower straw so this would be an interesting study on potential extraction crops. For these reasons, wheat, barley, oat, oilseed rape, bagasse, miscanthus and sunflower straw were screened for wax content and composition. Figure 4.1 shows pictures of the biomass being studied. All the samples were milled to increase the bulk density following the procedure described in Section 6.2.2. The moisture content of the air-dried milled straw was determined using the method in Section 6.2.1 and it was

ensured that the moisture was no greater than 10% (w/w). All the milled straw was stored in paper sacks in a dry, cool place to keep the samples ventilated.

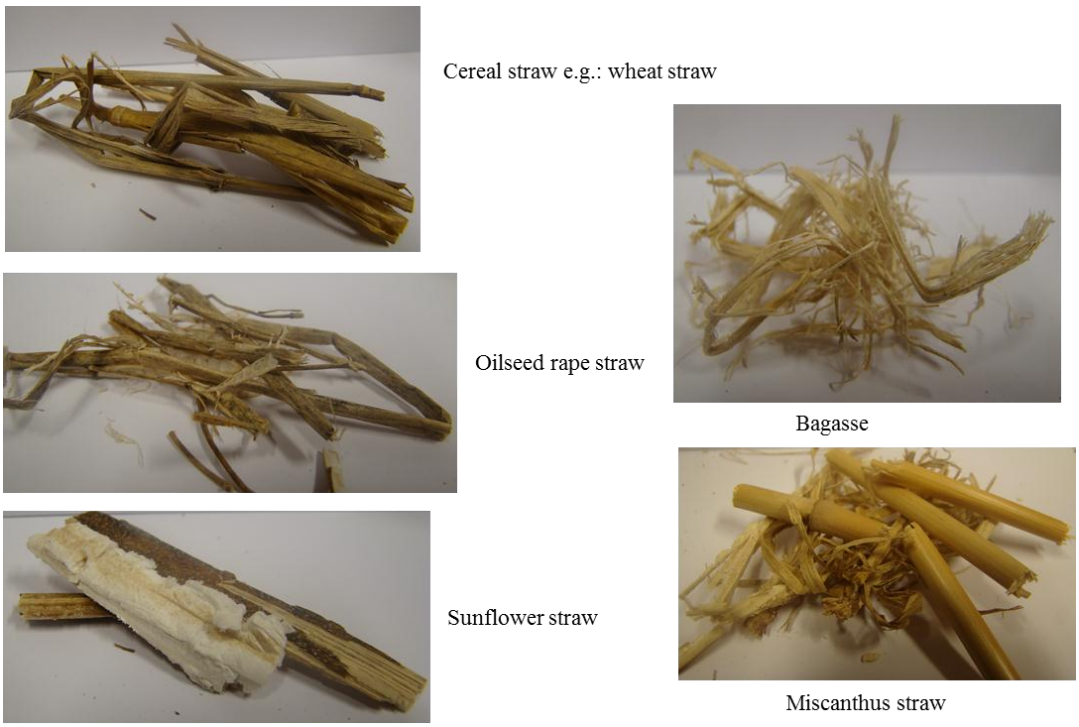


Figure 4.1: Straw samples (wheat, oilseed rape, miscanthus, bagasse and sunflower) used for wax screening (originally in colour)

4.2.2 Identification of chemical compositions from different raw materials

Hexane and ethanol were chosen as the two solvents for the biomass screen due to the distinctly different polarities. The comparison of *n*-alkanes extracted with $scCO_2$ was also demonstrated by wheat straw discussed in Chapter 3. Ethanol is a relatively green polar solvent which is capable of extracting more polar molecules hence the increased yield shown in Chapter 2. With these two solvent systems, a full extraction yield and composition profile for each biomass was obtained. As a large number of straw materials are required to be screened, the multi-point FexIKA® extractor was chosen as the extraction technique as many extractions can be carried out simultaneously. The detail of this extraction method is discussed in Section 6.2.4. Figure 4.2 shows pictures of the hexane and ethanol wax extracts. From the colour and the texture of each extract, it was apparent that the compositions are different. The colour of the

hexane extracts are usually lighter than the ethanol extracts as it is more selective so therefore contains less co-extracted pigments.

Figure 4.2: Hexane and ethanol extracts of the seven different straws (originally in colour)

Table 4.1 shows the list of straws that are under investigation and their descriptions. The yields for the hexane and ethanol extracts are displayed in Figure 4.3 and the biomass label corresponds to the biomass in Table 4.1.

Table 4.1: Description of straw

Straw code	Straw description
WS1	Winter wheat straw (*Triticum aestivum*) (Claire 07)
WS2	Winter wheat straw (*Triticum aestivum*) (Claire 08)
WS3	Winter wheat straw (*Triticum aestivum*) (Charger 08)
WS4	Winter wheat straw (*Triticum aestivum*) (Oakley 08)
WS5	Spring wheat straw (*Triticum aestivum*) (Hereward 08)
BS1	Winter barley straw (*Hordeum vulgare*) (Carat 08)
BS2	Spring barley straw (*Hordeum vulgare*) (Optic 08)
OS	Oat straw (Mixed varieties 08) (*Avena sativa*)
RS1	High docosenoic acid oilseed rape straw (*Brassica napus*)
RS2	Low docosenoic acid oilseed rape straw (*Brassica napus*)
SS	High octadecenoic acid sunflower straw *(Helianthus annuus)*
MS	Miscanthus straw (*Miscanthus giganteus*)
Bag	Bagasse straw (*Saccharum officinarum*)

The hexane yields indicate mainly the lipophilic fraction of the extract and were found to be between 0.3 – 1.4% with oat straw having the highest yield at 1.4%. All the cereal and oilseed rape straws have a consistent hexane yield about 1%. The sunflower, miscanthus and bagasse have very low hexane yields and this could be due to the larger cross-section of the straw meaning that the surface area of cuticular wax would be smaller per gram sample being extracted. For example, it can be observed in Figure 4.1, the sunflower straw has a very large cellulosic inner core which is completely wax free so it would be interesting to separate the straw surface from its inner core for future extractions. All the ethanol yields were considerably higher than the hexane yields as expected and these extracts are suspected to contain more polar compounds such as sugar molecules. In order to determine which of the straw are potentially good as new sources of wax, and can be scaled up for commercialisation, the chemical composition is determined and quantified to give a full wax assessment. Wheat straw (Oakley 08), barley straw (Carat 08) and high docosenoic acid oilseed rape straw were chosen for further analysis to represent their species types. The identification and quantification were carried out by following the procedures

described in Sections 6.3.1 and 6.3.2 respectively. Figures 4.4, 4.5, 4.6, 4.7, 4.8, 4.9 and 4.10 show the GC chromatograms of the hexane extracts for wheat, barley, oat, oilseed rape, sunflower, miscanthus and bagasse straw respectively.

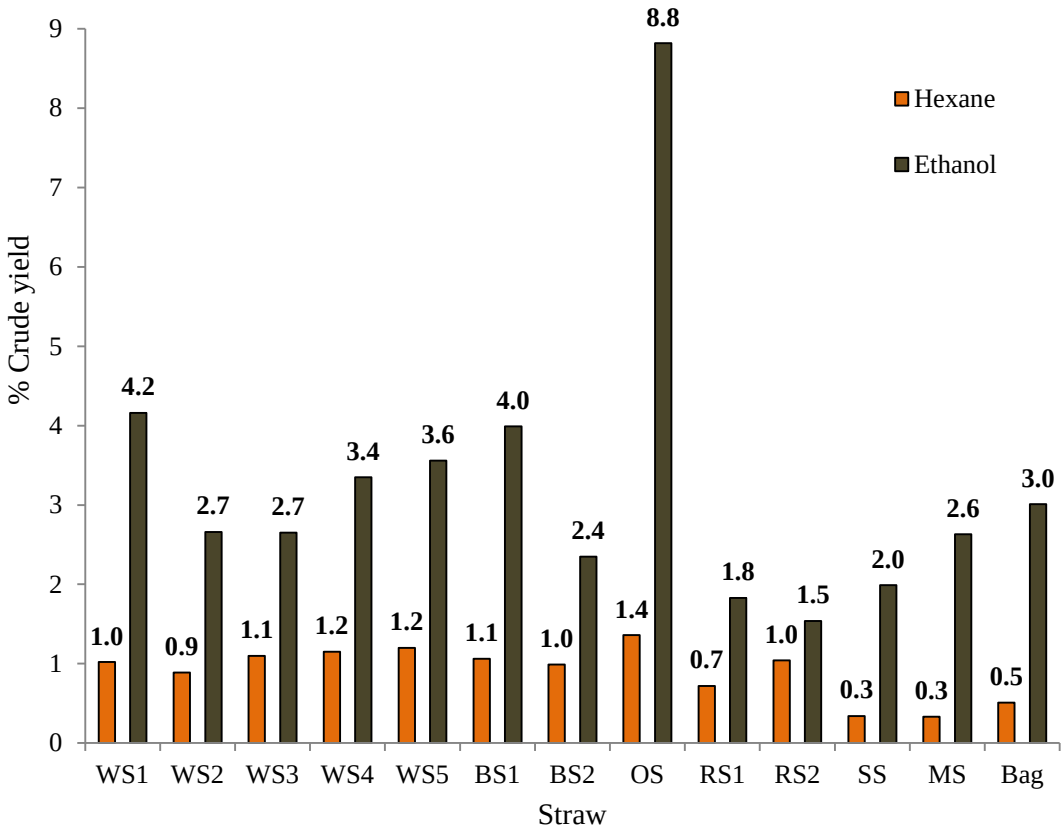
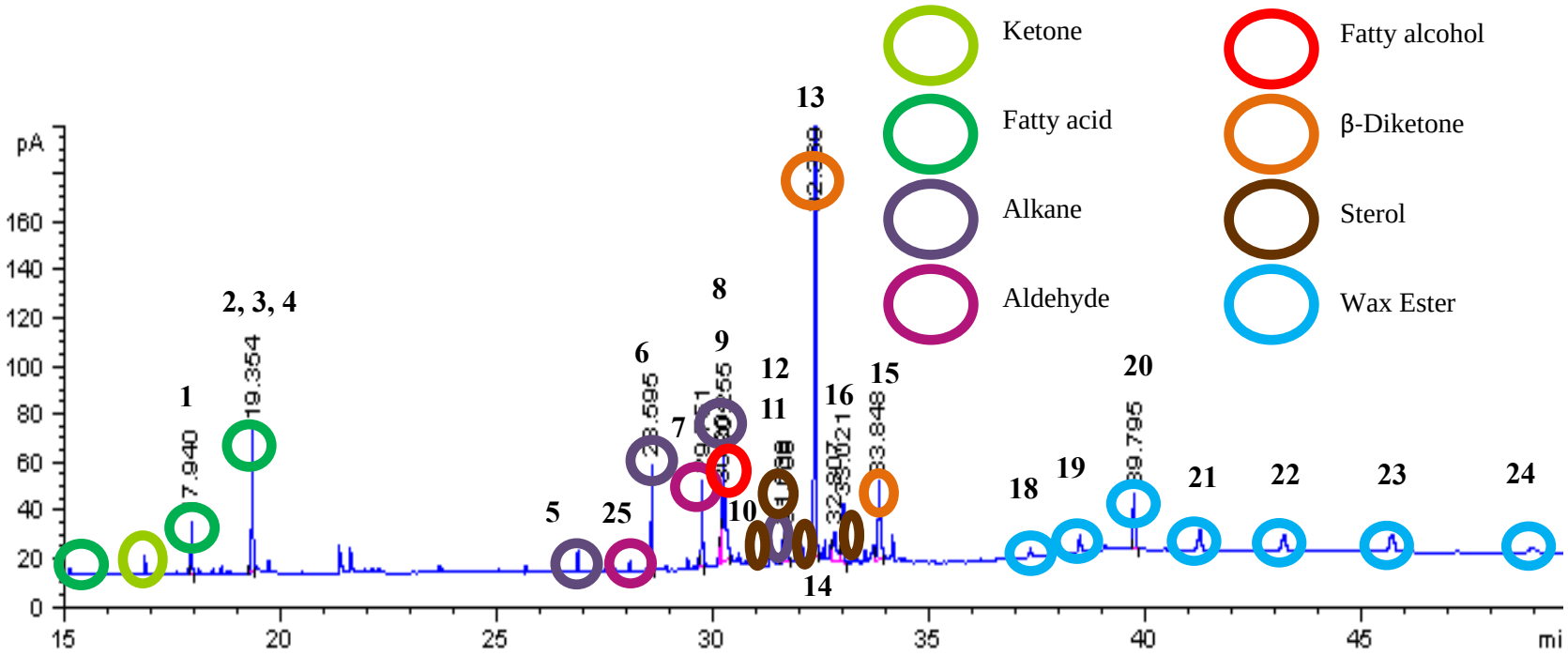
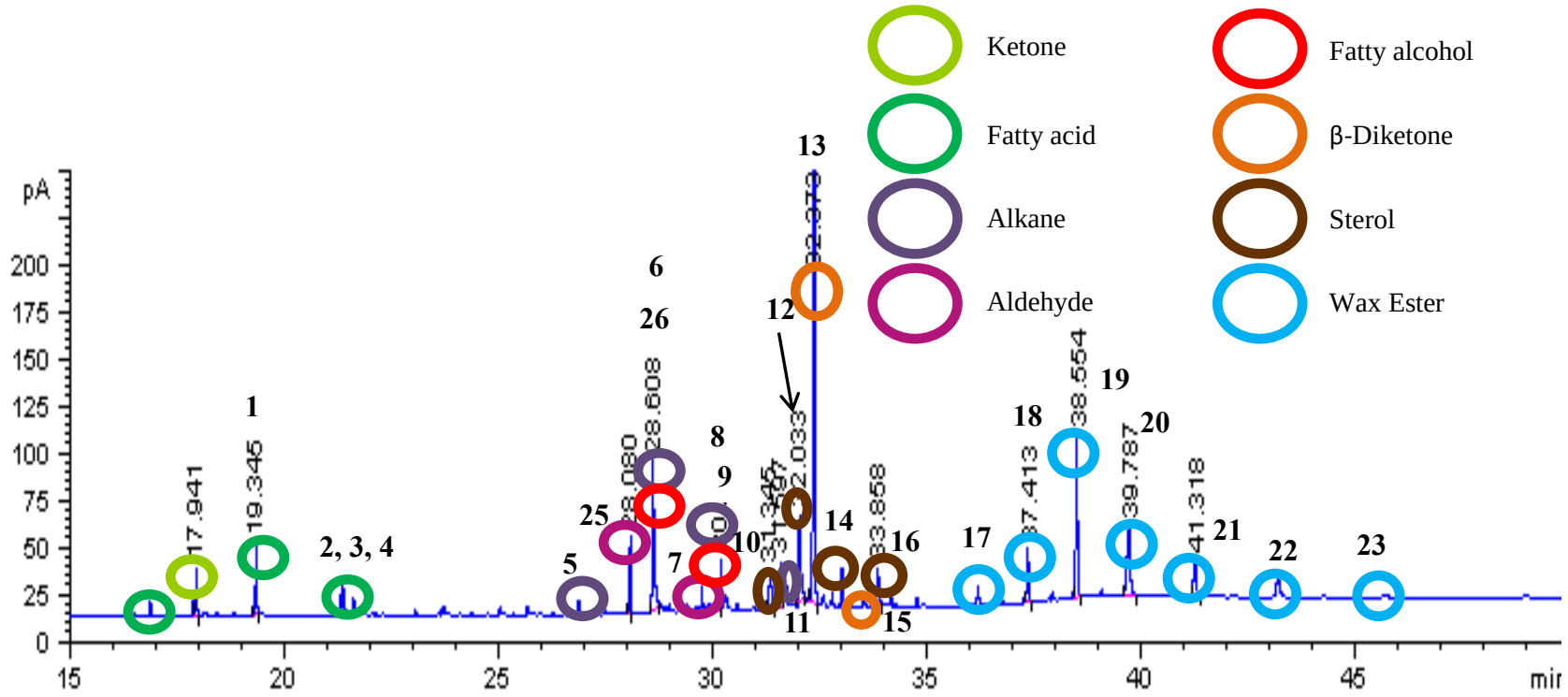


Figure 4.3: Percentage crude yield of hexane and ethanol extracts of different straws (originally in colour)



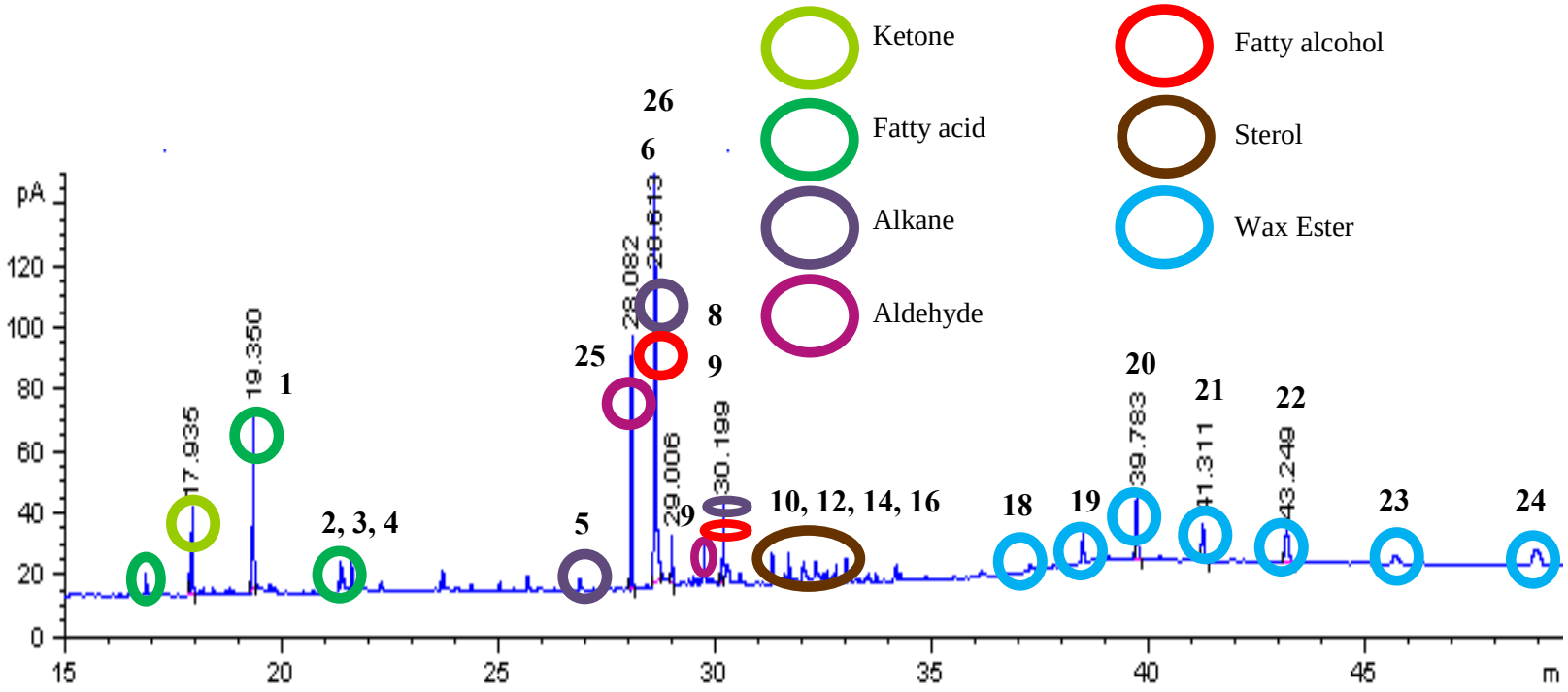
1	Hexadecanoic acid ($C_{16:0}$)	**7**	Octacosanal (C_{28})	**13**	14,16-hentriacontanedione (C_{31})	**20**	Octacosanyl octadecanoate (C_{46})
2	Octadecadienoic acid ($C_{18:2}$)	**8**	Octacosanol (C_{28})	**14**	β-Sitosterol	**21**	Octacosanyl eicosanoate (C_{48})
3	Octadecenoic acid ($C_{18:1}$)	**9**	Hentriacontane (C_{31})	**15**	16,18-triatriacontanedione (C_{33})	**22**	Octacosanyl docosanoate (C_{50})
4	Octadecanoic acid ($C_{18:0}$)	**10**	Campesterol	**16**	Δ^4-Sitosten-3-one	**23**	Octacosanyl tetracosanoate (C_{52})
5	Heptacosane (C_{27})	**11**	Campesterol	**18**	Hexacosanyl hexadecanoate (C_{42})	**24**	Octacosanyl hexacosanoate (C_{54})
6	Nonacosane (C_{29})	**12**	Triatriacontane (C_{33})	**19**	Octacosanyl hexadecanoate (C_{44})	**25**	Hexacosanal (C_{26})

Figure 4.4: GC chromatogram of hexane extract for wheat straw (Oakley 08) (WS4) (originally in colour)



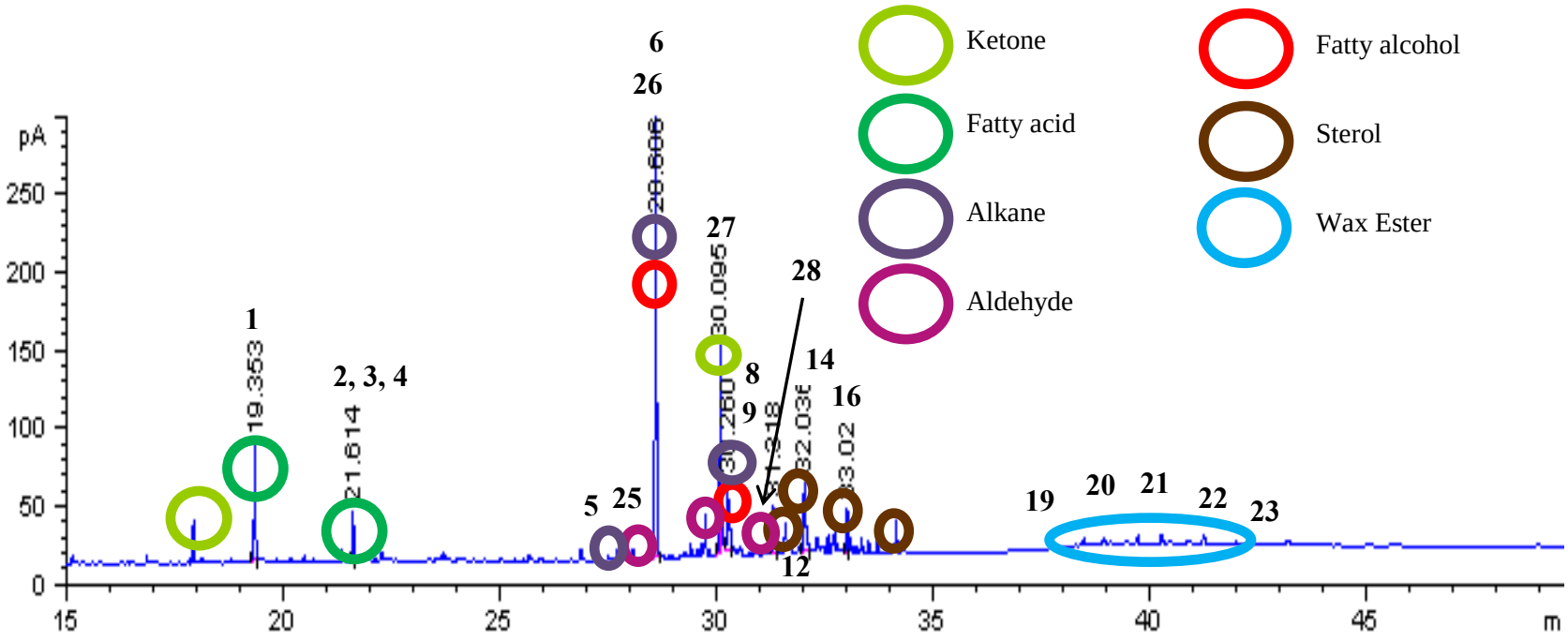
1	Hexadecanoic acid ($C_{16:0}$)	**7**	Octacosanal (C_{28})	**13**	14,16-hentriacontanedione (C_{31})	**19**	Octacosanyl hexadecanoate (C_{44})
2	Octadecadienoic acid ($C_{18:2}$)	**8**	Hentriacontane (C_{31})	**14**	β-Sitosterol	**20**	Octacosanyl octadecanoate (C_{46})
3	Octadecenoic acid ($C_{18:1}$)	**9**	Octacosanol (C_{28})	**15**	16,18-triatriacontanedione (C_{33})	**21**	Octacosanyl eicosanoate (C_{48})
4	Octadecanoic acid ($C_{18:0}$)	**10**	Campesterol	**16**	Δ^4-Sitosten-3-one	**22**	Octacosanyl docosanoate (C_{50})
5	Heptacosane (C_{27})	**11**	Triatriacontane (C_{33})	**17**	Tetracosanyl hexadecanoate (C_{40})	**23**	Octacosanyl tetracosanoate (C_{52})
6	Nonacosane (C_{29})	**12**	Stigmasterol	**18**	Hexacosanyl hexadecanoate (C_{42})	**25**	Hexacosanal (C_{26})
						26	Hexacosanol (C_{26})

Figure 4.5: GC chromatogram of hexane extract for barley straw (Carat 08) (BS1) (originally in colour)



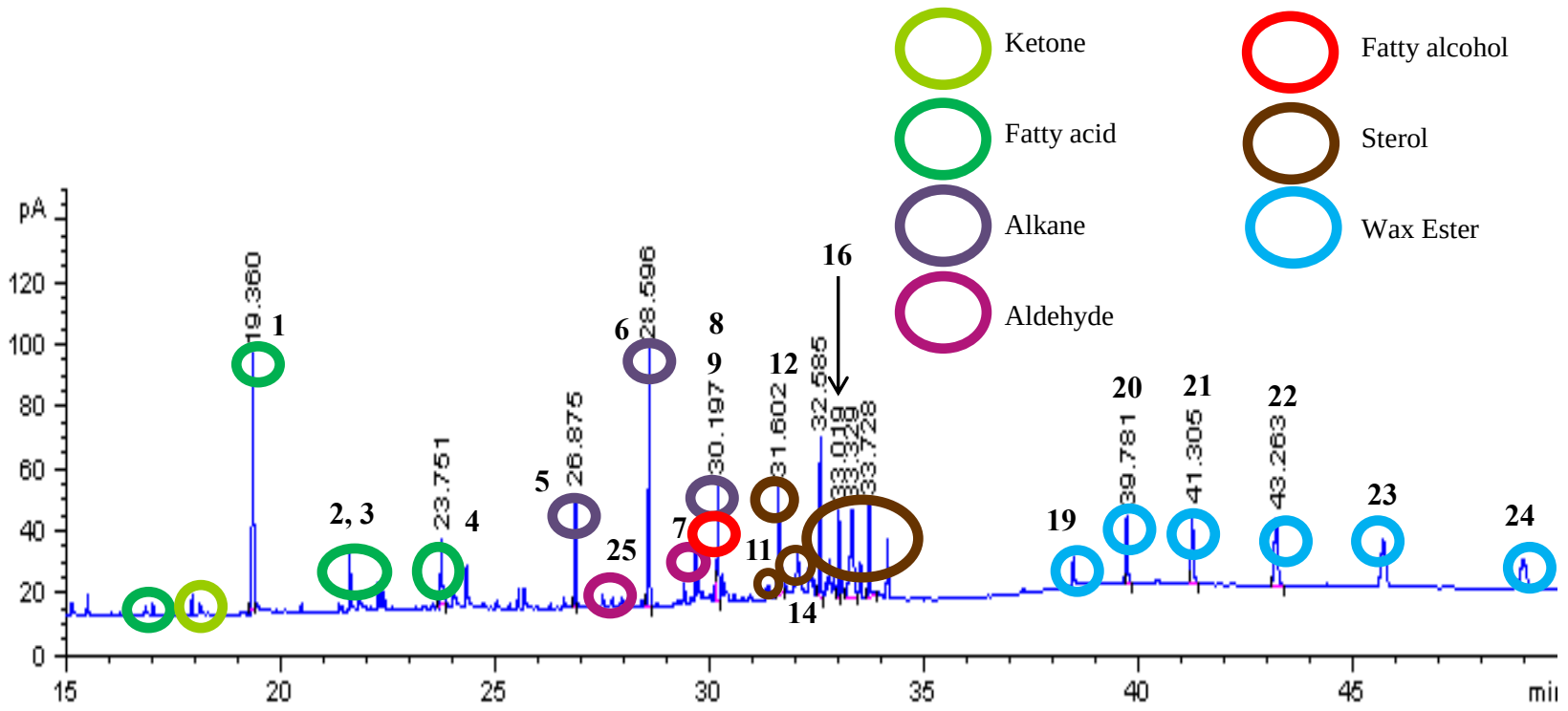
1	Hexadecanoic acid ($C_{16:0}$)	**6**	Nonacosane (C_{29})	**12**	Stigmasterol	**21**	Octacosanyl eicosanoate (C_{48})
2	Octadecadienoic acid ($C_{18:2}$)	**7**	Octacosanal (C_{28})	**14**	β-Sitosterol	**22**	Octacosanyl docosanoate (C_{50})
3	Octadecenoic acid ($C_{18:1}$)	**8**	Hentriacontane (C_{31})	**16**	Δ^4-Sitosten-3-one	**23**	Octacosanyl tetracosanoate (C_{52})
4	Octadecanoic acid ($C_{18:0}$)	**9**	Octacosanol (C_{28})	**18**	Hexacosanyl hexadecanoate (C_{42})	**24**	Octacosanyl hexacosanoate (C_{54})
5	Heptacosane (C_{27})	**10**	Campesterol	**19**	Octacosanyl hexadecanoate (C_{44})	**25**	Hexacosanal (C_{26})
				20	Octacosanyl octadecanoate (C_{46})	**26**	Hexacosanol (C_{26})

Figure 4.6: GC chromatogram of hexane extract for oat straw (OS) (originally in colour)



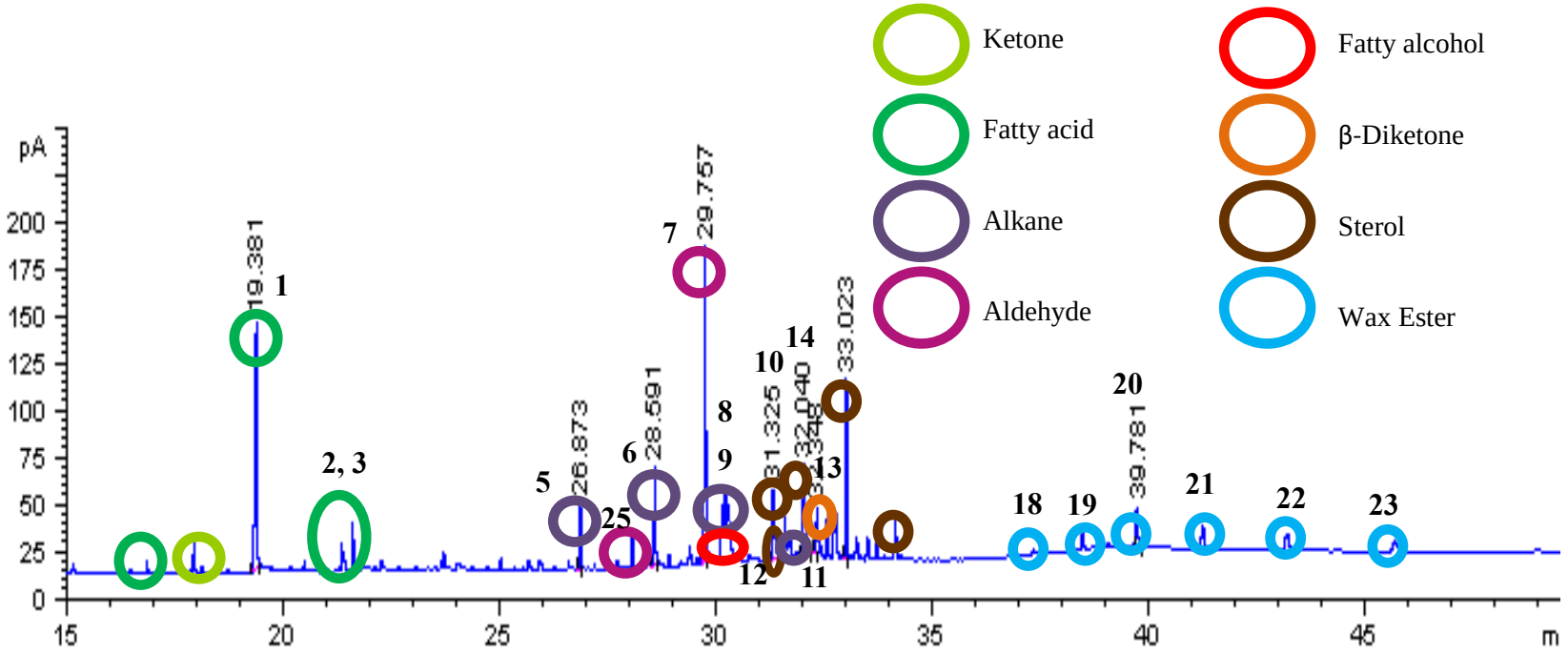
1	Hexadecanoic acid ($C_{16:0}$)	**6**	Nonacosane (C_{29})	**14**	β-Sitosterol	**22**	Octacosanyl docosanoate (C_{50})
2	Octadecadienoic acid ($C_{18:2}$)	**7**	Octacosanal (C_{28})	**16**	Δ^4-Sitosten-3-one	**23**	Octacosanyl tetracosanoate (C_{52})
3	Octadecenoic acid ($C_{18:1}$)	**8**	Hentriacontane (C_{31})	**19**	Octacosanyl hexadecanoate (C_{44})	**25**	Hexacosanal (C_{26})
4	Octadecanoic acid ($C_{18:0}$)	**9**	Octacosanol (C_{28})	**20**	Octacosanyl octadecanoate (C_{46})	**26**	Hexacosanol (C_{26})
5	Heptacosane (C_{27})	**12**	Stigmasterol	**21**	Octacosanyl eicosanoate (C_{48})	**27**	Nonacosan-15-one (C_{29})
						28	Triacontanal (C_{30})

Figure 4.7: GC chromatogram of hexane extract for oilseed rape straw (RS1) (originally in colour)



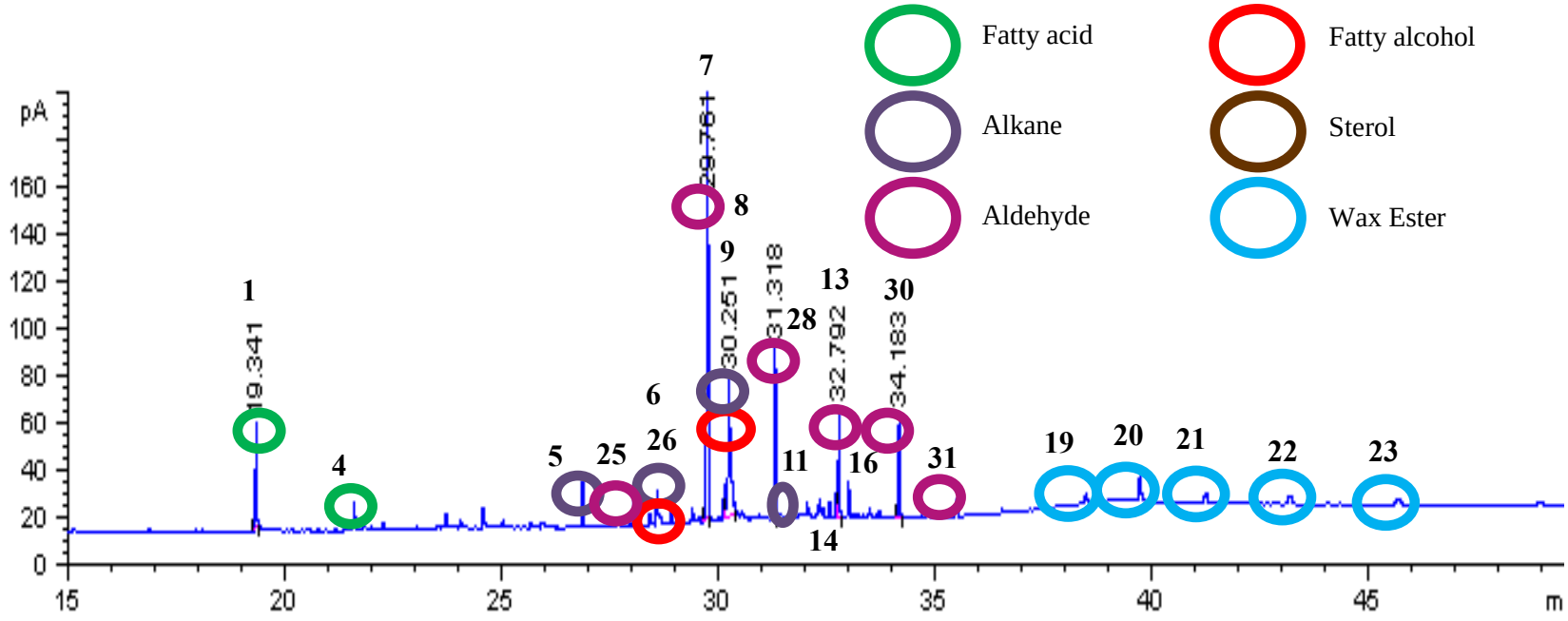
1	Hexadecanoic acid ($C_{16:0}$)	**6**	Nonacosane (C_{29})	**12**	Stigmasterol	**21**	Octacosanyl eicosanoate (C_{48})
2	Octadecadienoic acid ($C_{18:2}$)	**7**	Octacosanal (C_{28})	**14**	β-Sitosterol	**22**	Octacosanyl docosanoate (C_{50})
3	Octadecanoic acid ($C_{18:0}$)	**8**	Hentriacontane (C_{31})	**16**	Δ^4-Sitosten-3-one	**23**	Octacosanyl tetracosanoate (C_{52})
4	Eicosanoic acid ($C_{20:0}$)	**9**	Octacosanol (C_{28})	**19**	Octacosanyl hexadecanoate (C_{44})	**24**	Octacosanyl hexacosanoate (C_{54})
5	Heptacosane (C_{27})	**10**	Campesterol	**20**	Octacosanyl octadecanoate (C_{46})	**25**	Hexacosanal (C_{26})

Figure 4.8: GC chromatogram of hexane extract for sunflower straw (SS) (originally in colour)



1	Hexadecanoic acid ($C_{16:0}$)	**7**	Octacosanal (C_{28})	**12**	Stigmasterol	**20**	Octacosanyl octadecanoate (C_{46})
3	Octadecenoic acid ($C_{18:1}$)	**8**	Hentriacontane (C_{31})	**13**	14,16-hentriacontanedione (C_{31})	**21**	Octacosanyl eicosanoate (C_{48})
4	Octadecanoic acid ($C_{18:0}$)	**9**	Octacosanol (C_{28})	**14**	β-Sitosterol	**22**	Octacosanyl docosanoate (C_{50})
5	Heptacosane (C_{27})	**10**	Campesterol	**18**	Hexacosanyl hexadecanoate (C_{42})	**23**	Octacosanyl tetracosanoate (C_{52})
6	Nonacosane (C_{29})	**11**	Triatriacontane (C_{33})	**19**	Octacosanyl hexadecanoate (C_{44})	**25**	Hexacosanal (C_{26})

Figure 4.9: GC chromatogram of hexane extract for miscanthus straw (MS) (originally in colour)



1	Hexadecanoic acid ($C_{16:0}$)	**8**	Hentriacontane (C_{31})	**19**	Octacosanyl hexadecanoate (C_{44})	**25**	Hexacosanal (C_{26})
4	Octadecanoic acid ($C_{18:0}$)	**9**	Octacosanol (C_{28})	**20**	Octacosanyl octadecanoate (C_{46})	**26**	Hexacosanol (C_{26})
5	Heptacosane (C_{27})	**11**	Triatriacontane (C_{33})	**21**	Octacosanyl eicosanoate (C_{48})	**28**	Triacontanal (C_{30})
6	Nonacosane (C_{29})	**14**	β-Sitosterol	**22**	Octacosanyl docosanoate (C_{50})	**29**	Dotriacontanal (C_{32})
7	Octacosanal (C_{28})	**16**	Δ^4-Sitosten-3-one	**23**	Octacosanyl tetracosanoate (C_{52})	**30**	Tetratriacontanal (C_{34})
						31	Hexatriacontanal (C_{36})

Figure 4.10: GC chromatogram of hexane extract for bagasse (Bag) (originally in colour)

As discussed in Chapter 2, the solvent used during the extraction has very little influence on the chemical composition but the relative abundance of the wax compounds is highly dependent on the organic solvent. For this reason, only the chromatograms of the hexane extracts are shown. Table 4.2 shows a list of wax compounds that were identified in the straw waxes and highlights the new wax compounds found compared to wheat straw wax (Viscount 09) which was discussed in Chapter 2. The identification was carried out by using a combination of EI fragmentation pattern, FI mass spectra, NIST library and KI values.

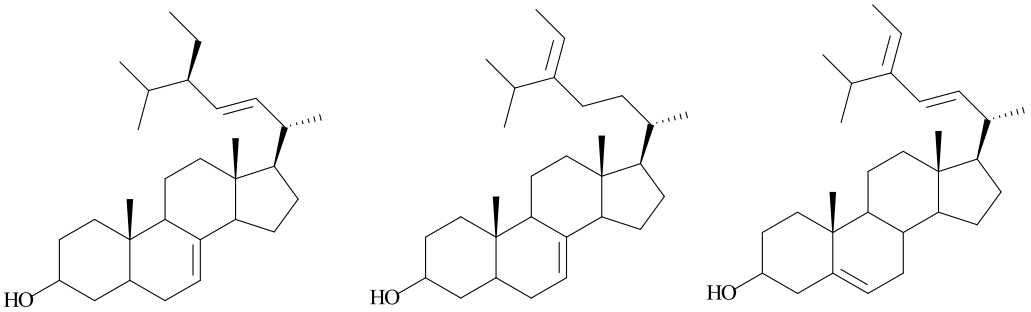
Table 4.2: List of wax compounds identified in straw waxes

Wheat straw wax (Viscount 09)	New wax molecules
Free fatty acids	Eicosanoic acid ($C_{20:0}$)
Hexadecanoic acid ($C_{16:0}$)	
Octadecadienoic acid ($C_{18:2}$)	
Octadecenoic acid ($C_{18:1}$)	
Octadecanoic acid ($C_{18:0}$)	
Hydrocarbons	
Heptacosane (C_{27})	
Nonacosane (C_{29})	
Hentriacontane (C_{31})	
Triatriacontane (C_{33})	
Fatty alcohols	
Octacosanol (C_{28})	Hexacosanol (C_{26})
Aldehydes	
Octacosanal (C_{28})	Hexacosanal (C_{26})
	Triacontanal (C_{30})
	Dotriacontanal (C_{32})
	Tetratriacontanal (C_{34})
	Hexatriacontanal (C_{36})
Wax esters	
Tetracosanyl hexadecanoate (C_{24}:C_{16})	
Wax ester 1 (C_{41})	
Hexacosanyl hexadecanoate (C_{26}:C_{16})	
Wax ester 2 (C_{43})	
Octacosanyl hexdecanoate (C_{28}:C_{16})	
Wax ester 3 (C_{45})	

Table 4.2 (continued)

Octacosanyl octadecanoate (C_{28}:C_{18})	
Wax ester 4 (C_{47})	
Octacosanyl eicosanoate (C_{28}:C_{20})	
Wax ester 5 (C_{49})	
Octacosanyl docosanoate (C_{28}:C_{22})	
Wax ester 6 (C_{51})	
Octacosanyl tetracosanoate (C_{28}:C_{24})	
Wax ester 3 (C_{53})	
Octacosanyl hexacosanoate (C_{28}:C_{26})	
Octacosanyl octacosanoate (C_{28}:C_{28})	
Sterols	
Campesterol	Sterol 3
Stigmasterol	Sterol 4
β-Sitosterol	Sterol 5
Sterol 1	Sterol 6
Δ^4-Sitosten-3-one	Sterol 7
Sterol 2	
ß-Diketones	
14,16-hentriacontanedione (C_{31})	
16,18-triatriacontanedione (C_{33})	
Ketones	
Ketone 1	Nonacosan-15-one (C_{29})

As seen in Table 4.2, there were a total of thirteen new molecules found in the seven different straw waxes. No work had been previously carried out on the extraction of sunflower straw but considerable amounts of sterols have been identified in sunflower oil. Campesterol, ß-sitosterol, stigmasterol, Δ^7-stigmasterol, Δ^7-avenasterol and Δ^5-avenasterol were all identified in high oleic sunflower oil so there is a possibility that these were also found in the sunflower straw.[281, 282] The sterols are all structurally similar which makes identification more difficult. The structures for campesterol, ß-sitosterol and stigmasterol were shown in Figure 2.30 in Chapter 2 and the structures for potential sterols Δ^7-stigmasterol, Δ^7-avenasterol and Δ^5-avenasterol found in sunflower straw are displayed as Figure 4.11.



Δ^7-Stigmasterol Δ^7-Avenasterol Δ^5-Avenasterol

Figure 4.11: Structures of sterols presented in sunflower oil

A new interesting molecule, nonacosan-15-one was found in oilseed rape straw wax as indicated in Figure 4.14. The aliphatic compounds identified in the cuticular waxes from the seven different straws all contain functional groups in the primary position except the β-diketones. Secondary fatty alcohols with odd-numbered carbon chain lengths of C_{23} - C_{33} are commonly found in plant epicuticular waxes and predominantly consist of C_{29} and C_{31}. The primary alcohols series are usually even-numbered as these molecules are derived from the complete reduction reaction of even-numbered fatty acids in the acyl reduction synthetic pathway. [80, 85] The secondary alcohols found in epicuticular waxes are derived from the alkane pathway hence the odd-numbered carbon chain length.[85] The biosynthetic schemes for both of these reactions are illustrated in Figure 1.11 in Chapter 1. Secondary alcohols are much less commonly found compared to primary alcohols and sometimes these secondary alcohols can undergo an oxidation reaction to form ketones so occasionally, a series of alkanes, secondary alcohols and ketones with the same chain length can be found.[85] In barley, a combination of heptacosan-9-ol, nonacosan-10, -11-ols and hentriacontain-10, -11, -12 and -13-ols have been reported.[283] It has been reported by a number of researchers that the addition of the hydroxyl group to the alkanes that forms the secondary alcohols usually occurs in the middle of the carbon chains but the location of the hydroxylation is strictly controlled by enzymes.[64, 283, 284, 285, 286] The secondary alcohols mostly occur freely in plant waxes but it has been reported to be esterified with fatty acids in rose and pine waxes.[65, 286]

In oilseed rape straw wax, nonacosan-15-one was identified as well as nonacosane but no nonacosan-15-ol was found. Kolattukudy *et al.* proved the biosynthetic relationship between these three related wax compounds in broccoli leaf waxes.[64, 287] In the study, both nonacosan-14 and -15-ols were identified at approximately 4:6 ratio however the study showed there is a high preferential oxidation to the ketone with 92% of the ketones is nonacosan-15-one in the wax.[64] The oxidation of the secondary alcohols shows that the reaction is tightly controlled and regulated by enzymes. [64] In oilseed rape straw wax, this could explain why no nonacosan-15-ol was identified as oxidation at the 15th position is highly preferable. This suggested that in oilseed rape straw wax, nonacosane undergoes hydroxylation to form the nonacosan-15-ol which is then quickly oxidised to nonacosan-15-one so in the GC analysis, only the alkane and ketone were found. The identification of nonacosan-15-one was carried out using the EI fragmentation pattern and FI mass spectrum. Figure 4.12 shows the EI fragmentation pattern of nonacosan-15-one. As EI is a harsh ionisation technique, the molecular ion was not observed. Characteristic fragment ions at m/z = 225 and m/z = 241 were seen in the EI mass spectrum. Another characteristic ion was also observed when the high m/z region was magnified; fragment ion of m/z = 240 was also present. The fragmentation pattern for secondary alcohols is very similar to ketones however in ketones, there is a cleavage of the C – C bond adjacent to the carbonyl group which results in a distinct fragment ion of m/z = 225 as shown in Figure 4.14. The formation of m/z = 240 is as a result of McLafferty rearrangement and fragment ion m/z = 241 is generated via McLafferty rearrangement then followed by the migration of a hydrogen atom which is shown in Figure 4.15. Other fragment ions presented in the mass spectrum include m/z = 43, 57, 71 and 85 which are from the alkyl chain as shown in Table 2.7 in Chapter 2. This series of ions showed a difference of 14 mass units in the molecule which suggested the presence of alkyl chain. As the published literature KI for nonacosan-15-one is not available to determine the chain length of the molecule, the molecular ion is required. The molecular ion was observed from the FI mass spectrum which is shown in Figure 4.13. Table 4.3 shows the fragment ions and its percentage base peak intensity as well as the calculated KI value.

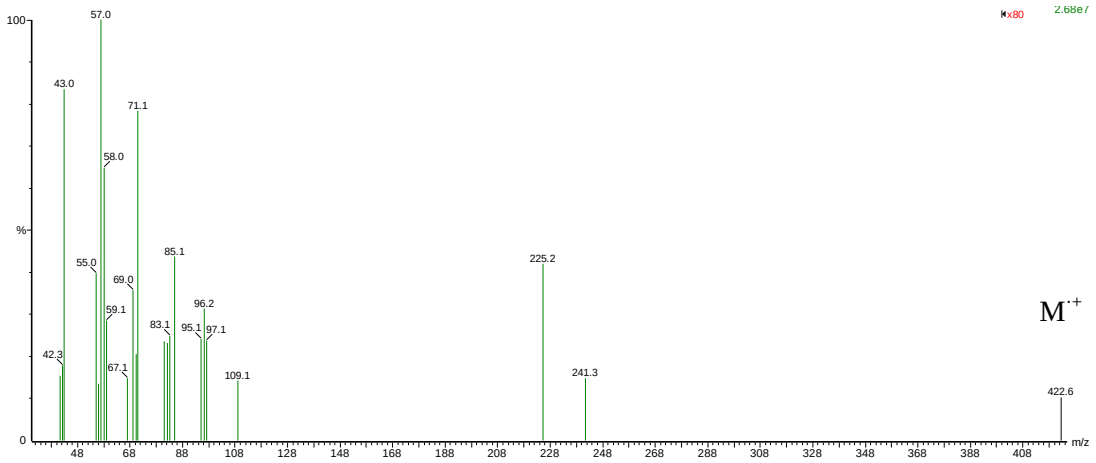


Figure 4.12: EI mass spectrum of nonacosan-15-one (magnified eight times from m/z 400 – 425) (originally in colour)

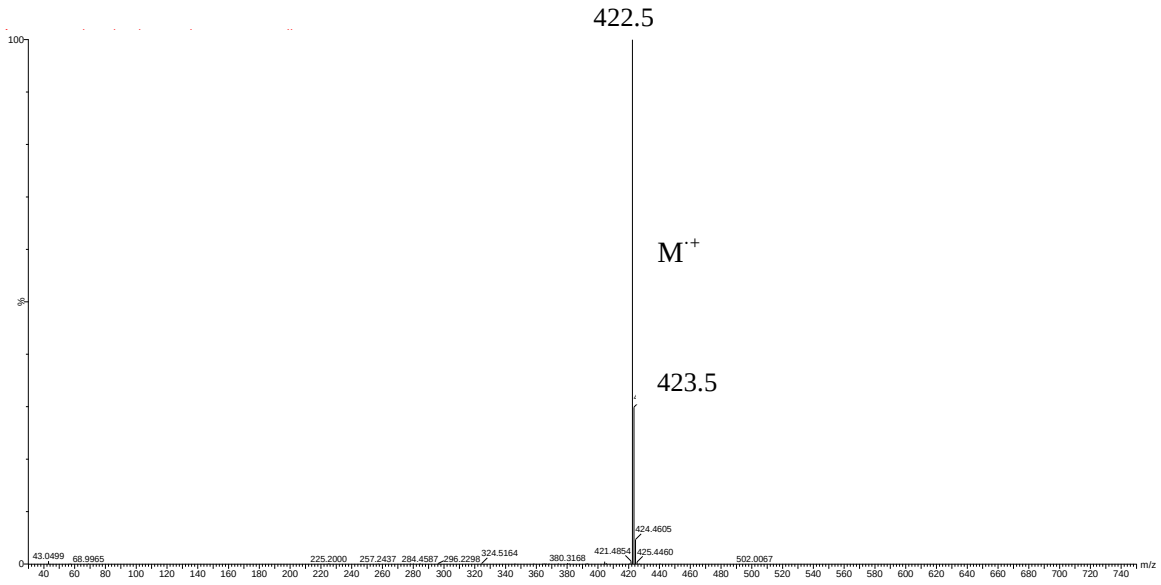


Figure 4.13: FI mass spectrum of nonacosan-15-one

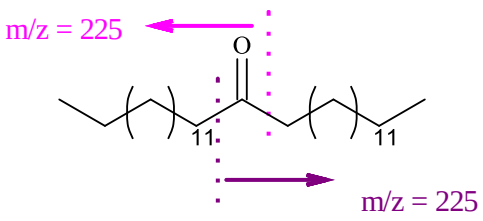


Figure 4.14: Characteristic fragment ions m/z = 225 for nonacosan-15-one (originally in colour)

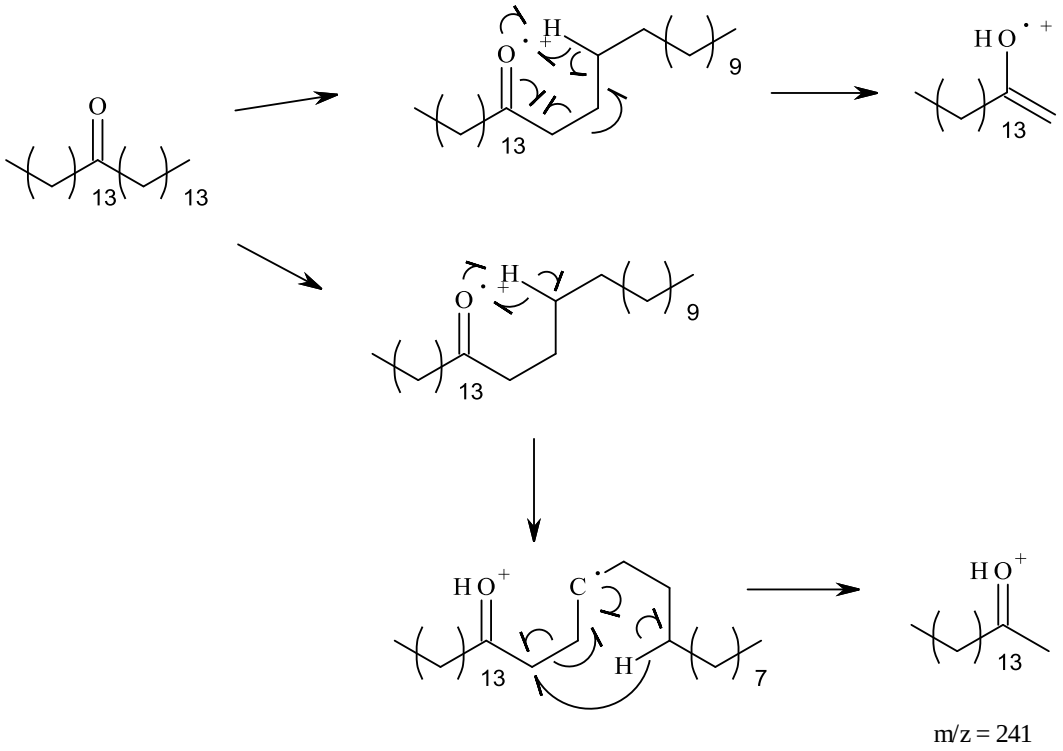


Figure 4.15: Characteristic fragment ions m/z = 240 and m/z = 241 for nonacosan-15-one

Table 4.3: Identification of noncosan-15-one in oilseed rape straw including the EI fragmentation pattern with percentage base peak intensity and KI value

Identification	M^+	EI Fragmentation	Calculated KI
Ketone			
Nonacosan-15-one	422.7	57(100), 43(84), 71(79), 58(65), 85(44), 225(42), 96(33), 59(29), 83(25), 81(24)	3088

4.2.3 Quantification of hexane and ethanol extraction of raw materials

The hexane and ethanol straw wax extracts were quantified using calibration data presented in Section 2.5.1 and the method described in Section 6.3.2. The quantities of the different wax components in the extracts are normalised to percentage in the extracts and in µg per gram of biomass. The quantification data for the hexane

extracts are shown in Tables 4.4 and 4.5 whereas the data for the ethanol extracts are shown in Tables 4.6 and 4.7. No purification or fractionation was carried out prior to quantification analysis therefore the results shown are data from the crude extracts. The two solvent extracts were quantified in order to gain an insight into the quantities of each wax group from each different type of straw. As expected and observed previously in Chapter 2, the total lipophilic fraction of the ethanol extracts is very small, and in the case of bagasse ethanol extract, only 6% of the extract was lipophilic compounds which suggests that bagasse consists of a high percentage of non-volatile polar compounds. The percentage of identified compounds for oat and miscanthus ethanol extracts were both less than 10% which indicated a high proportion of non-volatile polar compounds.

The fatty acid levels present in the different straw hexane extracts ranged from 2.3 – 8.5% which equates to about 120 – 640 $\mu g g^{-1}$ of fatty acids in the straws. Miscanthus, oilseed rape and sunflower straw extracts contain a high proportion of fatty acids. It is expected that there may be high levels of free fatty acids in oilseed rape and sunflower extracts as these are both oilseed crops that contain large amounts of free fatty acids in their seeds. Sunflower straw extract is the only wax that consists of a considerable amount of eicosanoic acid (C_{20}). Sunflower straw wax is particularly interesting as it also contains a high percentage of sterols. Sunflower straw contained eleven different sterols and their derivatives, of which four are identified. Campesterol, stigmasterol and β-sitosterol are the typical phytosterols found across all seven different straws. Interestingly, a high level of wax esters is also present in the sunflower straw as over 18% of the extract is aliphatic wax esters. Currently, sunflower straw is being developed for use as a major component of particle boards to replace wood-based panels so extraction of sunflower straw for sterols and wax esters prior to particle board manufacture means adding value to the low cost agricultural by-product.[288] Sunflower appears to be a good potential biomass for scale up extraction due to its high sterol and wax ester content in its straw but the crude yield of sunflower straw hexane extract is only a very low 0.3%. With such low extraction yield, it is possible that extraction is not very economically viable as there would be less kg of product extracted per kg of CO_2. However, if the sunflower straw was to be sorted and pre-treated to remove the large cellulosic core and then solely extract the straw surfaces then it may improve the economics but a full economic analysis must be carried out

due to the extra cost in processing step. As an extra pre-treatment step will have to be added, the overall economics of the whole process needs to be assessed. Sunflower straw wax does not contain any fatty alcohols which is an important group of compounds in many cosmetics applications such as cosmetic creams but oat straw wax showed an exceptionally high fatty alcohol content.[289] Above 1700 $\mu g g^{-1}$ of fatty alcohols can be found in oat straw which is 2.5 times higher than the second highest, barley straw. About 17% of the oat straw hexane extract was fatty alcohols which makes it the biggest identified wax group found in oat. The results are in agreement with results from oat leaf wax where 45% of the identified compounds is fatty alcohols.[100] Wax esters are the main wax group found in sunflower straw as almost of the half of the quantified hexane extract is found to be wax esters with chain lengths of up to at least 56 carbons. This could potentially be a good source of biomass for the extraction high wax esters-based waxes. It was reported that at least 65% of the sunflower seed wax was found to be aliphatic wax esters with the most predominant being wax esters with 42 total alkyl chain length.[290]

Wheat and barley which showed a high hexane yield of about 1% do not have distinctive high levels of a specific wax group but both of these cereal straws are the only biomass to contain β-diketones which are interesting molecules with potential to be metal chelators.[93, 202] Both of the hexane extracts contain at least 2000 $\mu g g^{-1}$ of β-diketones with the predominant β-diketone being 14,16-hentriacontanedione. Bagasse hexane extract contains the lowest amount of identified compounds and aldehydes were mainly found in this extract with carbon chain length from $C_{26} - C_{36}$ which is in agreement with data from sugarcane wax.[291] Most of the other straw waxes only contain long chain aldehydes with chain lengths $C_{26} - C_{30}$. Aldehydes are usually the intermediates between alcohols and fatty acids. The low amount of identified compounds in the bagasse extracts suggests that the majority of the compounds were too non-volatile to be analysed by GC therefore it would be interesting to carry out HPLC or GPC fractionation on the extract and identify the higher molecular weight compounds. Derivatisation can also be carried out on the waxes to improve peak shape and increase volatility. No wax esters have been reported previously in sugarcane wax however, large triglycerides of $C_{51} - C_{57}$ have been identified.[291] The study of sugarcane wax showed that the wax contains mainly aldehydes, fatty alcohols and *n*-alkanes which are the three main groups identified. Aldehydes can be useful in

the flavour industry and from the identified compounds; the major wax group in the extract is the long chain aldehydes which means the purity is high, and therefore less downstream processing is required for the recovery of these aldehydes.

Considering the biomass availability, percentage crude yield and the chemical composition profile (high in sterols, fatty alcohols and wax esters), wheat, barley and oat straw were chosen for the scale up trials. These three agricultural crops are the top three cereals grown in the UK and as the chemical compositions were somewhat similar, wax blending at a later stage can be considered for formulations. Wheat and barley straw were selected due to the extracts containing unique β-diketones as well as relatively high levels of other valuable wax groups such as wax esters, sterols and fatty alcohols. Oat straw wax showed exceptionally high quantities of wax esters, sterols and fatty alcohols which have many important applications such as cosmetics.

Table 4.4: Quantification of hexane straw waxes (% in extracts)

Identification	Hexane (% in extract) (n ≥ 2)						
	Wheat	Barley	Oat	Oilseed Rape	Sunflower	Miscanthus	Bagasse
Tetradecanoic acid (C_{14})	0.4 ± 0.07	0.4 ± 0.02	0.3 ± 0.02	0.2 ± 0.02	0.1 ± 0.06	0.2 ± 0.02	*Tr*
Hexadecanoic acid (C_{16})	3.3 ± 0.5	1.8 ± 0.1	3.1 ± 0.3	3.8 ± 0.2	3.8 ± 0.3	6.7 ± 0.9	1.8 ± 0.03
Octadecadienoic acid ($C_{18:2}$)	0.7 ± 0.02	0.5 ± 0.02	0.5 ± 0.03	0.3 ± 0.08	0.1 ± 0.07	*Tr*	*Tr*
Octadecenoic acid ($C_{18:1}$)	*Tr*	1.1 ± 0.01	0.2 ± 0.05	0.2 ± 0.01	*Tr*	0.8 ± 0.05	*Tr*
Octadecanoic acid ($C_{18:0}$)	0.5 ± 0.01	0.4 ± 0.04	0.6 ± 0.01	1.4 ± 0.2	0.8 ± 0.02	0.8 ± 0.05	0.5 ± 0.08
Eicosanoic acid (C_{20})	*Tr*	*Tr*	*Tr*	*Tr*	1.3 ± 0.1	*Tr*	*Tr*
Total free fatty acids	**4.9 ± 0.6**	**4.2 ± 0.28**	**4.7 ± 0.4**	**6.0 ± 0.5**	**6.1 ± 0.55**	**8.5 ± 1.0**	**2.3 ± 0.11**
Heptacosane (C_{27})	0.5 ± 0.1	0.4 ± 0.01	0.2 ± 0.02	0.4 ± 0.07	1.5 ± 0.08	1.3 ± 0.4	0.9 ± 0.1
Nonacosane (C_{29})	2.3 ± 0.4	7.4[a] ± 0.2	11.0[a] ± 0.3	16.2 ± 1.1	3.3 ± 0.2	2.5 ± 0.3	1.0[a] ± 0.2
Hentriacontane (C_{31})	1.9[a] ± 0.3	1.4[a] ± 0.3	1.4[a] ± 0.1	4.5 ± 0.2	1.8 ± 0.08	1.8 ± 0.08	5.3[a] ± 0.3
Triatriacontane (C_{33})	1.0 ± 0.02	1.0 ±0.2	*Tr*	*Tr*	*Tr*	1.0 ± 0.05	0.4 ± 0.02
Total hydrocarbons	**5.6[a] ± 0.82**	**10.2[a] ± 0.71**	**12.6[a] ± 0.42**	**25.9 ± 1.4**	**6.6 ± 0.4**	**6.5 ± 0.83**	**7.5 ± 0.69**
Hexacosanol (C_{26})	*Tr*	9.5[a] ± 0.9	14.2[a] ± 0.8	*Tr*	*Tr*	*Tr*	1.2[a] ± 0.04
Octacosanol (C_{28})	7.3[a] ± 0.2	1.9[a] ± 0.1	2.8[a] ± 0.1	*Tr*	*Tr*	3.2 ± 0.1	6.9[a] ± 0.2
Total fatty alcohols	**7.3[a] ± 0.2**	**11.4[a] ± 1**	**17.1[a] ± 0.9**	***Tr***	***Tr***	**3.2 ± 0.1**	**8.1 ± 0.24**
Hexacosanal (C_{26})	0.1 ± 0.02	1.2 ± 0.03	2.5 ± 0.1	13.7 ± 0.3	0.2 ± 0.02	0.5 ± 0.05	0.3 ± 0.1
Octacosanal (C_{28})	1.1± 0.05	0.4 ± 0.02	0.4 ± 0.05	3.8 ± 0.2	0.8 ± 0.08	6.1 ± 1.3	5.6 ± 0.4
Triacontanol (C_{30})	*Tr*	*Tr*	*Tr*	2.2 ± 0.03	*Tr*	*Tr*	1.7 ± 0.02
Dotriacontanal (C_{32})	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	1.1 ± 0.08
Tetratriacontanal (C_{34})	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	1.2 ± 0.06
Hexatriacontanal (C_{36})	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	0.2 ± 0.02
Total aldehydes	**1.3 ± 0.07**	**1.5 ± 0.05**	**2.9 ± 0.15**	**19.7 ± 0.53**	**0.9 ± 0.1**	**6.6 ± 1.4**	**10.1 ± 0.68**
Tetracosanyl hexadecanoate (C_{24}:C_{16})	0.3 ± 0.02	1.0 ± 0.1	0.4 ± 0.04	*Tr*	*Tr*	0.2 ± 0.01	*Tr*
Hexacosanyl hexadecanoate (C_{26}:C_{16})	0.8 ± 0.2	3.1 ± 0.3	0.4 ± 0.12	*Tr*	0.7 ± 0.08	0.7 ± 0.09	0.3 ± 0.1

Table 4.4 (continued)

Octacosanyl hexadecanoate (C_{28}:C_{16})	0.8 ± 0.2	3.1 ± 0.3	1.1 ± 0.03	0.5 ± 0.05	2.2 ± 0.08	2.1 ± 0.09	1.1 ± 0.08
Wax ester 3 and 4	*Tr*	*Tr*	*Tr*	1.3 ± 0.4	*Tr*	*Tr*	*Tr*
Octacosanyl octadecanoate (C_{28}:C_{18})	3.2 ± 0.4	9.0 ± 0.5	2.8 ± 0.9	0.6 ± 0.09	2.8 ± 0.8	1.5 ± 0.04	0.6 ± 0.09
Octacosanyl eicosanoate (C_{28}:C_{20})	1.7 ± 0.03	7.7 ± 2.1	1.8 ± 0.4	0.8 ± 0.04	3.6 ± 1.2	1.6 ± 0.02	0.6 ± 0.06
Wax ester 5	*Tr*	*Tr*	*Tr*	0.4 ± 0.01	*Tr*	*Tr*	*Tr*
Octacosanyl dosanoate (C_{28}:C_{22})	2.1 ± 0.3	2.3 ± 0.4	0.8 ± 0.2	0.5 ± 0.02	3.4 ± 0.9	1.1 ± 0.03	0.5 ± 0.01
Octacosanyl tetracosanoate (C_{28}:C_{24})	0.7 ± 0.08	0.7 ± 0.3	1.8 ± 0.08	0.5 ± 0.03	2.5 ± 0.6	0.5 ± 0.02	0.5 ± 0.03
Octacosanyl hexacosanoate (C_{28}:C_{26})	0.5 ± 0.09	0.6 ± 0.1	1.1 ± 0.02	0.4 ± 0.08	1.5 ± 0.4	0.2 ± 0.06	0.4 ± 0.04
Octacosanyl octacosanoate (C_{28}:C_{28})	1.3 ± 0.1	0.4 ± 0.07	0.8 ± 0.4	*Tr*	1.7 ± 0.1	0.4 ± 0.1	1.6 ± 0.5
Total wax esters	**12.1 ± 1.4**	**28.0 ± 5.22**	**12.9 ± 2.2**	**4.8 ± 0.76**	**18.4 ± 4.1**	**8.4 ± 0.3**	**5.8 ± 0.81**
Campesterol	1.2 ± 0.3	2.9 ± 0.09	0.9 ± 0.03	*Tr*	0.2 ± 0.03	3.6 ± 0.2	*Tr*
Stigmasterol	1.4 ± 0.2	2.8 ± 0.1	0.7 ± 0.08	3.5 ± 0.3	5.0 ± 0.1	2.1 ± 0.1	*Tr*
β-sitosterol	1.0 ± 0.08	6.1 ± 0.3	0.9 ± 0.2	6.3 ± 0.4	3.0 ± 0.2	5.3 ± 0.6	0.8 ± 0.02
Sterol 1	0.7 ± 0.02	*Tr*	*Tr*	*Tr*	3.2 ± 0.08	5.3 ± 1.8	*Tr*
Δ^4-Sitosten-3-one	2.0 ± 0.14	1.9 ± 0.1	0.6 ± 0.1	2.4 ± 0.4	1.7 ± 0.1	*Tr*	1.1 ± 0.08
Sterol 2	0.9 ± 0.03	*Tr*	0.6 ± 0.05	1.6 ± 0.1	1.2 ± 0.1	1.6 ± 0.2	*Tr*
Sterol 3	*Tr*	*Tr*	1.0 ± 0.2	*Tr*	1.4 ± 0.4	*Tr*	*Tr*
Sterol 4	*Tr*	*Tr*	*Tr*	*Tr*	1.0 ± 0.01	*Tr*	*Tr*
Sterol 5	*Tr*	*Tr*	*Tr*	*Tr*	4.2 ± 0.1	*Tr*	*Tr*
Sterol 6	*Tr*	*Tr*	*Tr*	*Tr*	0.8 ± 0.02	*Tr*	*Tr*
Sterol 7	*Tr*	*Tr*	*Tr*	*Tr*	2.0 ± 0.04	*Tr*	*Tr*
Total sterols	**7.2** ± 0.8	**13.7** ± 0.6	**4.6** ± 0.69	**13.8** ± 1.2	**10.6** ± 1.4	**17.9** ± 2.9	**1.9** ± 0.1
14,16 hentriacontanedione (C_{31})	14.8 ± 3.4	19.5 ± 2.9	*Tr*	*Tr*	*Tr*	2.0 ± 0.2	*Tr*
16,18 triatriacontanedione (C_{33})	3.0 ± 0.3	2.3 ± 0.4	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*
Total β-diketones	**17.8** ± 3.7	**21.8** ± 3.3	***Tr***	***Tr***	***Tr***	**2.0** ± 0.2	***Tr***
Nonacosan-15-one (C_{29})	*Tr*	*Tr*	*Tr*	5.0 ± 1.4	*Tr*	*Tr*	*Tr*
Total ketone	***Tr***	***Tr***	***Tr***	**5.0** ± 1.4	***Tr***	***Tr***	***Tr***

Table 4.4 (continued)

Total identified	**38.4** ± 6.8	**69.2** ± 10.9	**55.0** ± 4.9	**75.3** ± 5.8	**42.6** ± 6.3	**53.2** ± 7.8	**35.7** ± 2.6

[a] = co-elution and values shown are the combined quantities, *Tr* = Trace levels < LOQ, LOQ shown in Section 2.5.1

Table 4.5: Quantification of hexane straw waxes (μgg^{-1} of biomass)

Identification	Hexane μgg^{-1} of biomass (n ≥ 2)						
	Wheat	Barley	Oat	Oilseed Rape	Sunflower	Miscanthus	Bagasse
Tetradecanoic acid (C_{14})	41 ± 7.2	46 ± 2.3	42 ± 2.8	11 ± 1.1	3 ± 1.8	6 ± 0.6	*Tr*
Hexadecanoic acid (C_{16})	380 ± 57.6	194 ± 10.8	422 ± 40.8	276 ± 14.5	128 ± 10.1	222 ± 29.8	94 ± 1.6
Octadecadienoic acid ($C_{18:2}$)	84 ± 2.4	48 ± 1.9	71 ± 7.1	25 ± 6.7	5 ± 3.5	*Tr*	*Tr*
Octadecenoic acid ($C_{18:1}$)	*Tr*	115 ± 10.5	30 ± 1.5	15 ± 0.4	*Tr*	25 ± 1.7	*Tr*
Octadecanoic acid ($C_{18:0}$)	54 ± 1.1	46 ± 4.6	78 ± 2.6	102 ± 14.6	26 ± 0.7	28 ± 1.8	24 ±3.8
Eicosanoic acid (C_{20})	*Tr*	*Tr*	*Tr*	*Tr*	44 ± 3.4	*Tr*	*Tr*
Total free fatty acids	**559** ± 68.4	**449** ± 29.9	**643** ± 54.7	**430** ± 35.8	**207** ± 18.7	**282** ± 33.8	**117** ± 5.6
Heptacosane (C_{27})	55 ± 11	41 ± 1	30 ± 3	27 ± 4.7	52 ± 2.8	43 ± 13.2	44 ± 4.9
Nonsacosane (C_{29})	265 ± 46.1	782[a] ± 21.1	1498[a] ± 40.9	1170 ± 79.4	112 ± 6.8	81 ± 9.7	49[a] ± 11.3
Hentriacontane (C_{31})	215[a] ± 33.9	153[a] ± 32.8	192[a] ± 13.7	322 ± 14.3	60 ± 3	59 ± 2.6	270[a] ± 17.3
Triatriacontane (C_{33})	113 ± 2.3	108 ± 21.6	*Tr*	*Tr*	*Tr*	33 ± 1.7	22 ± 1.1
Total hydrocarbons	**649[a]** ± 95	**1084[a]** ± 75.5	**1720[a]** ± 57.3	**1865** ± 98.7	**224** ± 12.6	**216** ± 27.6	**384[a]** ± 35.3
Hexacosanol (C_{26})	*Tr*	1011[a] ±	1937[a] ±	*Tr*	*Tr*	*Tr*	63[a] ± 2.1
Octacosanol (C_{28})	843[a] ±	198[a] ±	249[a] ±	*Tr*	*Tr*	104 ±	350[a] ± 10.1
Total fatty alcohols	**843[a]** ±	**1209[a]** ±	**2186[a]** ±	***Tr***	***Tr***	**104** ±	**412[a]** ± 12.2
Hexacosanal (C_{26})	20 ± 4	174 ± 4.4	524 ± 21	990 ± 21.7	5 ± 0.5	17 ± 1.7	15 ± 5
Octacosanal (C_{28})	202 ± 9.2	62 ± 3.1	83 ± 10.4	272 ± 14.3	26 ± 2.6	200 ± 42.6	288 ± 20.6
Triacontanal (C_{30})	*Tr*	*Tr*	*Tr*	157 ± 2.1	*Tr*	*Tr*	86 ± 1

Table 4.5 (continued)

Dotriacontanal (C_{32})	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	54 ± 3.9
Tetratriacontanal (C_{34})	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	62 ± 3.1
Hexatriacontanal (C_{36})	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	10 ± 1
Total aldehydes	**222 ± 12**	**236 ± 7.9**	**607 ± 31.4**	**1419 ± 38.2**	**31 ± 3.4**	**217 ± 44.4**	**515 ± 34.7**
Tetracosanyl hexadecanoate (C_{24}:C_{16})	39 ± 2.6	110 ± 11	58 ± 5.8	*Tr*	*Tr*	6 ± 0.3	*Tr*
Hexacosanyl hexadecanoate (C_{26}:C_{16})	94 ± 24.7	326 ± 358	145 ± 43.5	*Tr*	25 ± 2.9	25 ± 3.2	18 ± 6
Octacosanyl hexdecanoate (C_{28}:C_{16})	366 ± 91.5	955 ± 95.5	385 ± 10.5	33 ± 3.3	75 ± 2.7	71 ± 3	58 ± 4.2
Wax ester 3 and 4	*Tr*	*Tr*	*Tr*	88 ± 29.8	*Tr*	*Tr*	*Tr*
Octacosanyl octadecanoate (C_{28}:C_{18})	190 ± 23.8	811 ± 44.2	248 ± 79.7	41 ± 6.2	95 ± 27.1	51 ± 1.4	31 ± 4.7
Octacosanyl eicosanoate (C_{28}:C_{20})	180 ± 3.2	352 ± 96	302 ± 73.8	60 ± 3	123 ± 41	51 ± 0.6	31 ± 3.1
Wax ester 5	*Tr*	*Tr*	*Tr*	26 ± 0.7	*Tr*	*Tr*	*Tr*
Octacosanyl dosanoate (C_{28}:C_{22})	240 ± 35.4	239 ± 41.6	107 ± 30.8	36 ± 1.4	116 ± 30.7	37 ± 1	26 ± 0.5
Octacosanyl tetracosanoate (C_{28}:C_{24})	80 ± 9.1	70 ± 30	248 ± 11	37 ± 2.2	85 ± 20.4	16 ± 0.6	25 ± 1.5
Octacosanyl hexacosanoate (C_{28}:C_{26})	54 ± 9.7	68 ± 15.9	155 ± 2.8	26 ± 5.2	52 ± 13.9	8 ± 1	21 ± 2.1
Octacosanyl octacosanoate (C_{28}:C_{28})	150 ± 11.5	39 ± 6.8	113 ± 56.5	*Tr*	58 ± 3.4	13 ± 3.3	83 ± 25.9
Total wax esters	**1394 ± 163.5**	**2970 ± 553.7**	**1761 ± 303.1**	**346 ± 54.8**	**627 ± 139**	**277 ± 9.9**	**294 ± 41.1**
Campesterol	138 ± 35.7	312 ± 9.7	119 ± 4	*Tr*	7 ± 1.1	118 ± 7.9	*Tr*
Stigmasterol	160 ± 26.3	297 ± 12.7	90 ± 10.3	252 ± 20.9	170 ± 4.8	69 ± 3.3	*Tr*
β-sitosterol	112 ± 9	649 ± 28.7	122 ± 31.2	455 ± 31.8	102 ± 8.2	175 ± 18.5	42 ± 1.1
Sterol 1	83 ± 2.4	*Tr*	*Tr*	*Tr*	108 ± 2.7	176 ± 59.7	*Tr*
Δ^4-Sitosten-3-one	228 ± 16	200 ± 13.7	84 ± 14	173 ± 29.6	57 ± 4	*Tr*	55 ± 4
Sterol 2	105 ± 3.5	*Tr*	76 ± 6.3	117 ± 5.9	42 ± 4.6	52 ± 7.2	*Tr*
Sterol 3	*Tr*	*Tr*	139 ± 27.8	*Tr*	47 ± 13.8	*Tr*	*Tr*
Sterol 4	*Tr*	*Tr*	*Tr*	*Tr*	32 ± 0.3	*Tr*	*Tr*
Sterol 5	*Tr*	*Tr*	*Tr*	*Tr*	143 ± 4.4	*Tr*	*Tr*
Sterol 6	*Tr*	*Tr*	*Tr*	*Tr*	28 ± 0.7	*Tr*	*Tr*
Sterol 7	*Tr*	*Tr*	*Tr*	*Tr*	67 ± 1.3	*Tr*	*Tr*

Table 4.5 (continued)

Total sterols	**827 ± 93**	**1457 ± 64.9**	**631 ± 94.7**	**996 ± 88.1**	**360 ± 45.8**	**591 ± 95.6**	**97 ± 5.1**
14,16 hentriacontanedione (C_{31})	1707 ± 392.1	1931 ± 287.2	*Tr*	*Tr*	*Tr*	68 ± 7.8	*Tr*
16,18 triatriacontanedione (C_{33})	343 ± 35.4	228 ± 38.7	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*
Total β-diketones	**2051 ± 427.5**	**2160 ± 325.9**	***Tr***	***Tr***	***Tr***	**68** ± 7.8	***Tr***
Nonacosan-15-one (C_{29})	*Tr*	*Tr*	*Tr*	363 ± 101.6	*Tr*	*Tr*	*Tr*
Total ketone	***Tr***	***Tr***	***Tr***	**363** ± 101.6	***Tr***	***Tr***	***Tr***
Total identified	**6544 ± 1160.5**	**9565 ± 1506.6**	**7547 ± 680**	**5419 ± 414.5**	**1449** ± **212.6**	**1582 ± 232.5**	**1820 ± 132.5**

[a] = co-elution and values shown are the combined quantities, *Tr* = Trace levels < LOQ, LOQ shown in Section 2.5.1

Table 4.6: Quantification of ethanol straw waxes (% in extract)

Identification	Ethanol (% in extract) (n ≥ 2)						
	Wheat	Barley	Oat	Oilseed Rape	Sunflower	Miscanthus	Bagasse
Tetradecanoic acid (C_{14})	0.3 ± 0.04	0.8 ± 0.03	*Tr*	*Tr*	*Tr*	0.8 ±	0.3 ± 0.04
Hexadecanoic acid (C_{16})	2.7 ± 0.1	2.8 ± 0.1	0.9 ± 0.01	4.1 ± 0.03	1.6 ± 0.03	1.3 ±	0.6 ± 0.05
Octadecadienoic acid ($C_{18:2}$)	0.4 ± 0.1	1.1 ± 0.1	0.1 ± 0.03	3.5 ± 0.2	*Tr*	*Tr*	*Tr*
Octadecenoic acid ($C_{18:1}$)	1.0 ± 0.08	1.6 ± 0.2	0.4 ± 0.1	3.7 ± 0.2	*Tr*	0.1 ± 0.01	0.1 ± 0.01
Octadecanoic acid ($C_{18:0}$)	0.4 ± 0.02	0.6 ± 0.07	0.2 ± 0.06	1.3 ± 0.07	0.3 ± 0.04	0.1 ± 0.02	0.1 ± 0.02
Eicosanoic acid (C_{20})	*Tr*	*Tr*	*Tr*	*Tr*	0.4 ± 0.07	*Tr*	*Tr*
Total free fatty acids	**4.8** ± 0.4	**6.8** ± 0.59	**1.6** ± 0.22	**12.6** ± 0.55	**2.3** ± 0.14	**2.4** ± 0.17	**1.1** ± 0.12
Heptacosane (C_{27})	0.2 ± 0.01	0.2 ± 0.02	2.3 ± 0.1	*Tr*	0.4 ± 0.03	0.2 ± 0.03	0.1 ± 0.02
Nonacosane (C_{29})	0.9 ± 0.07	0.1[a] ± 0.03	1.1[a] ± 0.04	3.7[a] ± 0.2	0.7 ± 0.02	0.6 ± 0.01	0.1 ± 0.03
Hentriacontane (C_{31})	2.7 ± 0.04	0.6[a] ± 0.01	0.3 ± 0.02	1.6[a] ± 0.06	0.3 ± 0.01	0.2 ± 0.01	1.4 ± 0.09
Triatriacontane (C_{33})	0.4 ± 0.03	0.4 ± 0.05	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*
Total hydrocarbons	**4.2** ± 0.15	**1.4** ± 0.1	**3.7** ± 0.16	**5.2** ± 0.23	**3.6** ± 0.06	**1.0** ± 0.05	**1.7** ± 0.14

Table 4.6 (continued)

Hexacosanol (C_{26})	*Tr*	4.9[a] ± 0.2	1.4[a] ± 0.1	4.7[a] ± 0.1	*Tr*	*Tr*	*Tr*
Octacosanol (C_{28})	3.5 ± 0.2	0.8[a] ± 0.09	0.3 ± 0.02	2.0[a] ± 0.09	*Tr*	0.4 ± 0.03	*Tr*
Total fatty alcohols	**3.5** ± 0.21	**5.7[a]** ± 0.32	**1.7[a]** ± 0.14	**6.8[a]** ± 0.21	***Tr***	**0.4** ± 0.03	***Tr***
Hexacosanal (C_{26})	*Tr*	0.1 ± 0.02	0.3 ± 0.04	*Tr*	*Tr*	*Tr*	0.1 ± 0.03
Octacosanal (C_{28})	*Tr*	*Tr*	*Tr*	*Tr*	0.3 ± 0.01	0.5 ± 0.04	1.1 ± 0.12
Triacontanal (C_{30})	*Tr*	*Tr*	*Tr*	0.3 ± 0.03	*Tr*	*Tr*	0.4 ± 0.09
Dotriacontanal (C_{32})	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	0.2 ± 0.02
Tetratriacontanal (C_{34})	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	0.2 ± 0.03
Total aldehydes	***Tr***	**0.1** ± 0.02	**0.3** ± 0.04	**0.3** ± 0.03	**0.3** ± 0.01	**0.5** ± 0.04	**1.9** ± 0.29
Tetracosanyl hexadecanoate (C_{24}:C_{16})	*Tr*	0.3 ± 0.01	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*
Hexacosanyl hexadecanoate (C_{26}:C_{16})	0.3 ± 0.04	1.0 ± 0.02	0.2 ± 0.03	0.4 ± 0.02	0.3 ± 0.01	0.1 ± 0.02	*Tr*
Octacosanyl hexdecanoate (C_{28}:C_{16})	1.0 ± 0.03	1.2 ± 0.03	0.4 ± 0.02	1.0 ± 0.03	0.3 ± 0.02	0.3 ± 0.04	*Tr*
Octacosanyl octadecanoate (C_{28}:C_{18})	0.8 ± 0.01	0.9 ± 0.02	0.2 ± 0.02	*Tr*	0.4 ± 0.02	0.3 ± 0.05	*Tr*
Octacosanyl eicosanoate (C_{28}:C_{20})	0.5 ± 0.02	0.8 ± 0.01	0.2 ± 0.08	*Tr*	0.4 ± 0.05	0.2 ± 0.06	*Tr*
Octacosanyl dosanoate (C_{28}:C_{22})	0.7 ± 0.03	0.3 ± 0.09	0.1 ± 0.01	*Tr*	0.3 ± 0.06	0.1 ± 0.08	*Tr*
Octacosanyl tetracosanoate (C_{28}:C_{24})	0.2 ± 0.01	0.2 ± 0.08	0.1 ± 0.04	*Tr*	0.2 ± 0.08	*Tr*	*Tr*
Octacosanyl hexacosanoate (C_{28}:C_{26})	0.2 ± 0.01	*Tr*	*Tr*	*Tr*	0.2 ± 0.09	*Tr*	*Tr*
Octacosanyl octacosanoate (C_{28}:C_{28})	0.4 ± 0.04	*Tr*	*Tr*	*Tr*	0.2 ± 0.01	*Tr*	*Tr*
Total wax esters	**4.1** ± 0.19	**4.7** ± 0.26	**1.2** ± 0.2	**1.4** ± 0.05	**4.4** ± 0.34	**1.0** ± 0.25	***Tr***
Campesterol	0.6 ± 0.04	1.3 ± 0.1	*Tr*	0.5 ± 0.04	0.3 ± 0.003	0.5 ± 0.04	*Tr*
Stigmasterol	0.8 ± 0.05	1.3 ± 0.02	*Tr*	1.8 ± 0.5	1.3 ± 0.04	0.4 ± 0.02	*Tr*
β-sitosterol	1.7 ± 0.09	3.9 ± 0.3	0.4 ± 0.03	0.7 ± 0.01	0.8 ± 0.02	1.0 ± 0.03	0.5 ± 0.02
Sterol 1	0.3 ± 0.08	0.6 ± 0.02	*Tr*	*Tr*	0.8 ± 0.02	0.8 ± 0.09	*Tr*
Δ^4-Sitosten-3-one	1.1 ± 0.02	*Tr*	*Tr*	*Tr*	0.6 ± 0.03	*Tr*	0.4 ± 0.03
Sterol 2	0.5 ± 0.01	*Tr*	*Tr*	0.4 ± 0.01	0.3 ± 0.1	*Tr*	0.3 ± 0.04
Sterol 3	*Tr*	*Tr*	*Tr*	*Tr*	0.3 ± 0.1	*Tr*	*Tr*

Table 4.6 (continued)

Sterol 4	*Tr*	*Tr*	*Tr*	*Tr*	0.2 ± 0.1	*Tr*	*Tr*
Sterol 5	*Tr*	*Tr*	*Tr*	*Tr*	0.01 ± 0.01	*Tr*	*Tr*
Sterol 6	*Tr*	*Tr*	*Tr*	*Tr*	0.2 ± 0.1	*Tr*	*Tr*
Sterol 7	*Tr*	*Tr*	*Tr*	*Tr*	0.6 ± 0.2	*Tr*	*Tr*
Total sterols	**5.1** ± 0.29	**7.1** ± 0.44	**0.4** ± 0.03	**3.4** ± 0.55	**5.3** ± 0.71	**2.7** ± 0.18	**1.2** ± 0.09
14,16 hentriacontanedione (C_{31})	4.9 ± 0.03	3.9 ± 0.08	*Tr*	*Tr*	*Tr*	0.1 ± 0.02	*Tr*
16,18 triatriacontanedione (C_{33})	1.1 ± 0.05	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*
Total β-diketones	**6.1** ± 0.08	**3.9** ± 0.08	***Tr***	***Tr***	***Tr***	**0.1** ± 0.02	***Tr***
Nonacosan-15-one (C_{29})	*Tr*	*Tr*	*Tr*	1.5 ± 0.004	*Tr*	*Tr*	*Tr*
Total ketone	***Tr***	***Tr***	***Tr***	**1.5** ± 0.004	***Tr***	***Tr***	***Tr***
Total identified	**27.7** ± 1.32	**29.7** ± 1.81	**6.7** ± 0.69	**31.3** ± 1.62	**15.9** ± 1.53	**8.0** ± 0.82	**5.9** ± 0.72

[a] = co-elution and values shown are the combined quantities, *Tr* = Trace levels < LOQ, LOQ shown in Section 2.5.1

Table 4.7: Quantification of ethanol straw waxes (μgg^{-1} of biomass)

Identification	Ethanol (μgg^{-1} of biomass) (n ≥ 2)						
	Wheat	Barley	Oat	Oilseed Rape	Sunflower	Miscanthus	Bagasse
Tetradecanoic acid (C_{14})	87 ± 11.6	159 ± 6	*Tr*	*Tr*	*Tr*	221 ± 24.9	98 ± 13
Hexadecanoic acid (C_{16})	910 ± 40.4	565 ± 24.2	753 ± 8.4	742 ± 5.4	325 ± 6.1	336 ± 31	182 ± 15.2
Octadecadienoic acid ($C_{18:2}$)	145 ± 50.8	209 ± 26.6	102 ± 6.1	645 ± 38.7	*Tr*	*Tr*	*Tr*
Octadecenoic acid ($C_{18:1}$)	325 ± 26	314 ± 45.1	351 ± 22.8	681 ± 44.2	*Tr*	37 ± 3.1	33 ± 3.3
Octadecanoic acid ($C_{18:0}$)	135 ± 6.8	113 ± 13.2	208 ± 62.4	240 ± 12.9	65 ± 8.7	36 ± 10.8	34 ± 6.8
Eicosanoic acid (C_{20})	*Tr*	*Tr*	*Tr*	*Tr*	71 ± 12.4	*Tr*	*Tr*
Total free fatty acids	**1602** ± 133.5	**1359** ± 117.9	**1414** ± 194.4	**2308** ± 100.7	**461** ± 28.1	**630** ± 65.6	**346** ± 37.7
Heptacosane (C_{27})	72 ± 3.6	48 ± 4.8	973 ± 42.3	*Tr*	71 ± 5.3	50 ± 7.5	43 ± 8.6
Nonsacosane (C_{29})	311 ± 24.2	29[a] ± 8.7	292[a] ± 108.8	671[a] ± 30.8	140 ± 4	152 ± 2.5	44 ± 13.2

Table 4.7 (continued)

Hentriacontane (C_{31})	904 ± 13.4	117[a] ± 11.7	*Tr*	288[a] ± 10.8	62 ± 2.1	54 ± 2.7	418 ± 94.1
Triatriacontane (C_{33})	119 ± 8.9	79 ± 3	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*
Total hydrocarbons	**1407** ± 50.3	**273[a]** ± 21.5	**1266[a]** ± 54.3	**959[a]** ± 16	**710** ± 11.8	**256** ± 12.8	**505** ± 41.6
Hexacosanol (C_{26})	*Tr*	984[a] ± 46.2	1256[a] ± 107.7	867[a] ± 22.1	*Tr*	*Tr*	*Tr*
Octacosanol (C_{28})	1169 ± 70.1	152[a] ± 17.1	241 ± 16.1	373[a] ± 16.8	*Tr*	93 ± 7	*Tr*
Total fatty alcohols	**1169** ± 70.1	**1136[a]** ± 63.8	**1497[a]** ± 123.3	**1240** ± 38.3	***Tr***	**93** ± 7	***Tr***
Hexacosanal (C_{26})	*Tr*	296 ± 59.2	180 ± 24	*Tr*	*Tr*	*Tr*	16 ± 4.8
Octacosanal (C_{28})	*Tr*	*Tr*	*Tr*	*Tr*	59 ± 2	127 ± 10.2	327 ± 35.7
Triacontanal (C_{30})	*Tr*	*Tr*	*Tr*	52 ± 5.2	*Tr*	*Tr*	107 ± 24.1
Dotriacontanal (C_{32})	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	48 ± 4.8
Tetratriacontanal (C_{34})	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	60 ± 9
Total aldehydes	***Tr***	**296** ± 59.2	**180** ± 24	**52** ± 5.2	**59** ± 2	**127** ± 10.2	**559** ± 8.5
Tetracosanyl hexadecanoate (C_{24}:C_{16})	*Tr*	57 ± 1.9	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*
Hexacosanyl hexadecanoate (C_{26}:C_{16})	103 ± 13.7	207 ± 4.1	*Tr*	81 ± 4.1	39 ± 1.3	21 ± 4.2	*Tr*
Octacosanyl hexdecanoate (C_{28}:C_{16})	336 ± 10	243 ± 6.1	191 ± 28.7	183 ± 5.5	58 ± 3.9	79 ± 10.5	*Tr*
Octacosanyl octadecanoate (C_{28}:C_{18})	253 ± 3.2	177 ± 3.9	380 ± 19	*Tr*	68 ± 3.4	67 ± 11.2	*Tr*
Octacosanyl eicosanoate (C_{28}:C_{20})	166 ± 6.6	150 ± 1.9	182 ± 18.2	*Tr*	77 ± 9.6	62 ±18.6	*Tr*
Octacosanyl dosanoate (C_{28}:C_{22})	229 ± 9.8	57 ± 7.1	182 ± 18.2	*Tr*	71 ± 14.2	32 ± 25.6	*Tr*
Octacosanyl tetracosanoate (C_{28}:C_{24})	81 ± 4.1	48 ± 9.2	66 ± 78.8	*Tr*	56 ± 22.4	*Tr*	*Tr*
Octacosanyl hexacosanoate (C_{28}:C_{26})	51 ± 2.6	*Tr*	101 ± 40.4	*Tr*	32 ± 14.4	*Tr*	*Tr*
Octacosanyl octacosanoate (C_{28}:C_{28})	145 ± 14.5	*Tr*	*Tr*	*Tr*	36 ± 5.1	*Tr*	*Tr*
Total wax esters	**1363** ± 63.2	**938** ± 49.9	**1102** ± 183.7	**264** ± 9.6	**436** ± 57.5	**261** ± 65.3	***Tr***
Campesterol	213 ± 14.2	256 ± 19.7	*Tr*	98 ± 7.8	65 ± 0.7	124 ± 9.9	*Tr*
Stigmasterol	282 ± 17.6	267 ± 4.1	*Tr*	335 ± 93.1	260 ± 8	100 ± 5	*Tr*
β-sitosterol	557 ± 29.5	775 ± 59.6	321 ± 24.1	119 ± 1.7	153 ± 3.8	261 ± 7.8	146 ± 5.8
Sterol 1	114 ± 30.4	113 ± 3.8	*Tr*	*Tr*	157 ± 17.7	222 ± 25	*Tr*

Δ^4-Sitosten-3-one	366 ± 6.7	*Tr*	*Tr*	*Tr*	111 ± 5.6	*Tr*	128 ± 9.6
Sterol 2	165 ± 3.3	*Tr*	*Tr*	71 ± 1.8	61 ± 20.3	*Tr*	96 ± 12.8
Sterol 3	*Tr*	*Tr*	*Tr*	*Tr*	57 ± 9	*Tr*	*Tr*
Sterol 4	*Tr*	*Tr*	*Tr*	*Tr*	42 ± 21	*Tr*	*Tr*
Sterol 5	*Tr*	*Tr*	*Tr*	*Tr*	45 ± 4.3	*Tr*	*Tr*
Sterol 6	*Tr*	*Tr*	*Tr*	*Tr*	112 ± 37.3	*Tr*	*Tr*
Sterol 7	*Tr*	*Tr*	*Tr*	*Tr*	45 ± 15	*Tr*	*Tr*
Total sterols	**1697** ± 96.5	**1412** ± 87.5	**321** ± 24.1	**623** ± 102.6	**1109** ± 150.7	**707** ± 47.1	**371** ± 28.2
14,16 hentriacontanedione (C_{31})	1763 ± 10.8	776 ± 15.9	*Tr*	*Tr*	*Tr*	34 ± 6.8	*Tr*
16,18 triatriacontanedione (C_{33})	406 ± 18.5	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*
Total β-diketones	**2169** ± 28.4	**776** ± 15.9	***Tr***	***Tr***	***Tr***	**34** ± 6.8	***Tr***
Nonacosan-15-one (C_{29})	*Tr*	*Tr*	*Tr*	280 ± 0.8	*Tr*	*Tr*	*Tr*
Total ketone	***Tr***	***Tr***	***Tr***	**280** ± 0.8	***Tr***	***Tr***	***Tr***
Total identified	**9408** ± 448.3	**6191** ± 377.3	**5779** ± 595.2	**560** ± 29	**2775** ± 267	**2109** ± 261.2	**1780** ± 217.2

[a] = co-elution and values shown are the combined quantities, *Tr* = Trace levels < LOQ, LOQ shown in Section 2.5.1

4.3 Production scale extraction of cereal straw waxes

4.3.1 Raw materials

Wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and oat (*Avena sativa*) were the three cereal straws selected for the scale up trials from the preliminary straw wax screen. Air-dried cereal straws were physically pre-treated by Sundown Products Limited as described in Section 6.2.2. The moisture levels of the straw pellets were confirmed to be less than 10% by following the procedure described in Section 6.2.1 prior to extraction. It must be noted that the varieties of the straws were mixed so there may be variation in any of the initial biomass screens, organic solvent or CO_2 extraction data compared with that discussed in Chapter 2 and Chapter 3. As well as variation in varieties, the raw material is a natural product so other variations such as regional and seasonal must be considered when comparing the results. Although much effort was made to keep the materials the same in the extraction process itself, these uncontrolled parameters could influence both positively and negatively the quality of the raw materials, therefore indirectly affecting the quality of the wax products. Deswarte investigated the wax content of wheat straw variety Sabre in 2003 and 2005, and highlighted the differences in extraction yield for two different harvesting years and suggested it was due to environmental factors. Environmental factors such as rainfall have shown to have a big influence on wax production in plants.[47, 292, 293] As shown in Section 4.2.2, straw of different varieties from the same season and region can also have different wax content and this is due to the genetics of the plants.[47] There are controllable factors that could also influence the wax content and chemical composition and these include storage conditions and pre-treatment methods.[47, 294] Deswarte showed that the particle size of wheat straw can affect the $scCO_2$ extraction efficiency as small particle sizes can aid the diffusion of CO_2 through the straw and solubilise the wax compounds.[16] As many factors can influence the wax content, chemical composition and extraction efficiency, the three different cereal straws used were all winter cereals grown in the East Midlands in the UK and were harvested in summer 2009. To minimise any variables, the straws all underwent the same physical pre-treatment and storage conditions described in Section 6.2.2. The processed straw pellets were first investigated at laboratory scale to ensure good extraction yield. It would be interesting to investigate the influence on straw waxes of various environmental factors and pre-treatment methods. From the preliminary solvent and CO_2 extraction study, it was concluded that supercritical CO_2 behaved very similarly to both hexane and heptane. [231]

4.3.2 Laboratory extraction trials at York Green Chemistry Centre

Prior to the laboratory extractions, the moisture level of wheat, barley and oat straw pellets were recorded as 9.5%, 8.4% and 9.4% respectively. The straw pellets were Soxhlet extracted using heptane following the procedure described in Section 6.2.3. The wax compounds were identified using GC-MS using the method described in Section 6.3.2 and the key wax compounds were quantified using calibration data discussed in Section 2.5.1 using the GC method described in Section 6.3.1. The percentage crude yield for wheat, oat and barley were 1.4%, 1.8% and 2.1% respectively. These yields were generally higher than the percentage heptane yield presented in the biomass screen in Section 4.2. This could be due to a number of reasons such as the variety of straw, particle size of straw pellets, and quality of straw. From this initial heptane laboratory extraction trial, the percentage recovery of wax from the straw showed promising results for supercritical CO_2 scale up trials. The chemical composition of the straw waxes were found to be almost identical which was not expected as from the straw wax screen, there were distinct differences between the three different cereal crops. The chemical composition profile is highly comparable with Figure 2.43 shown for wheat straw (Viscount 09) and no new molecules were identified. To investigate this further, quantitative analysis was carried out to calculate the percentage of individual wax compound in the extracts. Table 4.8 displays the normalised percentage wax compound in extract (100%) and as µg of wax compound per g of cereal straw. The quantities for the different wax groups are very similar with about 7 – 12% of free fatty acids, 7 – 9% of hydrocarbons, 2% of octacosanol, 1 – 2% of aldehydes, 9 – 10% of wax esters, 5 – 8% of sterols and 3 – 14% of 14,16-hentriacontanedione in the straw waxes. Barley straw showed the highest level of sterol content out of the three cereal straws which are in agreement of a sterol study on the three identical mature cereal straws.[187] However, the study showed that oat straw contains a higher sterol content compared to wheat straw which is not the case with the raw materials used for the industrial scale trials.[187] It was also reported that cholesterol was also identified as one of the main wax components but no cholesterol was found in any extracts from all seven biomasses.[187] The total identified compounds varied between 38 – 47% and taken into the crude extraction yield, the percentage of lipophilic fraction in wheat; oat and barley straw heptane extracts are 0.5%, 0.9% and 1% respectively. This showed that oat and barley straw have higher wax content and can yield a higher value product however, wheat straw is the most abundant and cheapest agricultural cereal crop in the UK so offers the greatest potential source of renewable wax.[26] All three

straws were used for the supercritical CO_2 scale up trial with no pre-treatment modification other than initial milling and pelleting.

Table 4.8: Quantification of laboratory scale heptane Soxhlet extraction wheat, barley and oat straw wax components

Identification	% in extract (n ≥ 2)			$\mu g g^{-1}$ of biomass (n ≥ 2)		
	Wheat	Barley	Oat	Wheat	Oat	Barley
Tetradecanoic acid (C_{14})	0.5 ± 0.02	0.7 ± 0.02	0.9 ± 0.02	66 ± 2.6	126 ± 3.6	184 ± 4.1
Hexadecanoic acid (C_{16})	1.6 ± 0.01	5.4 ± 0.12	4.4 ± 0.07	224 ± 1.4	974 ± 21.6	922 ± 14.7
Octadecadienoic acid ($C_{18:2}$)	1.1 ± 0.03	1.7 ± 0.01	0.2 ± 0.004	161 ± 4.4	301 ± 1.8	40 ± 1.1
Octadecenoic acid ($C_{18:1}$)	2.9 ± 0.08	3.6 ± 0.03	1.3 ± 0.03	403 ± 11.1	639 ± 5.8	274 ± 6.8
Octadecanoic acid ($C_{18:0}$)	0.6 ± 0.01	0.9 ± 0.04	0.7 ± 0.0	87 ± 1.5	154 ± 6.8	139 ± 4.6
Total free fatty acids	**6.7** ± 0.15	**12.2** ± 0.11	**7.4** ± 0.15	**942** ± 21.1	**2195** ± 19.8	**1559** ± 31.6
Heptacosane (C_{27})	0.6 ± 0.04	0.6 ± 0.02	0.6 ± 0.02	78 ± 5.2	100 ± 3.3	136 ± 5.3
Nonsacosane (C_{29})	1.7 ± 0.01	5.9 ± 0.14	4.0 ± 0.04	232 ± 1.4	1057 ± 6.3	840 ± 8.9
Hentriacontane (C_{31})	4.2 ± 0.1	2.0 ± 0.01	2.4 ± 0.03	593 ± 190	355 ± 1.8	502 ± 6.7
Triatriacontane (C_{33})	0.6 ± 0.01	0.5 ± 0.01	0.6 ± 0.02	90 ± 1.7	92 ± 1.8	127 ± 5.1
Total hydrocarbons	**7.1** ± 0.19	**8.9** ± 0.18	**7.6** ± 0.11	**994** ± 27.1	**1605** ± 32.5	**1605** ± 23.5
Octacosanol (C_{28})	2.0 ± 0.1	2.4 ± 0.07	2.0 ± 0.04	287 ± 17.2	440 ± 12.8	428 ± 8.8
Total fatty alcohol	**2.0** ± 0.12	**2.4** ± 0.07	**2.0** ± 0.04	**287** ± 17.2	**440** ± 12.8	**428** ± 8.8
Hexacosanal (C_{26})	0.2 ± 0.01	1.3 ± 0.05	0.5 ± 0.03	29 ± 1.6	240 ± 10	109 ± 6.5
Octacosanal (C_{28})	1.4 ± 0.03	0.9 ± 0.01	0.7 ± 0.04	191 ± 4.4	162 ± 2.2	148 ± 8.7
Total aldehydes	**1.6** ± 0.04	**2.2** ± 0.06	**1.2** ± 0.07	**219** ± 6	**401** ± 12.2	**257** ± 15.2
Tetracosanyl hexadecanoate (C_{24}:C_{16})	0.5 ± 0.02	0.6 ± 0.01	0.7 ± 0.01	64 ± 3	110 ± 1.8	151 ± 2.7
Hexacosanyl hexadecanoate (C_{26}:C_{16})	1.3 ± 0.05	1.4 ± 0.03	1.5 ± 0.04	184 ± 7.1	250 ± 5.4	309 ± 8.9
Octacosanyl hexdecanoate (C_{28}:C_{16})	1.6 ± 0.03	1.9 ± 0.02	2.0 ± 0.1	224 ± 4.8	338 ± 6.2	423 ± 23.3
Octacosanyl octadecanoate (C_{28}:C_{18})	1.4 ± 0.02	1.3 ± 0.01	1.7 ± 0.03	194 ± 3.2	230 ± 2.2	366 ± 6.9
Octacosanyl eicosanoate (C_{28}:C_{20})	1.2 ± 0.01	1.4 ± 0.02	1.4 ± 0.09	165 ± 15.3	245 ± 4.2	297 ± 19.5
Octacosanyl dosanoate (C_{28}:C_{22})	2.1 ± 0.03	1.1 ± 0.01	1.1 ± 0.02	287 ± 8.1	195 ± 2.4	234 ± 4.5

Table 4.8 (continued)

Octacosanyl tetracosanoate (C_{28}:C_{24})	0.9 ± 0.01	0.9 ± 0.02	0.5 ± 0.004	*Tr*	161 ± 4.1	102 ± 0.9
Octacosanyl hexacosanoate (C_{28}:C_{26})	*Tr*	0.5 ± 0.002	*Tr*	*Tr*	99 ± 0.5	*Tr*
Octacosanyl octacosanoate (C_{28}:C_{28})	*Tr*	0.8 ± 0.03	*Tr*	*Tr*	153 ± 6.5	*Tr*
Total wax esters	**8.9** ± 0.17	**9.9** ± 0.15	**9.0** ± 0.3	**1247** ± 41.5	**1781** ± 27.3	**1883** ± 66.7
Campesterol	1.0 ± 0.03	0.9 ± 0.02	1.1 ± 0.01	142 ± 4.5	164 ± 4.3	233 ± 2.8
Stigmasterol	1.0 ± 0.03	0.7 ± 0.03	1.0 ± 0.03	133 ± 4.2	119 ± 5.8	205 ± 7
β-sitosterol	3.0 ± 0.004	2.2 ± 0.01	2.3 ± 0.09	416 ± 0.6	388 ± 2.1	477 ± 19.5
Sterol 1	1.4 ± 0.03	0.6 ± 0.01	0.9 ± 0.02	192 ± 4.7	113 ± 2.4	181 ± 4.5
Δ^4-Sitosten-3-one	1.0 ± 0.02	0.9 ± 0.02	2.0 ± 0.03	137 ± 3.2	163 ± 4.3	428 ± 6.6
Sterol 2	0.9 ± 0.01	*Tr*	*Tr*	120 ± 1.6	*Tr*	*Tr*
Total sterols	**8.1** ± 0.12	**5.3** ± 0.09	**7.3** ± 0.18	**1140** ± 18.8	**947** ± 18.9	**1525** ± 40.4
14,16 hentriacontanedione (C_{31})	3.3 ± 0.1	5.8 ± 0.29	14.3 ± 0.3	459 ± 19.8	1047 ± 52.4	3005 ± 66
Total β-diketones	**3.3** ± 0.14	**5.8** ± 0.29	**14.3** ± 0.3	**459** ± 19.8	**1047** ± 52.4	**3005** ± 66
Total identified	**37.8** ± 0.93	**46.8** ± 0.95	**48.9** ± 1.2	**5287** ±151.5	**8415** ± 175.9	**10262** ± 243.4

Tr = Trace levels < LOQ, LOQ shown in Section 2.5.1

4.3.3 Production scale extraction trials at Evonik Industries

Previously, pilot scale $scCO_2$ extraction of wheat straw at 313 K and 30 MPa using 74 kg of raw materials led to a recovery of 5 kg of wet wax products (containing 5.6% water).[16] In collaboration with Botanix, the flow rate and extraction time were selected according to the company's recommendation which were 350 kgh^{-1} and 3.5 hours respectively.[16] This production scale extraction of wheat, barley and oat straw was carried out in collaboration with Evonik Industries using raw materials from Sundown Products Limited. The optimal operating supercritical CO_2 conditions were determined to be 323 K and a minimum of 35 MPa. High temperatures such as 373 K (Chapter 3) showed an increased co-extraction of non-wax components so for this reason a temperature of 323 K was selected. The flow rate and extraction time were adopted using Evonik's recommendation. As the preliminary studies were

carried out with four hour extraction time, it was suggested the production scale extraction should be a similar extraction time. Table 4.9 summarises the extraction conditions for the production scale $scCO_2$ extraction of three tonnes of straw waxes (one tonne wheat straw, one tonne oat straw and one tonne barley straw) using 2 x 200 L extractors. As the maximum operating pressure for the plant used is only 28 MPa, the operating extraction pressure for wax extraction was dropped to 26 MPa. The bulk densities, hexane Soxhlet extraction yields and moisture levels of the three straw waxes were completed by Evonik Industries prior to production scale extraction as part of the standard operating procedure. The bulk density of the straw pellets allows the estimation of number of batches required for total extraction therefore predicting the total extraction time. Due to the size of the extractors used, the extractions must be carried out in batches. Currently, all industrial extractions are carried out in batch processes. An extraction of 200 L can accommodate a minimum of 90 kg of straw pellets with bulk density of about 0.6 gcm^{-3}. Hexane Soxhlet extraction of the straw pellets was carried out to check for percentage wax recovery as hexane and $scCO_2$ extraction yields are typically very similar. Moisture level of the straw pellets was determined as $scCO_2$ can co-extract any water in the raw material therefore if the level of moisture is too high the physical properties of the CO_2 such as polarity can be modified. When the conditions from the production scale was compared to the optimisation carried out as described in Chapter 3, the estimated yield was highly comparable. This shows that the model in Chapter 3 is relatively accurate when predicting extraction yields and would be useful for future scale up trials.

During the process, one significant problem was encountered as the wax product has a high melting point which means the extract can solidify easily. After ten extractions without emptying the separator, the wax had solidified after the depressurisation valve and in the separator. The separator temperature was only set at 323 K so the wax was not molten. This problem was not apparent in laboratory scale extractions as the quantity of wax in the separator was not enough to block the pipes and also the separator was emptied after each extraction. To overcome the blockage problem, Evonik Industries decided that a ratio of 0.7 – 1.5 parts of rapeseed oil needed to be added as a carrier oil to clear the pipes as indicated in Table 4.10. This was carried out by injecting the oil into the high pressure line between the extractor and separator. This additional oil is not ideal as it leads to wheat straw wax diluted with rapeseed oil which means separation is required prior to any analysis and further processing. A total of five batches of wheat straw waxes (in addition to the 10 batches retrieved without oil) were diluted

with rapeseed oil and details for the weight of wheat straw and percentage yields for these batches are shown below.

Table 4.9: Summary of production scale $scCO_2$ extraction conditions

Straw	Wheat	Oat	Barley
Total weight of raw materials (kg)	895	1095	1080
Bulk density (gcm^{-3})	0.61 - 0.64	0.63	0.63
Extraction temperature (K)	323 - 343	323 - 343	323 - 343
Extraction pressure (MPa)	26	26	26
Flow rate (kg of CO_2 per kg of straw pellets)	50 - 60	50 - 60	50 - 60
Extraction time (hours)	4	4	4
Number of batches	10	12	12
Extraction crude yields (%)	1.3	1.8	2.5

Table 4.10: Summary of carrier oil-containing wheat straw waxes

Straw	Wheat	Wheat	Wheat	Wheat
Total weight of raw materials (kg)	95	95	190	90
Extraction temperature (K)	323 - 343	323 - 343	323 - 343	323 - 343
Extraction pressure (MPa)	26	26	26	26
Flow rate (kg of CO_2 per kg of straw pellets)	50 - 60	50 - 60	50 - 60	50 - 60
Extraction time (hours)	4	4	4	4
Wax: rapeseed oil (carrier oil) ratio	1:1.5	1:1.2	1:0.7	Low
Percentage yields (including carrier oil) (%)	7.28	8.12	4.24	2.0

Due to blockage problems from solidifying waxes, the separator temperature had to be increased to ensure the wax remains molten in the pipelines and separator so the wax can be recovered easily. The majority of the wheat straw wax was fully melted at about 343 K so the temperature of the separator for the remaining batches of straw was set to 343 K and this was shown to be a good way to retrieve the wax. In this scale up trial, the extraction pressure had to be lowered due to the limited capability of the plant and the separator temperature was increased to enable easier handling of the extracted wax.

The waxes collected from the $scCO_2$ production scale trials were different to those from $scCO_2$ laboratory scale waxes as the volume of co-extracted water was larger. Wet waxes were also recovered in the pilot scale extraction of wheat straw waxes.[16] Water is not co-extracted during solvent extractions so this is not a problem that needs to be dealt with when using traditional non-polar organic solvents. The co-extraction of water is discussed in more detail in Section 5.2.1. Figure 4.16 shows the percentage crude yields for the laboratory solvent extractions carried out both in York and in Evonik Industries. The graph also shows the supercritical CO_2 wet and dry yield from production scale extractions. The supercritical CO_2 yields looked highly comparable when the co-extracted water was included however, once the water is removed, the percentage crude yield dropped to a range of only 0.8 – 1.5%. These yields were much lower than expected as the solvent extraction trials were between 1.4 – 2.3%. One explanation for the difference in percentage yield could be due to the lower pressure used for the scale up. A drop in extraction pressure is equivalent to a decrease in CO_2 density and it was concluded in Chapter 3 that the percentage crude yield is highly dependent on the density of CO_2. The lower efficiency of the extraction may also be due to the presence of water altering the solubility of the different wax components in $scCO_2$. It is possible to carry out a second $scCO_2$ extraction using higher pressure in order to gain more understanding of the chemical composition of the "missing" yield. As the preliminary trials were carried out using hexane/ heptane, the straw residues after the production scale $scCO_2$ extraction were re-extracted using heptane Soxhlet extraction.

The $scCO_2$ extracted straw residues appeared almost identical to the virgin cereal straw pellets. However, the extracted straw residues were re-pelletised using the procedure described in Section 4.2.2 by Sundown Products Limited for animal feed. Figure 4.17 shows a comparison graph for the supercritical CO_2 and heptane re-extracted crude yields with heptane Soxhlet crude yields. The combined total yields for heptane re-extracted and supercritical CO_2 are highly comparable with heptane Soxhlet yields which suggests that the straw residues still contain unextracted waxes. This indicates that the CO_2 density used for the extraction was not high enough to recover the full quantity of the lipophilic fraction. In order to improve the wax recovery, the extraction pressure must be increased. Increasing the pressure will lead to an increase in the cost of the process significantly so it must be considered carefully by carrying out an economical assessment to investigate whether increase in yield is sufficient to justify the increased extraction pressure.

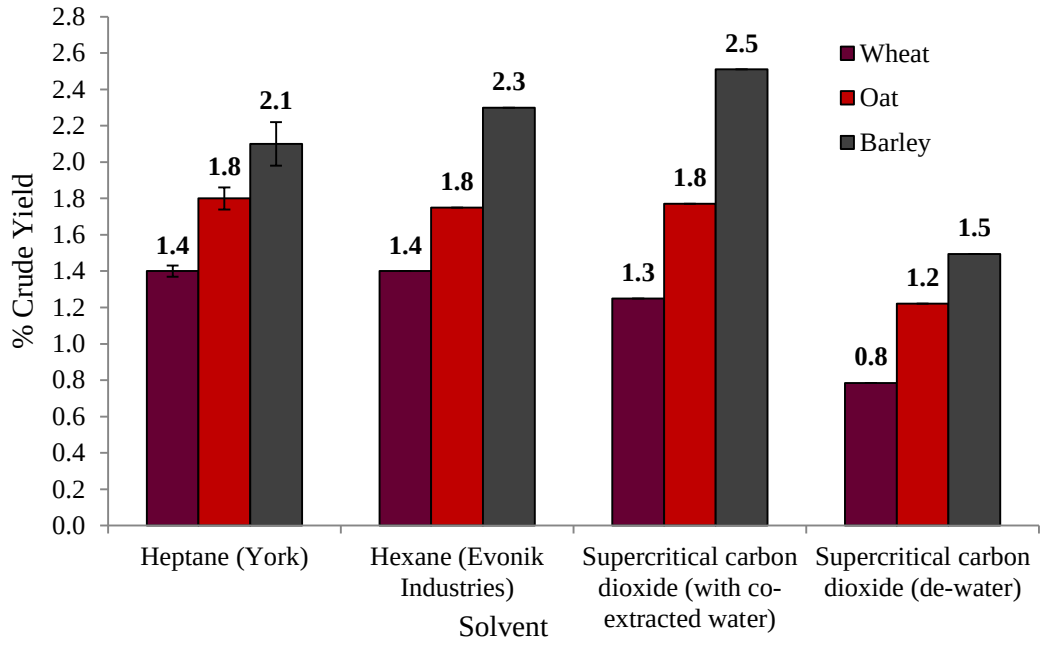


Figure 4.16: Laboratory heptane/ hexane Soxhlet and production scale supercritical CO_2 percentage crude yields (originally in colour)

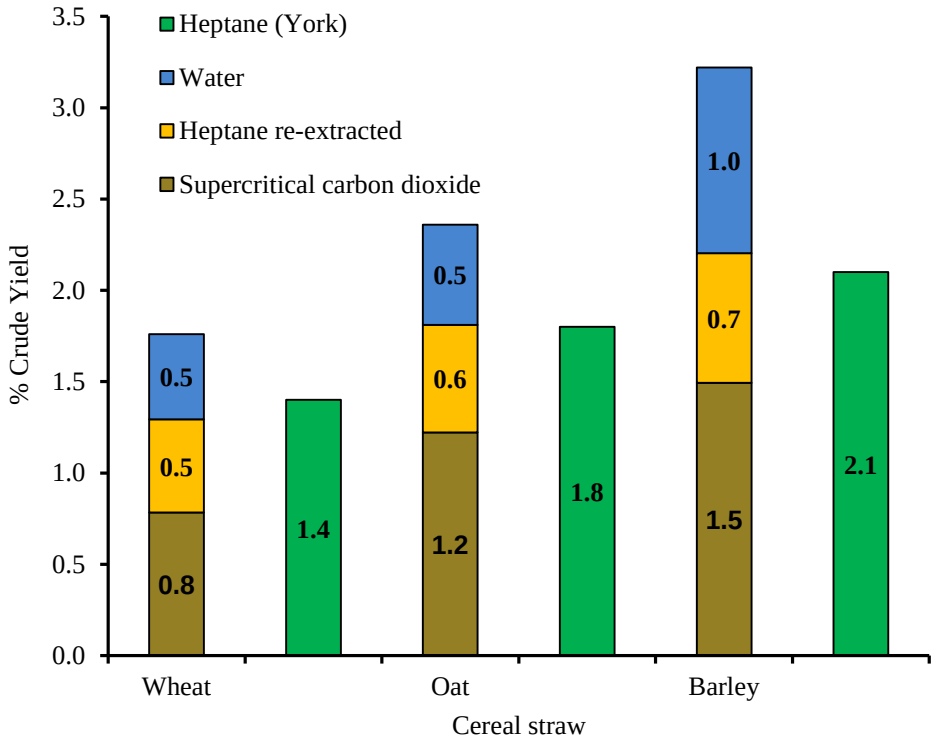


Figure 4.17: Comparison of supercritical CO_2 and heptane re-extracted yields against heptane Soxhlet yields (originally in colour)

Full identification and quantification of the straw waxes from the production scale supercritical CO_2 and laboratory scale heptane re-extracted Soxhlet extracts were carried out. The chromatograms for these extracts were identical to the heptane Soxhlet extracts which all looked almost identical to Figure 2.43 in Chapter 2, reinforcing the point that the chemical composition of the cereal straw extracts are highly comparable. The main differences between the extracts that give their unique physical properties are the relative abundance of the wax components. The calibration data for the quantification analysis used for these extracts are described in Section 2.5.1.

Table 4.11 shows the percentage of wax components in the extracts from the production scale $scCO_2$ extraction as well as the wax components in µg per gram of biomass. The recovery of barley straw wax from the $scCO_2$ extraction appeared to be above 100%. This could be due to the extra percentage added to the total due to the co-elution of hentriacontane (C_{31}) and octacosanol (C_{28}). The recoveries for all three straw waxes are high which suggested that the CO_2 conditions used were highly selective for the lipophilic components. The percentage yields for each wax groups for the three different straw waxes are similar under $scCO_2$ conditions. Generally as the percentage crude yield for barley straw is the highest, the high value wax groups such as hydrocarbons, sterols, wax esters, fatty alcohols and hydrocarbons were present at much higher levels in the straw. The quantity of wax esters found in the barley straw was 2.5 times higher than the wheat straw and, as discussed previously, wax esters are high value products that have many applications such as in cosmetics and wax polishes. The same trend can also be seen with hydrocarbons, fatty alcohols and β-diketones as barley straw contains around 2.5 times more of these wax groups which again have lots of potential high value applications. Based on the percentage crude yield and chemical composition, barley straw can be concluded to be the cereal straw with the greatest scale up potential even though the volume available is significantly less than wheat straw. As $scCO_2$ extraction requires high temperature and pressure, it is very energy intensive, so it is important that the maximum percentage crude yield is achieved from the process to make it more economically viable.

Table 4.12 shows the percentage of wax components in the extracts from heptane Soxhlet re-extraction of the straw residues and the wax components in µg per gram of biomass. As discussed earlier, there was a discrepancy between the heptane Soxhlet (laboratory scale) and $scCO_2$ (industrial scale) yields so this was investigated further. Again the chemical composition was identical so quantification analysis was carried out. Data showed that the wax components

remaining in the $scCO_2$ extracted straw residues were very rich in fatty acids. In the straw waxes, a range of 16% – 33% of the free fatty acids were not extracted with the $scCO_2$ conditions applied. Some wax esters, sterols and β-diketones were also present in the heptane re-extracted waxes showing that there is more potential to increase the percentage wax yield by manipulating the $scCO_2$ extraction conditions. Table 4.13 compares the wax components in µg per gram of biomass of heptane Soxhlet (laboratory scale) and the combined values of $scCO_2$ and heptane re-extracted Soxhlet. Small variation in wax composition can be due to random sampling, different ratios of straw leaf and stalk has been shown that by Deswarte that wheat straw leaf contains of higher wax content compared to stalk, therefore it may not be surprising to see different wax profiles.[16] Mazza compared the wax content of triticale straw extracted from both supercritical CO_2 and hexane and showed that the abundance of the main compounds were different.[90] It was suggested that this could be due to the intermolecular forces between hexane and $scCO_2$ being different. Hexane is a non-polar solvent so no hydrogen bonding can be formed whereas in $scCO_2$, polar and hydrogen bonding can occur. As demonstrated in Chapter 3, the relative abundance of main wax compounds can vary depending on $scCO_2$ conditions so it is not surprising that there is not an exact match of heptane and $scCO_2$ compositional profile. The combined $scCO_2$ and heptane yields were higher than heptane and $scCO_2$ yields alone which also suggested that the two solvents can form potentially different solute-solvent interactions. Mazza extracted flax straw using a combination of both hexane and $scCO_2$ and the solvents individually and also showed that the combined percentage hexane and $scCO_2$ crude yields were highest.[88] The β-diketones in the heptane Soxhlet extract of wheat straw wax was almost three times less than the combined values for $scCO_2$ and heptane re-extracted in which at least 98% was extracted by $scCO_2$. It is important to note that the total identified wax compounds were higher than heptane extracts which showed that despite the higher heptane yield, $scCO_2$ is more selective in the extraction of waxes. Since there was still a relatively high quantity of free fatty acids remaining in the straw residues as discussed earlier, this shows another added benefit to using $scCO_2$ as the solvent. The lower level of free fatty acids means less downstream processing is required to reduce the fatty acid contents of the products.

Table 4.11: Quantification of industrial scale $scCO_2$ extraction of cereal straw waxes

Identification	% in extract			$\mu g g^{-1}$ of biomass		
	Wheat	Oat	Barley	Wheat	Oat	Barley
Tetradecanoic acid (C_{14})	0.5	0.4	0.2	276	54	33
Hexadecanoic acid (C_{16})	3.5	5.8	4.7	110	713	699
Octadecadienoic acid ($C_{18:2}$)	1.4	2.5	1.9	172	303	278
Octadecenoic acid ($C_{18:1}$)	2.2	4.7	3.8	*Tr*	572	575
Octadecanoic acid ($C_{18:0}$)	0.5	1.3	0.9	37	159	129
Total free fatty acids	**8.1**	**6.0**	**11.5**	**634**	**731**	**1714**
Heptacosane (C_{27})	0.7	1.0	1.2	58	118	183
Nonacosane (C_{29})	2.4	8.6	11.6	187	1053	1726
Hentriacontane (C_{31})	6.8[a]	4.6[a]	6.6[a]	531[a]	562[a]	981[a]
Triatriacontane (C_{33})	*Tr*	0.4	*Tr*	*Tr*	*Tr*	*Tr*
Total hydrocarbons	**9.9[a]**	**14.6[a]**	**19.3[a]**	**776[a]**	**1783[a]**	**2889[a]**
Octacosanol (C_{28})	8.9[a]	11.9[a]	10.2[a]	698[a]	1454[a]	1519[a]
Total fatty alcohol	**8.9[a]**	**11.9[a]**	**10.2[a]**	**698[a]**	**1454[a]**	**1519[a]**
Hexacosanal (C_{26})	*Tr*	2.8	*Tr*	*Tr*	343	*Tr*
Octacosanal (C_{28})	0.3	1.8	2.2	39	222	327
Total aldehydes	**0.3**	**4.6**	**2.2**	**39**	**565**	**327**
Tetracosanyl hexadecanoate (C_{24}:C_{16})	0.6	*Tr*	*Tr*	45	*Tr*	*Tr*
Hexacosanyl hexadecanoate (C_{26}:C_{16})	1.0	1.4	0.8	80	168	127
Octacosanyl hexdecanoate (C_{28}:C_{16})	1.0	1.6	1.5	81	190	218
Octacosanyl octadecanoate (C_{28}:C_{18})	0.7	4.0	3.8	53	488	560
Octacosanyl eicosanoate (C_{28}:C_{20})	0.7	2.4	3.2	57	288	484
Octacosanyl dosanoate (C_{28}:C_{22})	0.6	2.4	2.8	45	288	417
Octacosanyl tetracosanoate (C_{28}:C_{24})	1.4	1.3	2.3	111	159	344
Octacosanyl hexacosanoate (C_{28}:C_{26})	6.0	1.1	1.3	473	136	191
Octacosanyl octacosanoate (C_{28}:C_{28})	0.7	0.4	1.3	58	55	188
Total wax esters	**12.8**	**14.5**	**16.9**	**1003**	**1771**	**2530**
Campesterol	2.4	2.5	3.0	184	310	455
Stigmasterol	2.4	1.6	2.1	187	191	320
β-sitosterol	6.3	4.4	5.8	490	532	873
Sterol 1	1.9	*Tr*	1.6	152	*Tr*	238
Δ^4-Sitosten-3-one	7.8	4.0	8.4	609	488	1252
Sterol 2	1.2	*Tr*	0.0	92	*Tr*	*Tr*
Total sterols	**21.9**	**12.5**	**21.0**	**1715**	**1521**	**3138**
14,16 hentriacontanedione (C_{31})	16.0	10.9	21.2	1257	1337	3164
16,18 triatriacontanedione (C_{33})	*Tr*	0.6	*Tr*	*Tr*	79	*Tr*
Total β-diketones	**16.0**	**11.6**	**21.2**	**1257**	**1416**	**3164**
Total identified	**67.5**	**75.7**	**102.3**	**5306**	**9240**	**15281**

[a] = co-elution and values shown are the combined quantities, *Tr* = Trace levels < LOQ, LOQ shown in Section 2.5.1

Table 4.12: Quantification of laboratory scale re-heptane Soxhlet extraction of cereal straw waxes

Identification	% in extract			$\mu g g^{-1}$ of biomass		
	Wheat	Oat	Barley	Wheat	Oat	Barley
Tetradecanoic acid (C_{14})	0.4	0.5	0.4	21	29	29
Hexadecanoic acid (C_{16})	2.8	3.0	2.4	145	174	170
Octadecadienoic acid ($C_{18:2}$)	0.4	0.5	0.4	20	29	29
Octadecenoic acid ($C_{18:1}$)	1.2	1.5	1.8	64	89	127
Octadecanoic acid ($C_{18:0}$)	0.9	0.6	0.7	44	35	47
Total free fatty acids	**5.7**	**6.1**	**5.7**	**295**	**356**	**401**
Heptacosane (C_{27})	0.3	0.2	0.2	15	11	12
Nonacosane (C_{29})	0.1	0.4	0.3	7	26	23
Hentriacontane (C_{31})	0.4[a]	0.5	0.3[a]	19[a]	30	22[a]
Triatriacontane (C_{33})	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*
Total hydrocarbons	**0.8[a]**	**1.1**	**0.8[a]**	**40[a]**	**67**	**58[a]**
Octacosanol (C_{28})	0.6[a]	0.7	0.4[a]	23[a]	38	29[a]
Total fatty alcohol	**0.6[a]**	**0.7**	**0.4[a]**	**23[a]**	**38**	**29[a]**
Hexacosanal (C_{26})	*Tr*	0.1	*Tr*	*Tr*	*5*	*Tr*
Octacosanal (C_{28})	*Tr*	0.2	*Tr*	*Tr*	*12*	*Tr*
Total aldehydes	***Tr***	**0.3**	***Tr***	***Tr***	***16***	***Tr***
Tetracosanyl hexadecanoate (C_{24}:C_{16})	0.4	0.6	0.7	*Tr*	*Tr*	48
Hexacosanyl hexadecanoate (C_{26}:C_{16})	0.4	0.6	0.8	22	34	55
Octacosanyl hexdecanoate (C_{28}:C_{16})	0.3	0.5	0.8	23	35	57
Octacosanyl octadecanoate (C_{28}:C_{18})	*Tr*	0.5	0.6	15	29	43
Octacosanyl eicosanoate (C_{28}:C_{20})	*Tr*	0.6	0.7	*Tr*	32	48
Octacosanyl dosanoate (C_{28}:C_{22})	*Tr*	0.6	0.4	*Tr*	35	31
Octacosanyl tetracosanoate (C_{28}:C_{24})	*Tr*	*Tr*	*Tr*	*Tr*	33	*Tr*
Total wax esters	**1.2**	**3.4**	**4.0**	**60**	**197**	**281**
Campesterol	0.6	0.5	0.4	31	30	30
Stigmasterol	0.5	0.5	0.4	25	29	29
β-sitosterol	1.5	2.0	1.4	79	118	102
Sterol 1	0.3	0.2	0.3	17	13	21
Δ^4-Sitosten-3-one	0.5	0.3	0.3	25	16	21
Total sterols	**3.5**	**3.5**	**2.8**	**178**	**206**	**202**
14,16 hentriacontanedione (C_{31})	0.5	1.4	1.6	23	81	141
Total β-diketones	**0.5**	**1.4**	**1.6**	**23**	**81**	**141**
Total identified	**12.2**	**16.4**	**15.3**	**619**	**962**	**1112**

[a] = co-elution and values shown are the combined quantities, *Tr* = Trace levels < LOQ, LOQ shown in Section 2.5.1

Table 4.13: Comparison of the chemical compositions from heptane Soxhlet, supercritical CO_2 and heptane re-extracted wax extracts

Identification	Heptane Soxhlet (n ≥ 2) µgg^{-1} of biomass			Supercritical CO_2 µgg^{-1} of biomass			Heptane re-extracted µgg^{-1} of biomass			sxCO_2 and heptane re-extracted µgg^{-1} of biomass		
	Wheat	Oat	Barley	Wheat	Oat	Barley	Wheat	Oat	Barley	Wheat	Oat	Barley
Tetradecanoic acid (C_{14})	66 ± 2.6	126 ± 3.6	184 ± 4.1	276	54	33	21	29	29	298	83	62
Hexadecanoic acid (C_{16})	224 ± 1.4	974 ± 21.6	922 ± 14.7	110	713	699	145	174	170	255	887	869
Octadecadienoic acid ($C_{18:2}$)	161 ± 4.4	301 ± 1.8	40 ± 1.1	172	303	278	20	29	29	192	332	307
Octadecenoic acid ($C_{18:1}$)	403 ± 11.1	639 ± 5.8	274 ± 6.8	*Tr*	572	575	64	89	127	64	661	701
Octadecanoic acid ($C_{18:0}$)	87 ± 1.5	154 ± 6.8	139 ± 4.6	37	159	129	44	35	47	81	194	176
Total free fatty acids	**942** ± 21.1	**2195** ± 19.8	**1559** ± 31.6	**595**	**1802**	**1714**	**295**	**356**	**401**	**890**	**2157**	**2115**
Heptacosane (C_{27})	78 ± 5.2	100 ± 3.3	136 ± 5.3	58	118	183	15	11	12	73	129	195
Nonsacosane (C_{29})	232 ± 1.4	1057 ± 6.3	840 ± 8.9	187	1053	1726	7	26	23	194	1079	1749
Hentriacontane (C_{31})	593 ± 190	355 ± 1.8	502 ± 6.7	531[a]	562[a]	981[a]	19[a]	30	22[a]	550[a]	591[a]	1003[a]
Triatriacontane (C_{33})	90 ± 1.7	92 ± 1.8	127 ± 5.1	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*
Total hydrocarbons	**994** ± 27.1	**1605** ± 32.5	**1605** ± 23.5	**776[a]**	**1783[a]**	**2889[a]**	**40[a]**	**67**	**58[a]**	**816[a]**	**1849[a]**	**2947[a]**
Octacosanol (C_{28})	287 ± 17.2	440 ± 12.8	428 ± 8.8	698[a]	1454[a]	1519[a]	23[a]	38	29[a]	722[a]	1492[a]	1547[a]
Total fatty alcohol	**287** ± 17.2	**440** ± 12.8	**428** ± 8.8	**698[a]**	**1454[a]**	**1519[a]**	**23[a]**	**38**	**29[a]**	**722[a]**	**1492[a]**	**1547[a]**
Hexacosanal (C_{26})	29 ± 1.6	240 ± 10	109 ± 6.5	Tr	343	Tr	*Tr*	5	*Tr*	*Tr*	348	Tr

Table 4.13 (continued)

Octacosanal (C_{28})	191 ± 4.4	162 ± 162	148 ± 8.7	39	*222*	327	*Tr*	12	*Tr*	39	234	327
Total aldehydes	**219** ± 6	**401** ± 12.2	**257** ± 15.2	**39**	**565**	**327**	***Tr***	**16**	***Tr***	**39**	**582**	**327**
Tetracosanyl hexadecanoate (C_{24}:C_{16})	64 ± 3	110 ± 1.8	151 ± 2.7	45	*Tr*	*Tr*	*Tr*	Tr	48	45	Tr	48
Hexacosanyl hexadecanoate (C_{26}:C_{16})	184 ± 7.1	250 ± 5.4	309 ± 8.9	80	168	127	22	34	55	102	202	182
Octacosanyl hexdecanoate (C_{28}:C_{16})	224 ± 4.8	338 ± 6.2	423 ± 23.3	81	190	218	23	35	57	104	224	276
Octacosanyl octadecanoate (C_{28}:C_{18})	194 ± 3.2	230 ± 2.2	366 ± 6.9	*53*	488	*560*	*15*	*29*	43	68	517	603
Octacosanyl eicosanoate (C_{28}:C_{20})	165 ±	245 ± 4.2	297 ± 19.5	57	288	484	*Tr*	32	48	57	319	532
Octacosanyl dosanoate (C_{28}:C_{22})	287 ±	195 ± 2.4	234 ± 4.5	45	288	417	*Tr*	35	31	45	324	448
Octacosanyl tetracosanoate (C_{28}:C_{24})	*Tr*	161 ± 4.1	102 ± 0.9	111	159	344	*Tr*	33	*Tr*	111	192	344
Octacosanyl hexacosanoate (C_{28}:C_{26})	*Tr*	99 ± 0.5	*Tr*	473	136	191	*Tr*	*Tr*	*Tr*	473	136	191
Octacosanyl octacosanoate (C_{28}:C_{28})	*Tr*	153 ± 6.5	*Tr*	58	55	188	*Tr*	*Tr*	*Tr*	58	55	188
Total wax esters	**1247** ±	**1781** ± 27.3	**1883** ± 66.7	**1003**	**1771**	**2530**	**60**	**197**	**281**	**1063**	**1968**	**2812**
Campesterol	142 ±	164 ± 4.3	233 ± 2.8	184	310	455	31	30	30	215	340	485
Stigmasterol	133 ±	119 ± 5.8	205 ± 7	187	191	320	25	29	29	212	220	348
β-sitosterol	416 ±	388 ± 2.1	477 ± 19.5	490	532	873	79	118	102	569	650	975
Sterol 1	192 ±	113 ± 2.4	181 ± 4.5	152	*Tr*	238	*17*	*13*	*21*	168	*Tr*	259
Δ^4-Sitosten-3-one	137 ±	163 ± 4.3	428 ± 6.6	609	488	1252	*25*	16	*21*	635	503	1272
Sterol 2	120 ±	*Tr*	*Tr*	92	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	270	*Tr*	*Tr*

Table 4.13 (continued)

Total sterols	**1140** ±	**947** ± 18.9	**1525** ± 40.4	**1715**	**1521**	**3138**	**178**	**206**	**202**	**1738**	**1602**	**3278**
14,16 hentriacontanedione (C_{31})	459 ±	1047 ± 52.4	3005 ± 66	1257	1337	3164	23	81	141	1281	1418	3305
16,18 triatriacontanedione (C_{33})	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	*Tr*	79	*Tr*	*Tr*	79	*Tr*
Total β-diketones	**459** ±	**1047** ± 52.4	**3005** ± 66	**1257**	**1337**	**3164**	**23**	**161**	**141**	**1280**	**1498**	**3305**
Total identified	**5287** ±	**8415** ± 175.9	**10262** ± 243.4	**6083**	**10232**	**15281**	**619**	**1025**	**1112**	**6564**	**10657**	**16004**

[a] = co-elution and values shown are the combined quantities, *Tr* = Trace levels < LOQ, LOQ shown in Section 2.5.1

4.4 Conclusion and future work

Previously, it was demonstrated that renewable and valuable waxes can be extracted using $scCO_2$ successfully and was chosen as a greener alternative for the extraction of straw waxes. Wheat straw wax had been shown to a provide valuable wax product but as there are a vast amount of waste straw worldwide, it was important to explore other potential biomass. A total of seven different straws were chosen for the biomass screen and the aim was to select three of the straws for further scale up. The epicuticular wax of wheat, barley, oat, oilseed rape, sunflower, miscanthus and bagasse were investigated. From the organic solvent study discussed in Chapter 2, hexane and ethanol Soxhlet extractions were selected to use as a quick screening method for the percentage crude yield and chemical composition. Due to the similarities in the chemical composition profiles, quantitative analysis was required in order to give a deeper insight into the epicuticular waxes of each biomass. It can be noted that as with all natural products, many natural variations will play a role such as seasonal variation therefore it was not necessary to identify all the wax compounds as it is most likely that the different batches of straw would result in a range of wax products. The aim of the screen was only to give an insight into the various waxes of the potential biomass. By considering different aspects such as biomass availability, percentage crude yield and chemical composition, wheat, barley and oat were selected for the $scCO_2$ scale up trials as laboratory scale work showed promising valuable wax products which can be used in many different markets.

Following successful laboratory study on the three selected cereal straws, the process was scaled up in collaboration with Sundown Products Limited and Evonik Industries. A total of three tonnes of wheat, barley and oat straw wax were extracted using $scCO_2$ in an industrial scale plant. Process conditions (temperature and pressure) requested were the optimised conditions investigated in Chapter 3 which were 323 K and 35 MPa. However, due to the limitations of the $scCO_2$ equipment at Evonik Industries, the extraction pressure was dropped to 28 MPa. The extraction temperature was between 333 K – 343K which was also higher than optimised conditions which means the CO_2 density was lower than expected. The extraction process was very successful except that blockage at the separator occurred due to the high melting point of the waxes. Oilseed rape oil was used as a carrier oil to lubricant the pipe work in order to retrieve the wax. Originally the temperature of the seprator was set at 323 K but from the blockages, it was apparent that the temperature is required to be higher than at laboratory scale so the latter extractions were carried using a

higher sepator temperature of 343 K. The percentage crude yields were lower than expected from the laboratory scale work but this was mainly due to the decrease in CO_2 density during the extraction process. For this reason, the straw residues were re-extracted using heptane. It was concluded that there was still a considerable amount of wax remaining in the straw residues so therefore it is important to increase the CO_2 density to maximise the extraction yield. Qualitative analysis was carried out on the straw waxes (both biomass screen and production scale trials) which found that the wax compounds were highly similar so therefore quantitative analysis was carried out so that extracts can be compared. A mixture of fatty acids, *n*-alkanes, sterols, fatty alcohols, wax esters, aldehydes and β-diketones were identified as straw wax extracts. As this is a complex mixture, the system could behave differently as the properties of the CO_2 are prone to changes as an increasing amount of wax compounds are dissolved into the supercritical fluid. The laboratory scale work demonstrated that small scale extraction can successfully predict the yield and chemical compoistion of scale up trials on larger $scCO_2$ extraction plants. The optimisation of $scCO_2$ conditions proved to be useful when selecting extraction conditions for the production scale trials but more work may be required for an optimised flow rate. Overall, the production scale up trials of straw waxes were successful and post-processing steps on the 60 kg of wax products are discussed in Chapter 5.

Chapter 5

Straw wax processing, physical properties and economic considerations

Oral presentation given at 1st NORthern Sustainable Chemistry meeting (NORSC), York, UK, October 2010

Poster winner at Plants as Providers as Fine Chemicals, Bangor, Wales, September 2011

5. STRAW WAX PROCESSING, PHYSICAL PROPERTIES AND ECONOMIC CONSIDERATIONS

5.1 Introduction

Wax extracts from the $scCO_2$ production scale extraction were firstly processed to remove the co-extracted water from the biomass. Fractionation by CO_2 and GPC and saponification were explored to create fractions containing different chemical compositions to aid future wax blending and formulations. The benefit of using CO_2 as a fractionation technique is that it can be incorporated with the $scCO_2$ extraction to make a one step process. Physical properties such as melting point are paramount for potential applications. Wet analytical methods such as acid value gives a greater understanding on the possible potential for commercialisation and apply to relevant products formulations. The chemical compositional profiles of the wax extracts are matched with the resulting physical properties to enable the tuning of properties by fractionation or chemical modification of the straw waxes.

In Chapter 4, the extraction of $scCO_2$ extraction was successfully carried out in production scale. The main advantage of using $scCO_2$ as the extraction solvent is its low environmental impact however, as the extraction of waxes requires high pressure it is an expensive technology which can raise many concerns industrially. For this reason, an economic assessment was carried out for the extraction of wax from cereal straw as part of the consideration of using $scCO_2$ extraction in future.

5.2 Straw wax processing

5.2.1 Co-extraction of water

Air-dried pelletised cereal straw contains residual water of less than 10% which is frequently co-extracted with the wax components under $scCO_2$ conditions as illustrated in Figure 5.1. The moisture content was calculated using Equation 6.1 and the moisture level before and after the extraction are shown in Table 5.1.

Table 5.1: Percentage moisture level of biomass before and after extraction

Cereal	% Moisture level	
	Before extraction	After extraction
Wheat	9.9	4.2
Oat	9.5	3.8
Barley	7.9	3.3

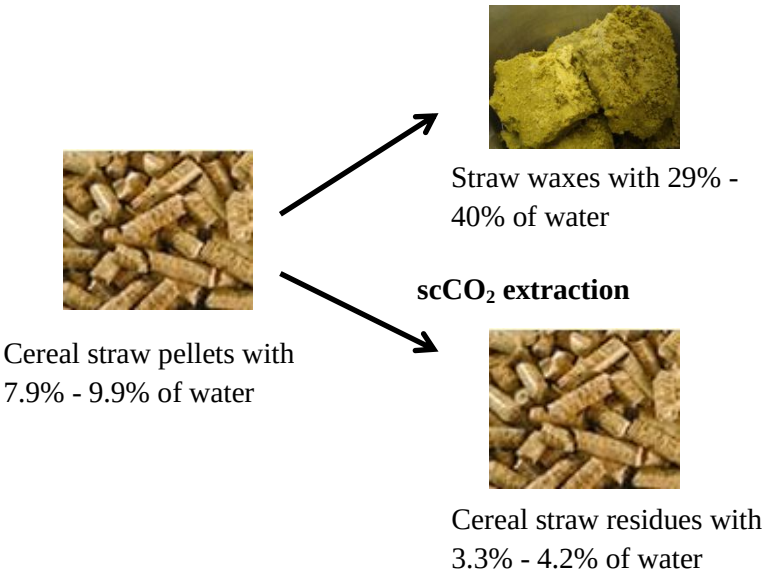


Figure 5.1: Co-extraction of water in industrial scale supercritical CO_2 extraction (originally in colour)

When CO_2 is applied to the biomass that contains water, there is a tendency for the CO_2 to form carbonic acid which can alter the solubility of different molecules in CO_2 by acting as a "co-solvent". In the laboratory trial, this is not so important as the quantity of water is not high enough to change the behaviour. However, this is especially crucial in terms of production scale extraction, as the presence of water can act as a modifier during the extraction process which can manipulate the polarity of the solvent and lead to a higher recovery of polar molecules.

Pilot scale extraction of rice bran oil also demonstrated the problems with the co-extraction of water from the biomass and centrifugation was adopted as a means to remove the water.[295] The problems of co-extraction of water only become apparent and problematic in scale ups when large quantities of water accumulated in the separator.[295] Evelein *et al.* studied the solubility of pure water in CO_2 and showed that

at 323 K and pressure from 20 – 60 MPa, the solubility of water in CO_2 is about 0.3%.[296] The interesting point to note from the study is that the solubility is almost independent of pressure when it is above 20 MPa.[296] This suggest that water is almost completely soluble with scCO_2 conditions of 323 K and 20 MPa.

The water from the wax extracts was separated manually by melting the waxes using the procedure described in Section 6.2.5 but, it would be advantageous if an additional separator is added to separate the water from the wax extract. Figure 5.2 shows the molten wax being separated from the coloured co-extracted water. All three co-extracted waters were coloured. Wheat and barley water were both light yellow and interestingly oat water was light pink as shown in Figure 5.3. The water retrieved from the DCM and aqueous liquid-liquid extractions with the solvent extracted wheat straw extracts discussed in Chapter 2 were also light yellow in colour. Any water soluble compounds in the cereal straw can be co-extracted with the water present in the biomass and this could include any pigments, phenolics, resin acids and flavonoids that occur in the plants. Carotenoids such as xanthophylls and flavonoids such as anthrocyanins known to produce colours have been identified in cereal crops previously.[297, 298] The co-extracted water can be utilised by identifying any valuable water soluble compounds. This would not only provide cleaner waste water but also adds value to a potentially large waste stream. It would be interesting to identify and quantify any commercially valuable compounds as it can also improve the economics of the overall process. Figure 5.4 shows the overall percentage of co-extracted water with the individual straw wax. Barley straw wax was the wettest as it contained double the quantities of co-extracted water compared to the wheat and oat straw waxes.

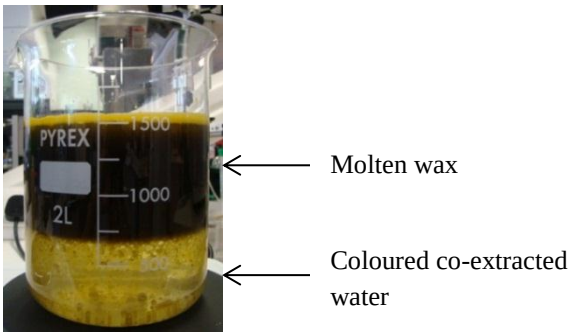
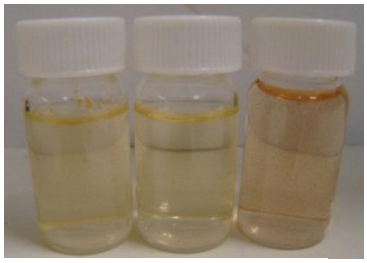


Figure 5.2: Co-extracted water separated from molten wheat straw wax (originally in colour)



Wheat Barley Oat

Figure 5.3: Coloured co-extracted water from cereal straw waxes (originally in colour)

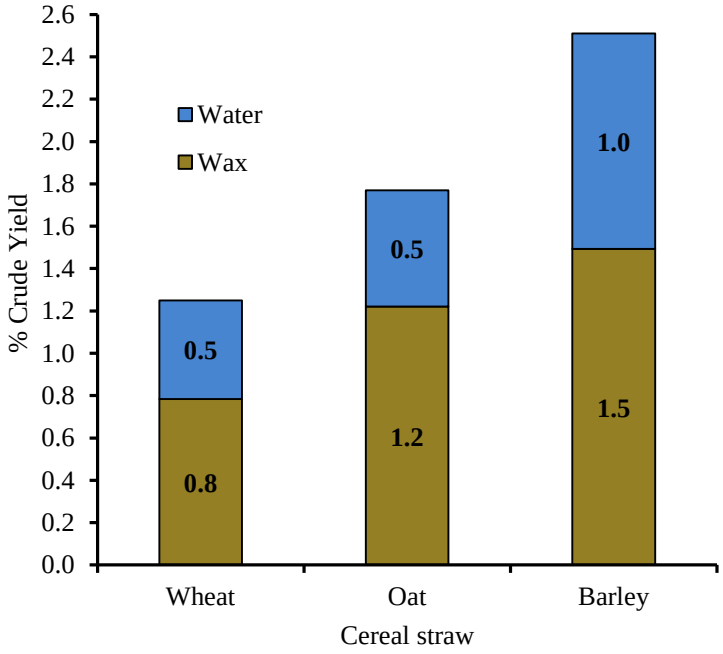


Figure 5.4: Percentage of co-extracted water within the cereal straw waxes (originally in colour)

5.2.2 Fractionation by supercritical carbon dioxide

From the qualitative and quantitative analysis of the three cereal straw waxes from the large scale trials, wheat and barley straw waxes showed similar compositional profiles. Wheat and oat straw waxes were selected to demonstrate that $scCO_2$ can be used to successfully fractionate as well as extract these renewable and valuable waxes. The use of $scCO_2$ to extract wheat straw waxes has been reported by Deswarte but this is the first time that straw waxes have been fractionated using $scCO_2$.[18] Reverchon *et al.* successfully conducted extraction and fractionation of lavender oil and waxes

using CO_2 where a high alkane fraction of the wax was separated. The wheat and oat straw waxes from the large scale trials were loaded onto glass beads and fractionated using a laboratory scale CO_2 extractor as described in Section 6.2.6. As there were only two separators, the conditions chosen for each of the separators were distinctly different. The extractor was set at 323 K and 35 MPa which is higher than the industrial extraction conditions in order to ensure that all the wax compounds are highly soluble at the specific temperature and pressure for fractionation efficiency. Separator 1 was set at 305 K and 7.5 MPa which is just above the critical point of CO_2 so that any wax compounds that are insoluble at the critical point of CO_2 would precipitate and remain in the separator. The second separator was at atmospheric pressure so that all the remaining wax compounds not precipitated in separator 1 could be captured. Figure 5.5 shows that the fractionated waxes were very different in colour, the waxes removed from separator 1 was darker in colour compared to the fraction retrieved from separator 2. The fractions separated at the critical point of CO_2 are also appeared harder than the fractions recovered at atmospheric pressure.

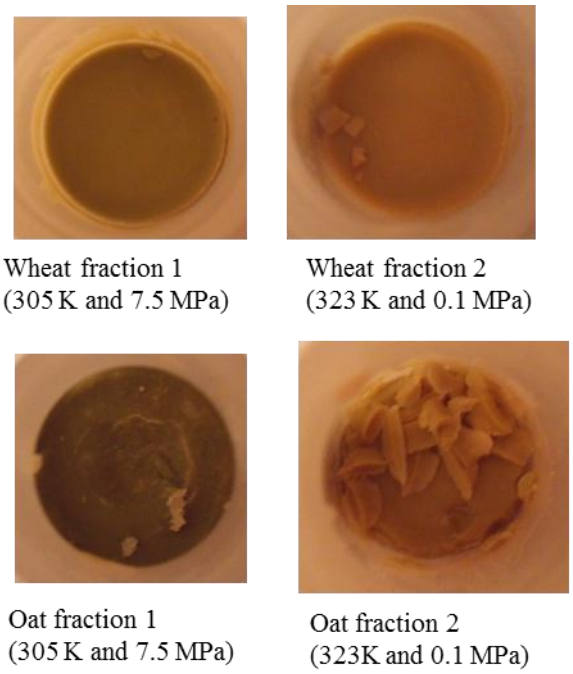


Figure 5.5: Wheat and oat wax fractions separated from $scCO_2$ fractionation (originally in colour)

Table 5.2 shows the percentage of waxes present in the extractor and the two separators after four hours of fractionation. Wheat straw wax was fractionated first and about 60 g of crude wheat straw wax was loaded into the 500 mL extractor and fractionated for four hours and as shown in Figure 5.6, about 34% of the wax still remained in the extractor which suggested that the quantities of wax loaded was too large. For this reason, the second fractionation with oat straw wax was reduced to about 50 g and also fractionated over a period of four hours and it was found that the fractionation was almost completed with only 4% of the crude wax remaining in the extractor. Fraction 1 for both of the waxes was the biggest which suggested almost 80% of the crude oat straw wax ended up in separator 1. This is expected as the temperature and pressure change in separator 1 results in the CO_2 density being reduced dramatically therefore many of the molecules will only be partially soluble or insoluble at the critical point and precipitate from the supercritical fluid. The fractionation can be improved by increasing the number of separators so that more different $scCO_2$ conditions can be incorporated into the fractionation process. By taking the advantage of the different solubilities of different wax compounds at various temperatures and pressures, a series of fractions of different composition can be achieved. The fractionation time and flow rate must also be optimised for any further scale up. The aim of this study was to demonstrate that the straw waxes can be extracted and fractionated in a single step process successfully. In a real extraction model, the extractor would be filled with biomass rather than the wax product but for the purpose of this demonstration, waxes were loaded into the extractor coated onto glass beads.

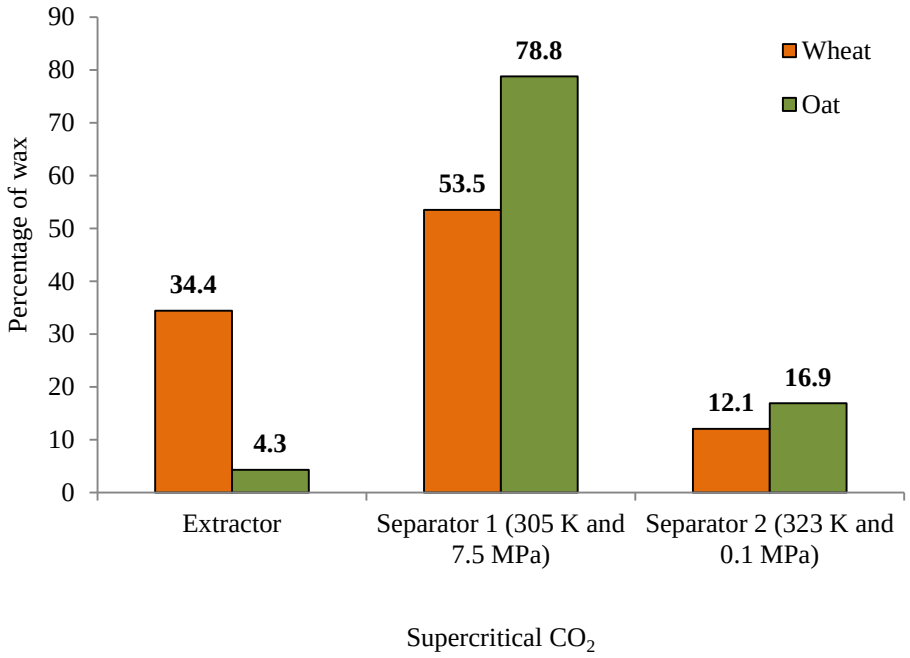


Figure 5.6: Percentage of wax present in each separator after $scCO_2$ fractionation (originally in colour)

Qualitative and quantitative analysis were carried out on the two fractions retrieved from separator 1 and 2 which is displayed as normalised percentage in the wax fractions in Table 5.2. The calibration used for the quantitative analysis was presented in Chapter 2. The wax compounds identified in each fraction were identical so it was necessary to carry out quantification analysis to give further details of the chemical composition of the extracts. It can be noted that the total identified is higher than 100% and this could be due to the co-elution of hentriacontane (C_{31}) and octacosanol (C_{28}) as the values shown are the total of the two wax compounds. Fraction 2 showed a higher proportion of β-diketones compared to fraction 1 for both straw waxes. In the oat straw wax, a significantly higher proportion of *n*-alkanes were separated as fraction 2 but strangely this was not the case with wheat straw wax. This may be because a significant amount of wax remained in the extractor and the *n*-alkanes were the hardest and last to extract. It would be interesting to repeat the fractionation of wheat straw wax with less wax in the extractor so the fractionation can go to completion. Nonetheless, this study does demonstrate that the use of $scCO_2$ can extract and fractionate the straw waxes simultaneously but conditions need to be optimised prior to any scale up trials.

Table 5.2: Identification and quantification of wheat and oat straw fractions from $scCO_2$ fractionation

Identification	% in extract			
	Wheat fraction 1	Wheat fraction 2	Oat fraction 1	Oat fraction 2
Tetradecanoic acid (C_{14})	0.2	2.1	0.3	2.8
Hexadecanoic acid (C_{16})	5.2	8.4	5.5	11.5
Octadecadienoic acid ($C_{18:2}$)	2.2	3.1	2.5	4.4
Octadecenoic acid ($C_{18:1}$)	4.5	5.9	4.7	7.6
Octadecanoic acid ($C_{18:0}$)	1.0	1.4	1.2	0.4
Total free fatty acids	**13.0**	**7.3**	**14.1**	**26.7**
Heptacosane (C_{27})	1.2	0.5	0.9	1.7
Nonsacosane (C_{29})	11.4	2.6	2.1	13.2
Hentriacontane (C_{31})	12.5[a]	0.9[a]	9.8[a]	6.5[a]
Triatriacontane (C_{33})	0.5	*Tr*	*Tr*	*Tr*
Total hydrocarbons	**25.6[a]**	**4.0[a]**	**12.8[a]**	**21.4[a]**
Octacosanol (C_{28})	6.8[a]	8.3[a]	12.6[a]	8.4[a]
Total fatty alcohol	**6.8[a]**	**8.3[a]**	**12.6[a]**	**8.4[a]**
Hexacosanal (C_{26})	0.5	1.3	1.8	2.6
Octacosanal (C_{28})	0.6	0.9	1.0	0.9
Total aldehydes	**1.1**	**2.2**	**2.8**	**3.4**
Tetracosanyl hexadecanoate (C_{24}:C_{16})	*Tr*	*Tr*	*Tr*	0.4
Hexacosanyl hexadecanoate (C_{26}:C_{16})	2.1	0.7	1.0	0.9
Octacosanyl hexdecanoate (C_{28}:C_{16})	2.4	1.4	1.8	1.0
Octacosanyl octadecanoate (C_{28}:C_{18})	3.9	1.6	5.4	1.3
Octacosanyl eicosanoate (C_{28}:C_{20})	6.3	0.8	2.8	0.4
Octacosanyl dosanoate (C_{28}:C_{22})	4.3	0.2	1.9	*Tr*
Octacosanyl tetracosanoate (C_{28}:C_{24})	1.9	0.2	0.5	*Tr*
Octacosanyl hexacosanoate (C_{28}:C_{26})	0.9	*Tr*	*Tr*	*Tr*
Octacosanyl octacosanoate (C_{28}:C_{28})	*Tr*	*Tr*	*Tr*	*Tr*
Total wax esters	**21.8**	**13.5**	**13.5**	**4.1**
Campesterol	3.1	2.7	2.1	2.0
Stigmasterol	3.0	2.8	1.8	2.2
β-sitosterol	7.9	6.7	5.0	5.5
Sterol 1	2.1	2.3	1.4	1.7
Δ^4-Sitosten-3-one	12.2	10.5	5.0	5.2
Sterol 2	*Tr*	*Tr*	*Tr*	*Tr*
Total sterols	**28.2**	**25.1**	**15.3**	**16.7**
14,16 hentriacontanedione (C_{31})	26.4	40.9	7.6	17.9
16,18 triatriacontanedione (C_{33})	0.4	0.6	0.6	1.2
Total β-diketones	**26.8**	**41.5**	**8.1**	**19.1**
Total identified	**123.3**	**101.8**	**79.3**	**99.8**

[a] = co-elution and values shown are the combined quantities, *Tr* = Trace levels < LOQ, LOQ shown in Section 2.5.1

5.2.3 Fractionation by gel permeation chromatography (GPC)

The GC method employed for the analysis of the straw waxes will only analyse molecules of up to about C_{60} so any larger non-volatile molecules would not be detected. Deswarte *et al*. managed to successfully separate the different wax groups in wheat straw waxes using column chromatography using a polarity gradient of eluting solvents and tracked the compounds using thin layer chromatography. High molecular weight molecules such as di-esters have previously been reported in wool wax using a short GC column and saponification however, the original structures are lost.[299] Initial work involved the use of an analytical scale GPC coupled with DAD and a series of polystyrene standards as described in Section 6.3.6 where all the wavelengths were being monitored. However, it is important to note that most of the wax compounds do not exhibit a UV chromophore so therefore further work must be carried out using another detector such as a light scattering detector for the complete analysis. The aim of this initial work was to establish a rough estimate of the molecular mass range for the waxes. Wool grease was used as a test sample for analysis for high molecular weight molecules. Figure 5.8 shows an example of the DAD signal from the GPC. As no high molecular weight esters are available commercially, the retention times were compared with the polystyrene standards for molecular mass range estimation. The polystyrene calibration was carried out using the method outlined in Section 6.3.6. The natural logarithm (Ln) was applied to the molecular weight of the polystyrene standards to create a linear plot with retention time for calibration purposes and this is shown as Figure 5.7.

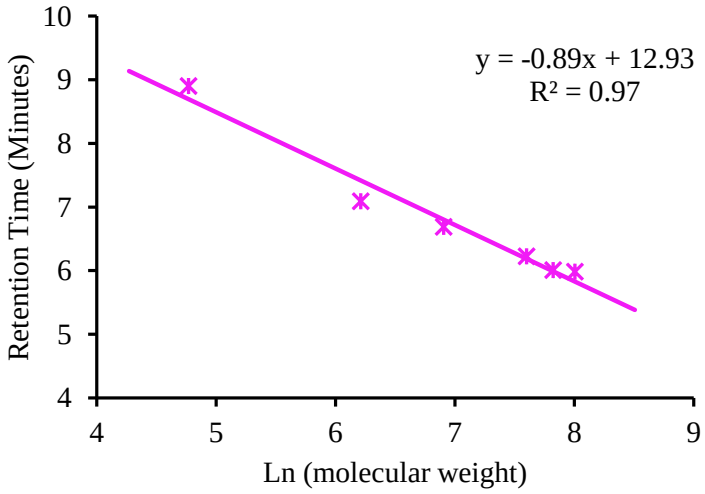


Figure 5.7: A plot of retention time against Ln (molecular weight) (originally in colour)

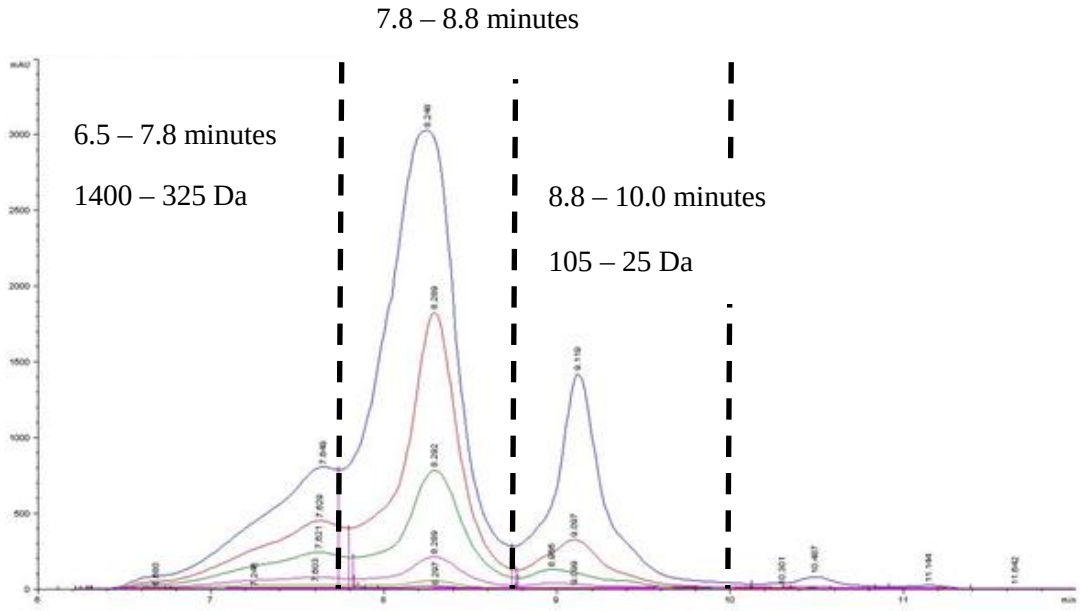


Figure 5.8: GPC trace showing DAD signal of wool grease (originally in colour)

Figure 5.8 provides promising results for the separation of complex mixtures such as wool grease with an estimated molecular mass range of up to 1400 Da. It was noted earlier that there may potentially be undetectable molecules due to the lack of chromophore for the detector used; however, this does show that high molecular weight molecules can be separated and fractionated. Based on the success of the analytical scale GPC work, the fractionation of wax by size was scaled up.

The wool grease was fractionated using a GPC preparative column originally designed for the separation of pesticides of wool wax described in Section 6.3.6. The three straw waxes were also fractionated using the same method as a comparison to identify high molecular weight molecules. Figure 5.9 shows the various fractions from wool grease and wheat straw wax. In the wool grease fractions, collection of molecules started at fraction 3 as a densely light brown solution and the colour decreases as the molecules get smaller. Straw wax fractions were different to wool grease as collection started at fraction 4 which indicated the wax contains lower molecular weight molecules in comparison. Unlike wool grease where the coloured compounds are the larger molecules within the complex mixture, in the straw waxes, the fractions were all coloured but in different degrees of yellow. In all three cereal straw waxes, three cloudy fractions (fractions 11, 12 and 13) were observed as shown in Figure 5.9 which

suggested that those molecules are not very soluble in DCM: hexane (1:1) solvent mix.

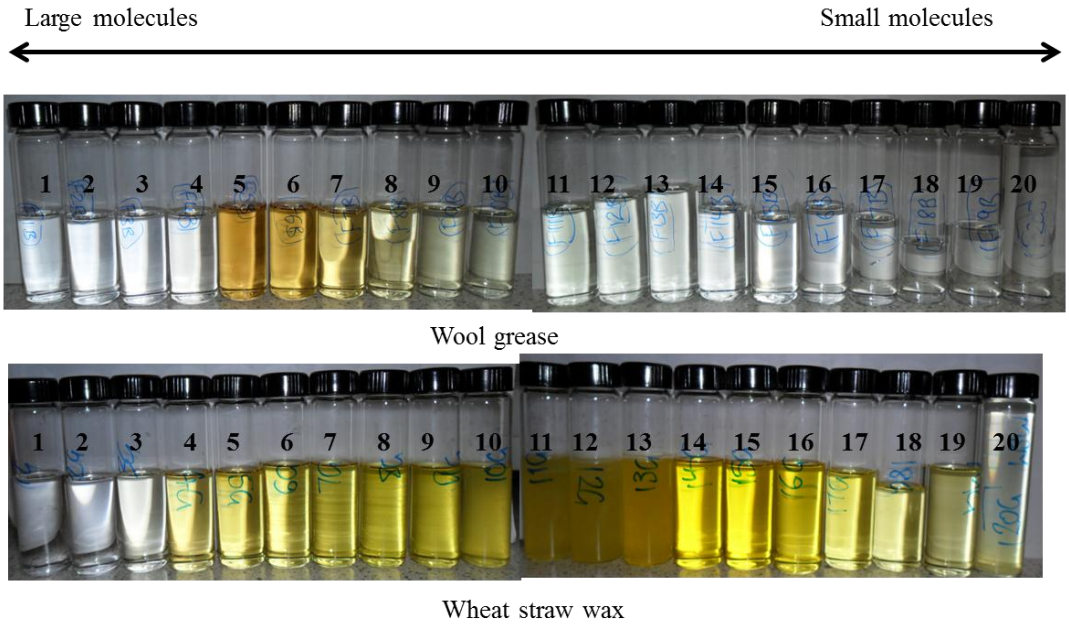


Figure 5.9: GPC separation of wool grease and wheat straw wax by size (originally in colour)

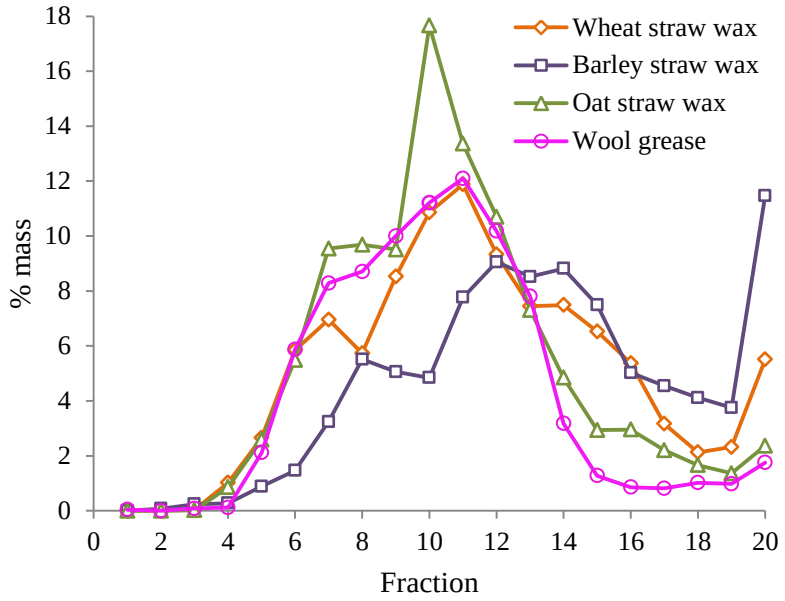


Figure 5.10: Mass balance for preparative GPC fractions (originally in colour)

It can be concluded that the fractionation of waxes was very successful as the recovery for all the waxes was over 95%. Figure 5.10 shows the mass balance for the various fractions of all four samples. It would be interesting to take all the fractions and carry out further compositional analysis but the aim of this fractionation using GPC is to separate any potential high molecular weight molecules for further analysis. In this case, wheat and oat straw waxes showed the highest amount of high molecular weight compounds as shown in figure 5.10. It can be observed that both of these waxes have similar mass balance curves compared to wool grease suggesting that there may possibly be di-esters in the waxes which would not have been seen by the standard GC method. For this reason, all the wheat and oat straw wax fractions were scanned using IR spectroscopy which is a quick technique to identify any high esters containing fractions. All the IR spectra for the two straw waxes are comparable and almost indistinguishable. Figure 5.11 shows the IR spectra for selected fractions in order to identify the main functional groups in the fractions.

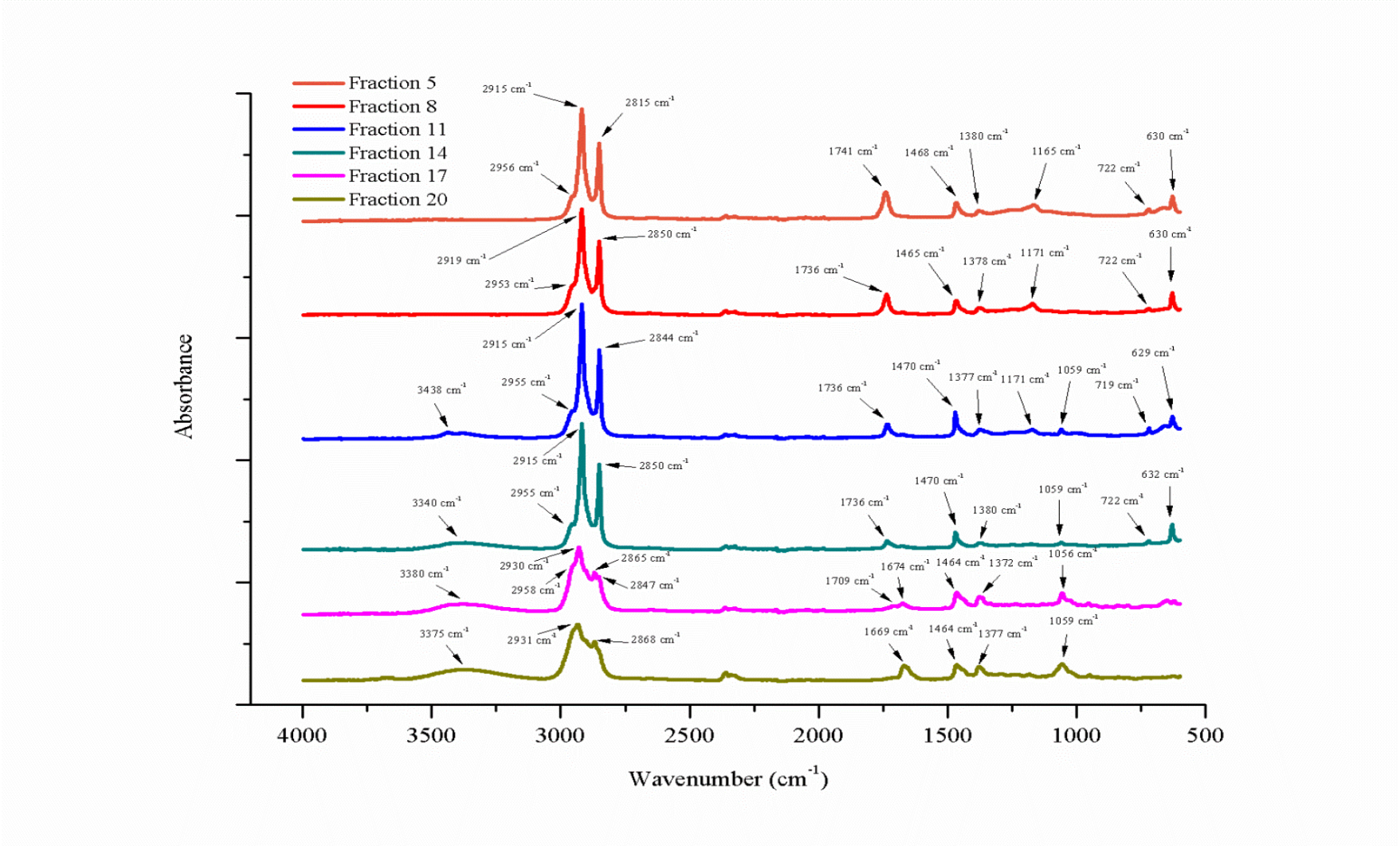


Figure 5.11: IR spectra (full) of selected wheat straw wax fractions (originally in colour)

The intense narrow peaks at around 2915 cm^{-1} and 2850 cm^{-1} can be assigned to the anti-symmetric and symmetric stretching bands of CH_2 respectively.[300] There is also a higher frequency shoulder at 2955 cm^{-1} which is due to the stretching of CH_3 groups.[300] These groups are highlighted due to the large number of molecules containing long aliphatic carbon chains within the complex mixture. However, as the molecular weight of the fractions changes, the intensity of these CH_2 and CH_3 stretches also changes and probably due to the reduced aliphatic carbon chains to other functional groups ratio. There is also a peak within the region of 1470 – 1430 cm^{-1} which represented CH_2 and CH_3 bends.[300] The peak at around 1378 cm^{-1} could be due to the C-H bend adjacent to a carbonyl group and the weak peak at 733 cm^{-1} is also due to CH_2 present in the aliphatic carbon chains.[301] There are peaks around 1160cm^{-1} and 1059 cm^{-1} which could be due to the C-O stretch from any phenolic and sugar molecules extracted.[302, 303] It is important to note that these bands are apparent in all the fractions. From fraction 11 and higher, a broad peak at around 3380 cm^{-1} started to appear which represents the presence of OH groups which are commonly found in the regions of 3100 – 3500 cm^{-1}.[301, 304] This could indicate the presence of fatty alcohol, sterols and fatty acids. In order to check for presence of fatty acids in the fraction, the carbonyl stretch must be presented. Figure 5.12 shows the IR spectra which highlights the carbonyl region as from the qualitative analysis, it was apparent that there are many compounds that contain the carbonyl group (esters, aldehydes, ketones, di-ketones and fatty acids). This region is particularly important as it helps to identify not only the high fatty acid fractions but also the high esters fractions.

As shown in Figure 5.12, the carbonyl stretch shifts to a lower frequency as fractionation proceeded i.e.: from large to small molecules. The carbonyl stretch at around 1740 cm^{-1} in fractions 5 and 8 could arise from both ester and aldehyde but it is more likely that these are ester carbonyl stretch bands due to the high molecular weight.[305] In fractions 11 and 14, the carbonyl stretch occurs at much lower frequency of 1734 cm^{-1} which suggests the presence of aldehydes.[304] There is also a weak shoulder around 1720 cm^{-1} which indicates the presence of ketones and carboxylic acids.[306] In fractions 14, 17 and 20, there is also a broad weak OH stretch as well as carboxylic acids stretch that showed a high probability of fatty acids. Peaks at the lower frequency of 1675 cm^{-1} could be due to 1,3-diketones which have shown to be

in relative high proportions in wheat straw waxes which has shown to occur around this region of the spectrum.[201] Similar IR spectra had been seen previously measured on wheat straw waxes by Dodson.[307]

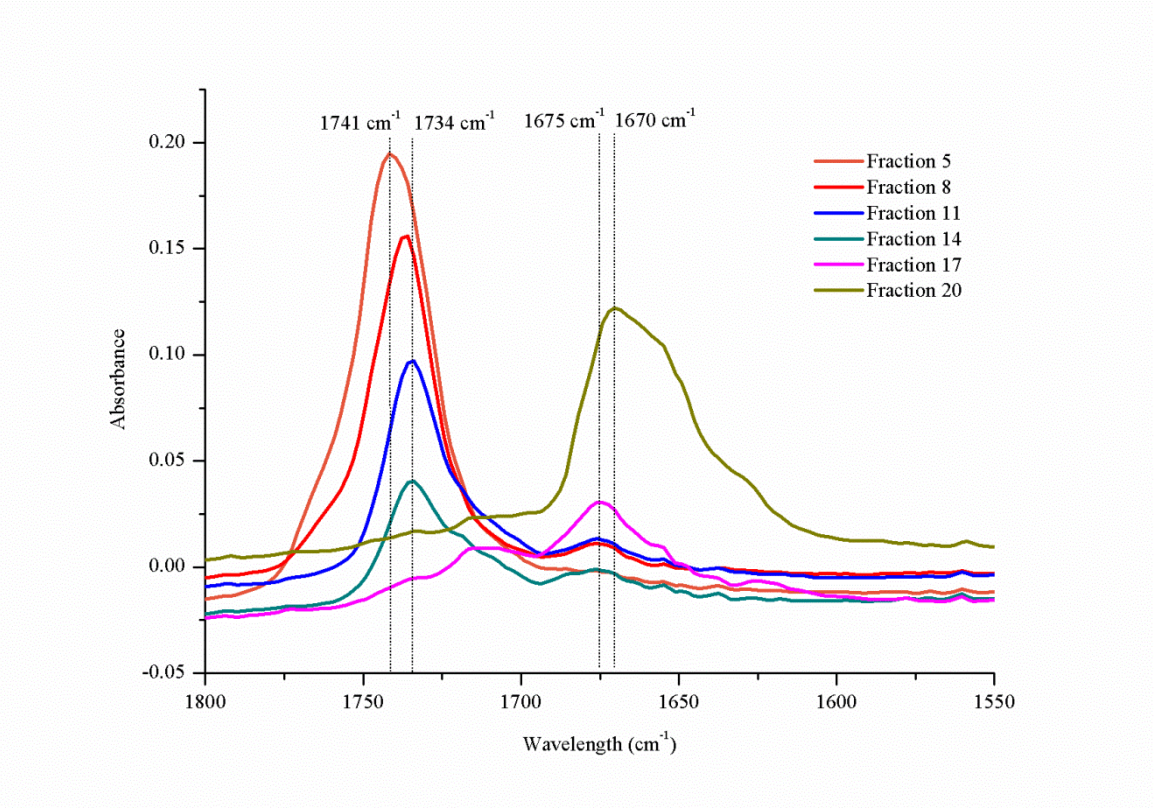


Figure 5.12: IR spectra (1800 – 1550 cm^{-1}) of selected wheat straw wax fractions (originally in colour)

The ester-rich fractions (first six fractions) were selected for MALDI-TOF analysis described in Section 6.3.7. This is a technique that has become increasingly popular in the analysis of large compounds with low polarity such as neutral lipids but it has been reported that there are difficulties in the analysis of compounds with long hydrocarbon chains.[308] In the case of straw waxes, a high proportion of the compounds consist of long aliphatic hydrocarbon chains and this could potentially be a problem. The most frequently used matrix for lipid molecules such as triglyceride is DHB but in order to improve the ionisation, various metal cations were introduced.[309, 310, 311] DHB was initially used as the matrix for this analysis however, no consistent results were gathered. Different ratios of wax samples to DHB were also tested and there was no improvement.[308] Cvačka *et al*. analysed natural wax esters and optimised

the analysis using various DHB and DHB metal salts, different sample preparation methods and different solvents. The optimised procedure was used and LiDHB was synthesised using the method described in Section 6.3.7.[308] The spectra quality was improved but there were still problems with uneven crystallisation of wax esters on the MALDI plate which resulted in "sweet spots" so therefore it was difficult to analyse using an automated laser. Further method development work is needed to give a high quality and reproducible analysis of wax esters and one area that has been highlighted is that due to the quick evaporation of organic solvent, there is a possible chance that not all the molecular ions are detected due to poor mixing of sample and matrix.[308]

As LiDHB was used as the chosen matrix, the MALDI spectra showed molecular ions with lithium $[M + {}^{6}Li]^{+}$. Hydrolysis of wax esters has been reported to occur by traces of water of crystallisation from ^{6}LiDHB but this would not have been observed using the chosen instrument unless the lithiated breakdown products were above that m/z value as the lowest detected m/z value is 600. Figure 5.13 shows the highest molecular weight fraction isolated from wool grease which indicated the biggest ester in the mixture has a $[M + {}^{6}Li]^{+}$ ion of 1771.5 meaning an ester of molecular weight 1764.6 Da if no hydrolysis has occurred. This is a breakthrough step in the analysis of large wax esters as typically the wax esters are hydrolysed to their basic building blocks prior to analysis. However, as there were no peaks observed above m/z of 1771.5, this suggested that no hydrolysis had occurred. As reported before, there are problems with hydrolysis of aliphatic wax esters with the hydrolysis not going to completion which means there will be wax esters as single compounds. The molecular ions of $[M + {}^{6}Li]^{+}$, lithiated fatty acid ions of $[R2COOH + {}^{6}Li]^{+}$ and $[R2COO{}^{6}Li + {}^{6}Li]^{+}$ and lithiated fatty alcohols $[R1OH + {}^{6}Li]^{+}$ and $[R2O{}^{6}Li + {}^{6}Li]^{+}$ would be observed for aliphatic wax esters if any hydrolysis had occurred. It is concluded that no hydrolysis had taken place and the ions observed would be solely $[M + {}^{6}Li]^{+}$.

In wool grease, there is evidence of di-acids and hydroxy acids and there is a high probability that the high molecular weight compounds identified are di-esters rather than mono-esters. Interestingly, the series of peaks shown in Figure 5.13 have a mass difference of 14 which indicated a difference in a CH_2 group between the various esters. This regular mass difference also suggested that the ester fraction is predominately aliphatic wax esters and not steryl esters. However, the MALDI

technique is classed as soft ionisation so no fragmentation would have been observed and it would be interesting to further separate the mixture or hydrolyse the mixture and carry out further MS analysis in order to assist in structure elucidation.

Figures 5.14 and 5.15 show the mass spectra for the biggest fractions in wheat and oat straw waxes. The crucial information from this data is that there are high molecular weight esters detected above molecular weight compounds of 800 Da. The biggest peak in wheat and oat straw waxes showed m/z values of 995.9 and 1498.1 respectively. As before these represent the molecular ions with lithium $[M + {}^{6}Li]^{+}$ which reflects a molecular weight of 989 and 1491.2 Da respectively. The initial chemical composition of the straw waxes using GC-MS showed a range of aliphatic wax esters range from C_{40} – C_{56} with molecular weight of 592.6 – 816.9 Da so therefore the $[M + {}^{6}Li]^{+}$ ions should be observed using this technique. These wax esters were observed and are highlighted in Figures 5.14 and 5.15. Table 5.3 shows a list of ions found from m/z of 600 and higher, indicated in previous work using GC-MS and their corresponding identification. Some of the wax esters can be identified using MALDI and this is highlighted in the table. Due to the poor quality in the low mass region of the spectra, the intensity of peaks was low and not reproducible. This again could potentially be due to crystallisation problems of wax esters on the MALDI plate for which further method development is needed. The unidentified large molecules could potentially be glycerides or bigger wax esters and currently, at this stage, with only limited $[M + {}^{6}Li]^{+}$ ion data and without any fragmentation pattern, it is difficult to deduce the exact structures of these compounds. The aim of the investigation was to fractionate the waxes by size, which was achieved successfully. However, it would be interesting to further separate and analyse the large molecules possibly by hydrolysis of the esters. This work has proved that fractionation of waxes by GPC is a good way of separating high molecular weight compounds and also showed the potential of using MALDI TOF MS as a wax ester analytical technique but further method optimisation is required.

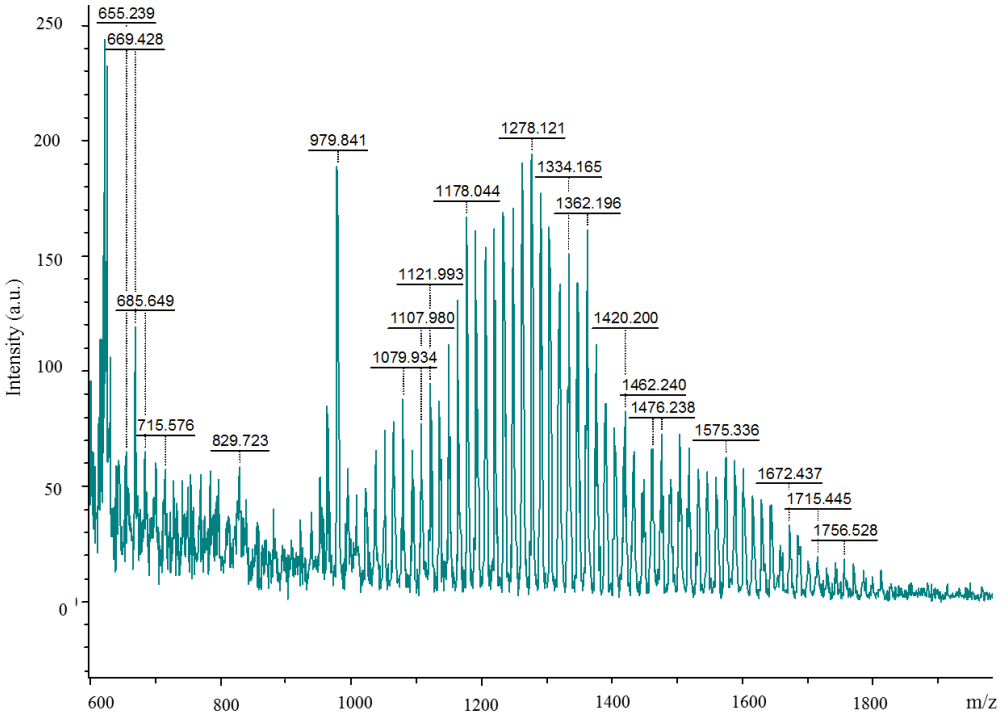


Figure 5.13: MALDI-TOF mass spectrum for wool grease fraction 5 (originally in colour

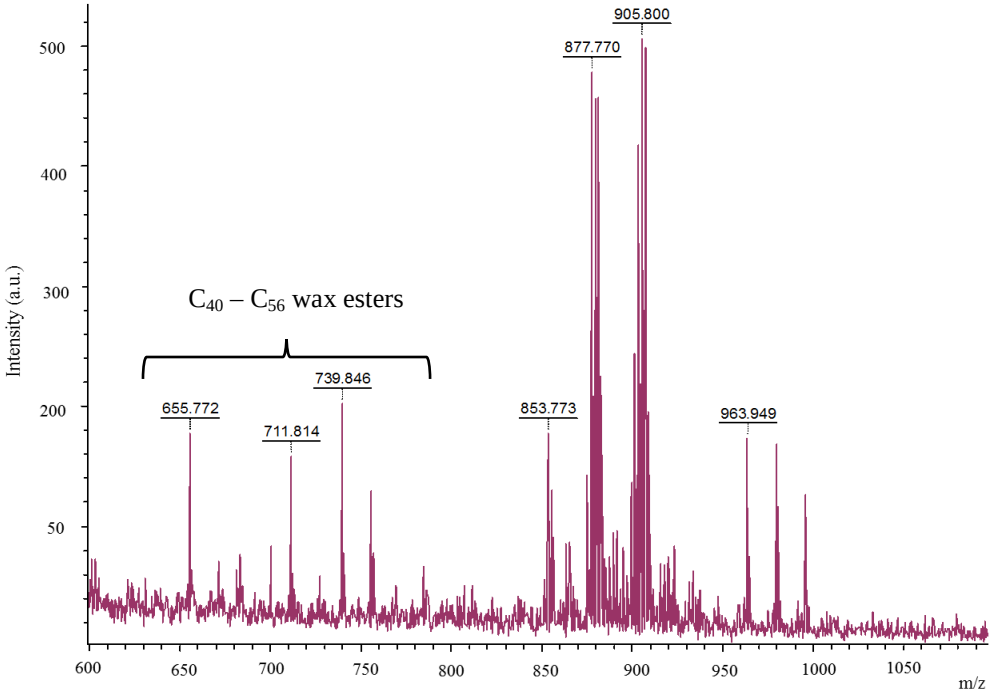


Figure 5.14: MALDI-TOF mass spectrum for wheat straw wax fraction 5 (originally in colour)

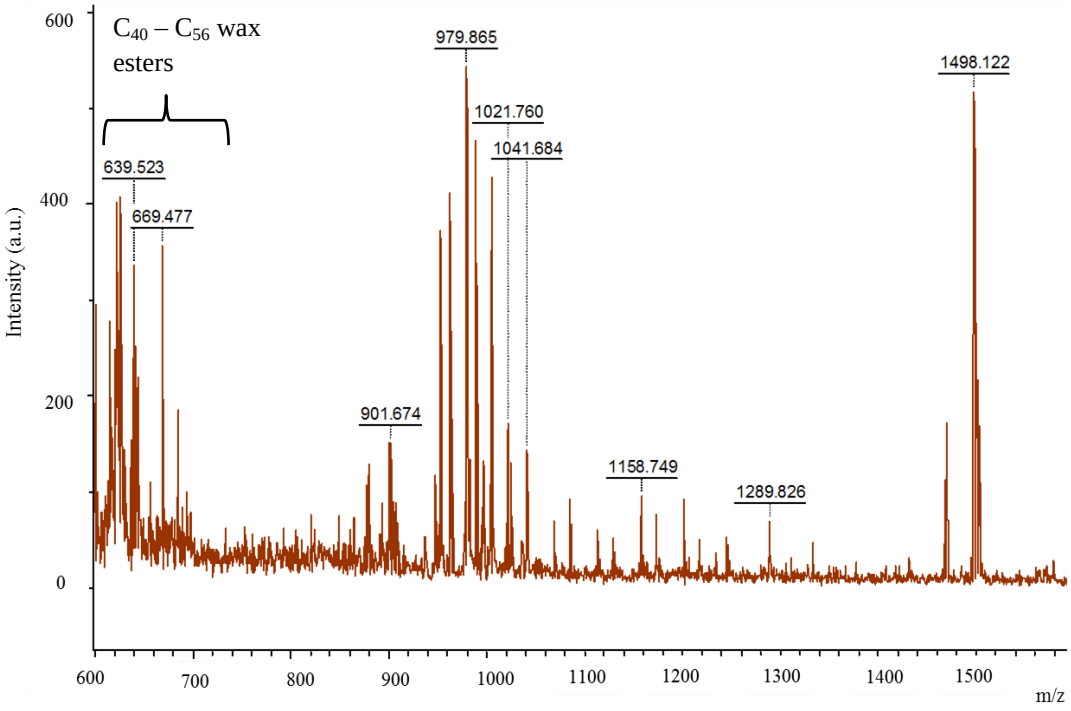


Figure 5.15: MALDI-TOF mass spectrum for oat straw wax fraction 5 (originally in colour)

5.2.4 Saponification

Saponification is a common process that is used to produce soap from fats. In the case of straw waxes, the glycerides and esters are both prone to saponification to form the sodium salts, glycerol and fatty alcohols as shown as the reaction scheme in Figure 5.16. Wheat straw wax from the scale up trial was saponified into wheat alcohol and acid fractions using the procedure shown in Section 6.2.7.

The aim of saponifying the crude wax into its fractions was to investigate the change in physical properties such as melting point and to check for any new undetected wax esters. During the saponification process, the yield is about 75% and within the recovered fractions 63% was wheat alcohols and 37% was wheat acids. The properties for the new fractions are discussed within the physical properties in Section 5.4. Due to the improved water absorption from the fatty alcohol fraction, the saponification process was scaled up in order to gain enough wheat alcohols for personal care applications trials which will be carried out by the applications team at Croda. The

two fractions varied not only in terms of physical properties, they also varied in colour and texture. The fractions were much softer in comparison with the crude wax. The Gardner colour of the wheat alcohol fraction was measured as 14 and the wheat acid fraction was above 18 as can be seen in Figure 5.17. This demonstrated that a simple chemical modification of crude wax can alter the wax and open opportunities for different applications.

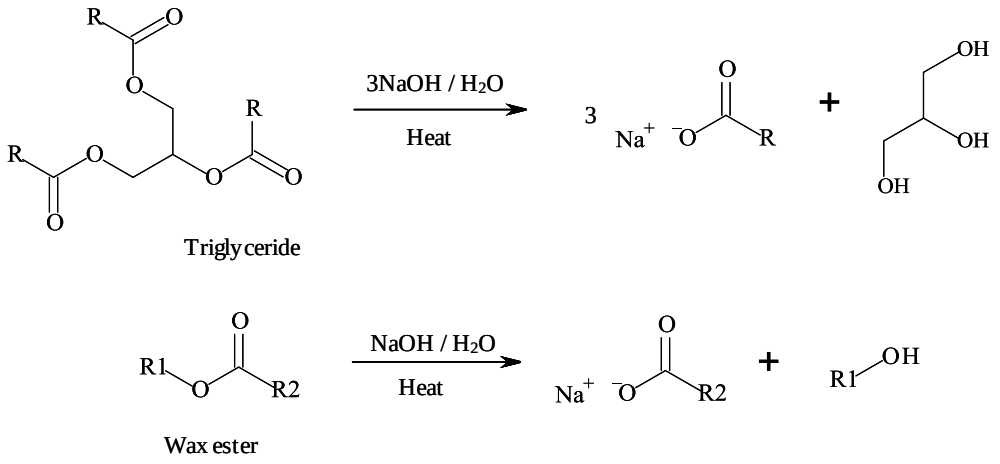


Figure 5.16: Saponification reaction scheme for triglycerides and wax esters

Wheat alcohol fraction Wheat acid fraction

Figure 5.17: Wheat alcohol and acid fractions of wheat straw wax (originally in colour)

The chemical composition of the fractions was analysed to check for successful saponification as shown in Figure 5.18. Interestingly, in the wheat alcohol fraction,

two extra peaks were observed, as highlighted. The other compounds found in the fractions have already been identified previously in Chapter 2 so these were expected to be seen in the chromatogram and this is shown as a summary in Table 5.5. However, hexacosanol (C_{26}) and hexacosanoic acid (C_{26}) were expected to be identified in the saponified fractions as hexacosanyl hexadecanoate (C_{42}) and octacosanyl hexacosanoate (C_{54}) were previously identified in the crude wheat straw wax in the scale up trial. As these compounds were present only at an extremely low level, the saponified products would also be present at low concentrations and may only show as very small peaks in the chromatogram. The two new peaks highlighted in the alcohol fraction were identified to be ketones with the carbonyl group on the second carbon which give a distinct fragmentation pattern as shown in Figure 5.18. Figure 5.19 shows the EI spectra with the large m/z region magnified as the molecular ion was lacking in the original trace. The identification was achieved by a combination of the EI fragmentation pattern and KI values. Table 5.4 shows the fragments and their percentage base peak intensity along with the calculated KI values against literature values and it appears to be a good match. The ketones differ structurally by only two carbon units and this is reflected in the 200 KI values difference between them. The pattern shown in Figure 5.19 has a base peak of m/z = 58 which is the result of a McLafferty rearrangement as shown in Figure 5.20. The ion m/z = 196 can be observed in the EI fragmentation pattern for 2-heptadecanone which is due to $[M - 58]^+$ ion as a result of the loss of the McLafferty rearrangement fragment ion. When the higher m/z region was magnified, the molecular ion was observed which confirmed the carbon chain length of the molecules. The regular fragment ions of 43, 57, 71 and 85 arise from the aliphatic alkyl chain as described in Table 2.7.

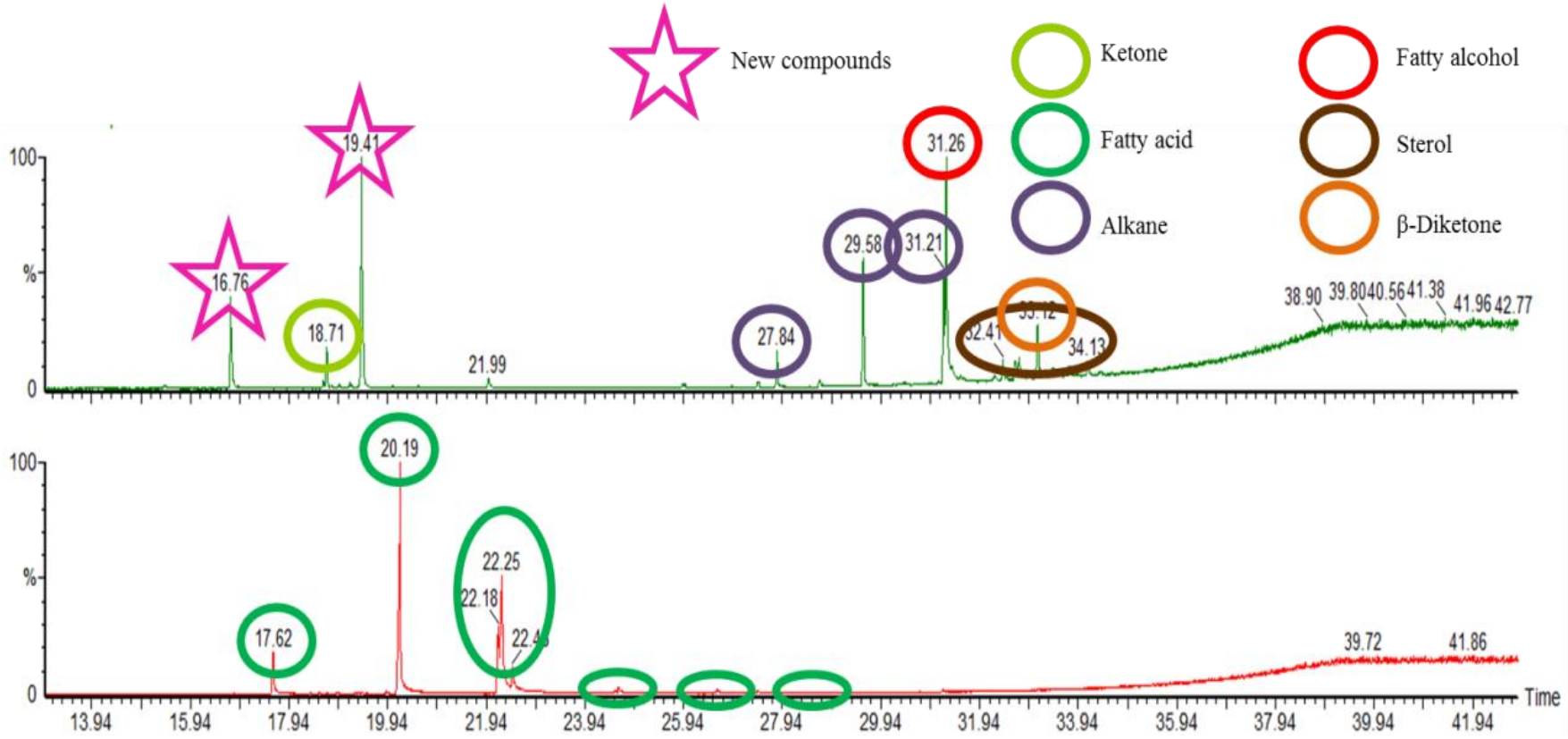


Figure 5.18: GC chromatograms of saponified wheat alcohol and acid fractions (originally in colour)

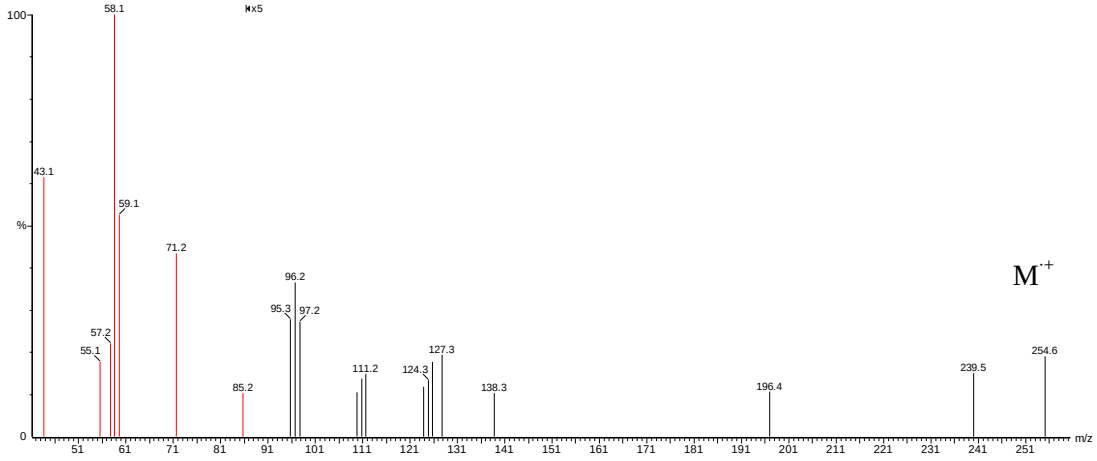


Figure 5.19: EI mass spectrum of 2-heptadecanone (magnified three times from m/z 90 – 260) (originally in colour)

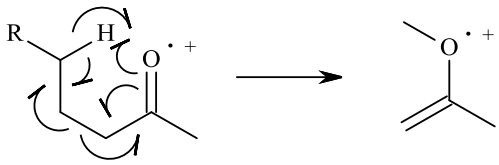


Figure 5.20: Formation of EI fragment ion of m/z = 58

Table 5.4: Identification of ketones presented in wheat alcohol fraction

Identification	M^+	EI Fragmentation	Calculated KI	Literature KI
Ketones				
2-Pentadecanone	226.4	58(100), 43(82), 59(52), 71(45), 57(26)	1708	1703[312]
2-Heptadecanone	254.5	58(100), 43(60), 59(53), 71(43), 57(21)	1916	1901[313]

The two new ketones are products resulting from the saponification of the wheat straw wax but ketones cannot be formed directly from a saponification process. The wax esters present in the straw wax must contain secondary alcohols as one of its basic components as secondary alcohols can be oxidised to ketones. In this case, the wax sample was saponified into its alcohols namely octacosanol, pentadecan-2-ol and heptadecan-2-ol but under the conditions used, the secondary alcohols have oxidised to ketones. Oxidation might have occurred in the presence of atmospheric oxygen but octacosanol was not oxidised as both octacosanal or octacosanoic acid were not

observed. To minimise oxidation of compounds in future, the sample can be stored under vacuum or an inert gas at all times.

This is the first time that secondary alcohols may have been identified and generally in plants, secondary alcohols occur freely rather than esterified.[80] Secondary alcohols found in cuticular waxes are all typically odd-numbered as they are biosynthesised via the alkane pathway.[82] The most abundant free secondary alcohols identified are usually from C_{23} – C_{33} and predominantly C_{29} and C_{31} and the functional groups are usually found in the middle of the carbon chain.[80] This group of compounds are not often found in *Gramineae* species but a mixture of heptacosan-9-ol, nonacosan-10 and -11-ols and hentriacontan-10, -11, -12 and -13-ols were identified in barley.[283] Shorter alkan-2-ols with 17 and 19 carbon chain lengths are reported to occur as aliphatic monomer that is covalently bound to the cutin and suberin polymer and has also been reported in barley.[314, 315] No alkan-2-ols were found in the barley straw wax from the scale up trial. However, it would be interesting to saponify the wax to look for any esterified alkan-2-ols and compare this with wheat straw wax. However, as the secondary alcohols in wheat straw wax are bound with various fatty acids to form wax esters, they appeared to have a shorter alkyl carbon chain. In Figure 2.26, the GC analysis showed the presence of a series of unidentified odd-numbered wax esters from C_{41} – C_{52}. As odd-numbered primary alcohols are not found in plant cuticular waxes, there is a high probability that these are formed from the odd-numbered secondary alcohols. Fatty acids are rarely present as odd-numbered because the elongation step in the biosynthetic pathway extends the carbon chain with the addition of C_2 moiety.[82, 85] From this reaction, the alcohol moieties of these wax esters are suggested to be either pentadecan-2-ol or heptadecan-2-ol. However, at this stage the exact structures of the series of odd-numbered wax esters cannot be found without the EI fragmentation pattern of the compounds so as an example the proposed structures for C_{41}wax esters are displayed as Figure 5.21. In order to acquire an accurate EI mass spectra of the odd esters, the concentration must be increased and the use of a shorter column (e.g.: 15 m) will be necessary to gain better resolution.

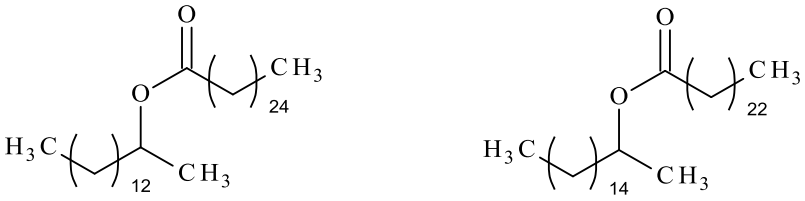


Figure 5.21: Proposed structures for odd-numbered C_{41} wax ester in wheat straw wax

Table 5.5: Wax compounds presented in crude wheat straw wax, wheat alcohol and acid fractions

Wax compounds	Crude	Wheat	
		Alcohol fraction	Acid fraction
Ketone 1	✓	✓	✓
Tetradecanoic acid (C_{14})	✓	×	✓
Hexadecanoic acid (C_{16})	✓	×	✓
Octadecanoic acid ($C_{18:0}$)	✓	×	✓
Octadecenoic acid ($C_{18:1}$)	✓	×	✓
Octadecadienoic acid ($C_{18:2}$)	✓	×	✓
Eicosanoic acid (C_{20})	×	×	✓
Docosanoic acid (C_{22})	×	×	✓
Tetracosanoic acid (C_{24})	×	×	✓
Octacosanol (C_{28})	✓	✓	×
Campesterol	✓	✓	×
Stigmasterol	✓	✓	×
β-sitosterol	✓	✓	×
Sterol 1	✓	✓	×
β-Sitosterone	✓	✓	×
Sterol 2	✓	✓	×
14,16-Hentriacontanedione (C_{31})	✓	✓	×
Octacosanal	✓	×	×
Hexacosanyl hexadecanoate (C_{26}:C_{16})	✓	×	×
Octacosanyl hexdecanoate (C_{28}:C_{16})	✓	×	×
Octacosanyl octadecanoate (C_{28}:C_{18})	✓	×	×
Octacosanyl eicosanoate (C_{28}:C_{20})	✓	×	×
Octacosanyl docosanoate (C_{28}:C_{22})	✓	×	×
Octacosanyl tetracosanoate (C_{28}:C_{24})	✓	×	×
Octacosanyl hexacosanoate (C_{28}:C_{26})	✓	×	×
Glycerides	✓	×	×
2-Pentadecanone (C_{15})	×	✓	×
2-Heptadecanone (C_{17})	×	✓	×

Another explanation for the presence of these ketones is the breakdown products of 14, 16-hentriacontanedione via a Retro-Claisen reaction shown in Figure 5.22. The nucleophile OH group from the NaOH in the saponification mixture can attack both carbonyl groups which would give a total of four breakdown products. In the wheat straw wax from the large scale trial, only the C_{31} β-diketone was present so therefore if this reaction was to proceed, it would result in tetradecanoic acid ($C_{14:0}$), hexadecanoic acid ($C_{16:0}$), 2-pentadecanone (C_{15}) and 2-heptadecanone (C_{17}) which were all identified. Interestingly, 14, 16-hentriaconatane was also found in the wheat alcohol fraction which means that if this reaction did occur, the reaction had not gone to completion. In order to conclude the origin of the 2-ketones, quantification would suggest whether the Retro-Claisen reaction had occurred.

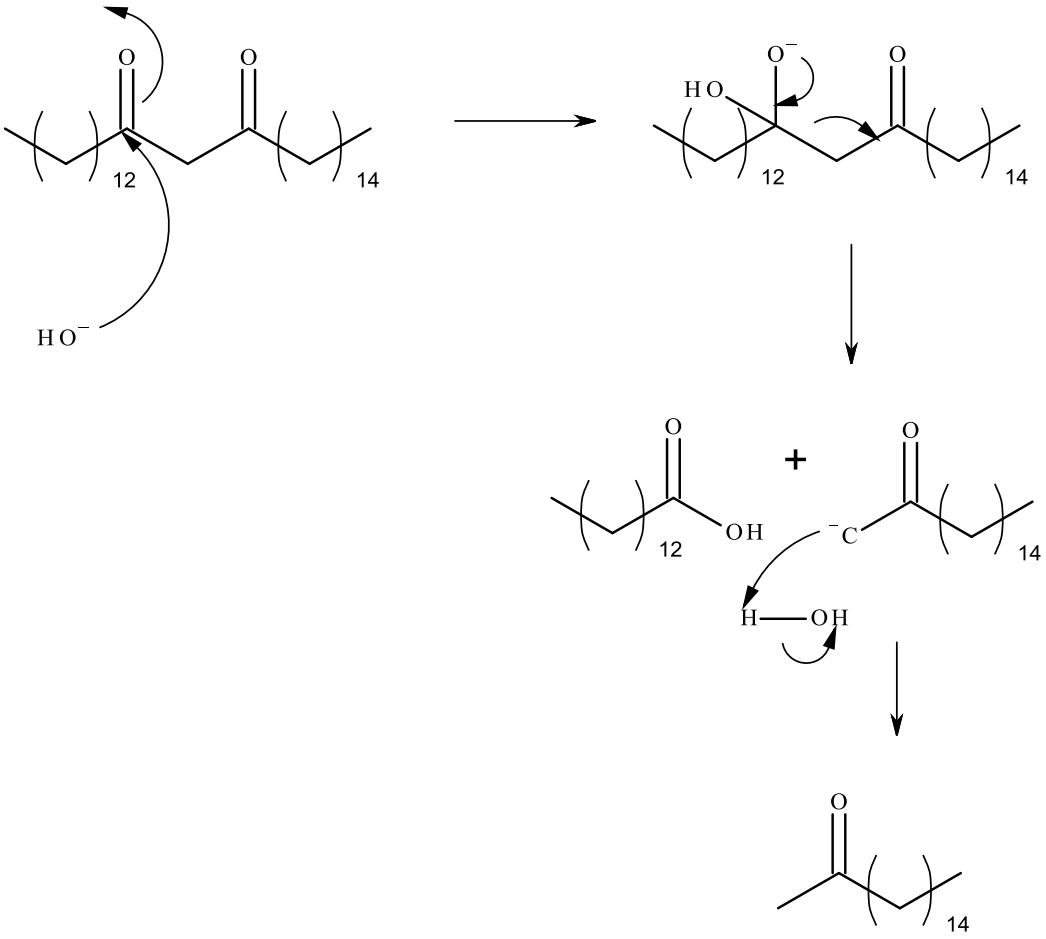


Figure 5.22: An example of the Retro-Claisen reaction on β-diketone

5.3 Physical properties

5.3.1 Melting point

DSC was used as to investigate phase transitions in the straw waxes and one of the most important physical properties of waxes is their melting point(s). The phase transition of melting is endothermic and therefore shows a negative heat flow. The decomposition temperature was first determined using STA by following the method described in Section 6.4.1. STA is a combination of TGA and DSC but in this case, the TGA thermogram was of interest. The decomposition temperature was determined so that during DSC analysis, the maximum temperature does not exceed its decomposition point. All the waxes were found to have a decomposition temperature above 463 K so that a temperature of 393 K was set for the maximum DSC temperature. Figure 5.23 shows an example of the STA thermogram of a wheat straw wax.

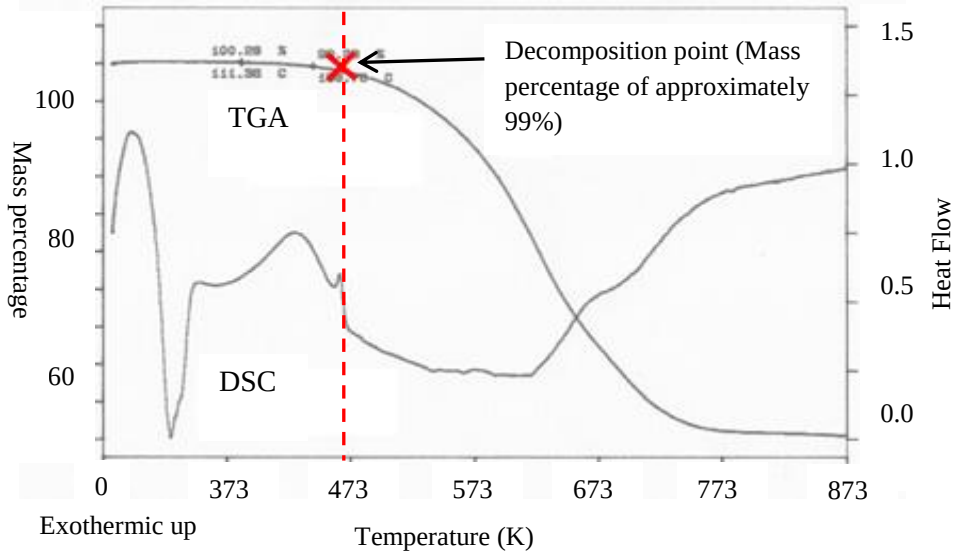


Figure 5.23: STA thermogram of wheat straw wax (originally in colour)

The samples analysed were initially heated and then cooled to remove any prior thermal character so all the thermograms shown are from the second heating cycle of the analysis. Lanolin, beeswax, candelilla wax and carnauba wax were analysed as they are popular waxes with very distinct applications. The DSC thermogram for the four commercial waxes is shown in Figure 5.24. These waxes are finished products and have been refined so their melting range is fairly sharp. Lanolin is a soft wax and

has a melting temperature of 313 K which is body temperature. This makes the wax especially good for skin care formulations. Beeswax, candelilla and carnauba waxes are much harder waxes and have much higher melting ranges which make excellent cosmetic ingredients for lip-sticks, mascara formulations and many others.[125, 316, 317] In the case of beeswax and carnauba wax, there are multiple peaks which indicate multiple groups of compounds going through thermal transition at different temperatures. There is still an opportunity to replace some of these waxes that are not UK-sourced which would be ideal in terms of reducing carbon footprint from transportation. According to recent market trends, especially in the personal care sector, there is a big consumer-driven trend for natural cosmetics products and the removal of animal derived ingredients so this is an opportunity to develop the market for new plant-derived waxes as the launching of such ingredients would be widely accepted.

The melting range for the straw waxes was determined in an attempt to correlate the composition and the melting range. As the chemical compositions of the extracts are too complex and often with over 100 different compounds, it is impossible to identify which single compound is corresponding to which particular thermal transition. Even if this was possible, it would not give useful information as it has been shown that different wax groups within complex mixtures interact and behave differently to expectations. Patel *et al.* analysed the melting points of wax ester and alkane mixtures and discovered that there are some packing disruptions when the two molecules are forced into interactions which was reflected in a 3 – 5 °C decrease in the melting points compared to expected.[274] Nemoto *et al.* carried out DSC on mixtures of ketones and *n*-alkanes and also showed interactions between the two molecules and a mixture of both physical properties depending on the ratio of the two.[318] However, it has been reported by Bonsor *et al.* that if the chain lengths for *n*-alkanes do not differ excessively, the mixtures would form mixed crystals and would exhibit straight forward predictable physical properties.[319] Gibbs also analysed the influence of melting points of two mixed *n*-alkanes and found that the melting point lies between the melting points of the two individual *n*-alkanes therefore making prediction possible.[320] Similar work was also carried out in the same study on mixtures of branched alkanes and *n*-alkanes and the co-crystallised molecules so making the melting points possible to calculate.[320] Since there is more than one wax group in the

straw waxes interactions between each group would occur and the melting points would be difficult to predict. However, despite these interactions between each wax group, it is apparent that the melting points are similar to the pure single components so Table 5.6 shows a list of wax components and their melting points.

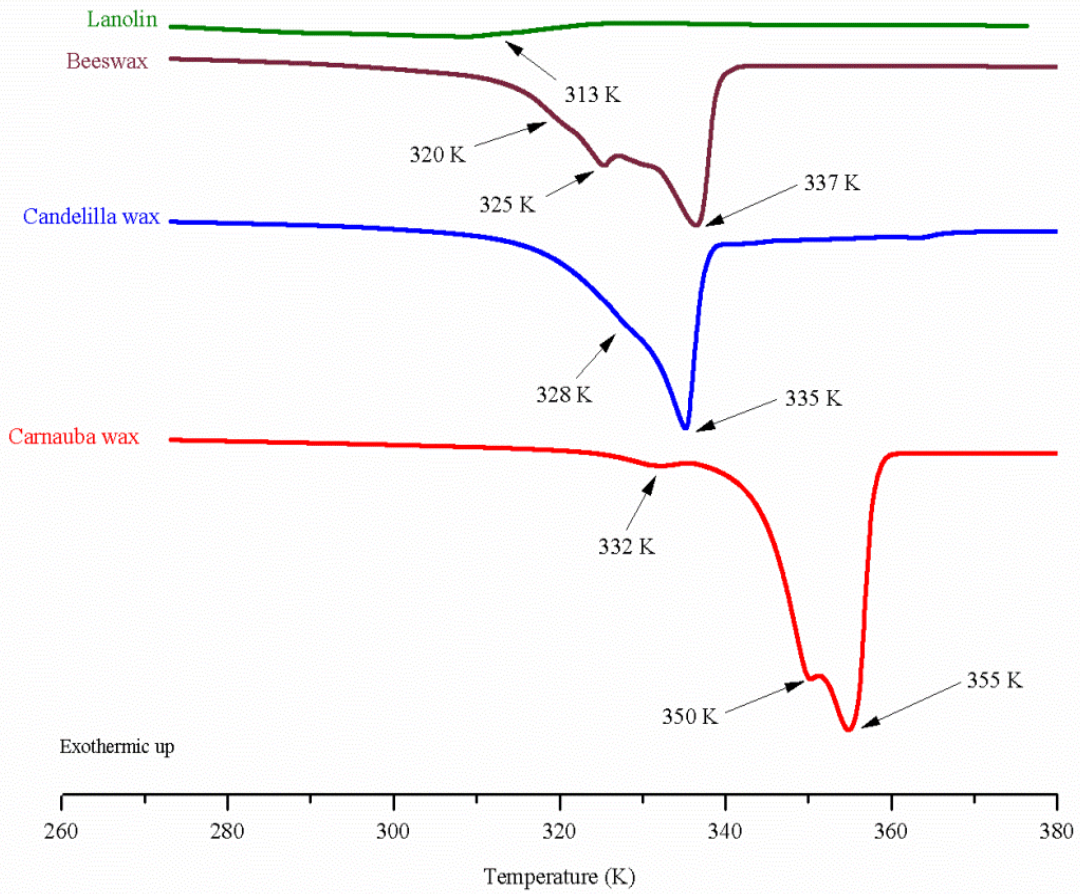


Figure 5.24: DSC thermograms of four commercial waxes (originally in colour)

If the DSC and GC analysis can be correlated then it would enable the physical properties to be tuned more easily. If physical properties can be tailored from extraction conditions by altering the conditions, specific unique wax products would be obtained which could fit well in certain applications. To understand more on the correlation, the DSC plots for the CO_2 and key solvent wheat straw extracts were determined and are shown in Figure 5.25 from low to high wax yield. The thermal transitions for the different $scCO_2$ extracts were also compared in order to gather extra

information on links between composition and melting ranges. The DSC thermograms are shown as Figure 5.26 in order of CO_2 density. It is important to note that the thermogram displayed for $scCO_2$ **B** in both figures are the same sample. The extraction conditions for Figures 5.25 and 5.26 can be found in Tables 3.3 and 3.6.The relative abundance of each wax group in each extract is listed in Table 5.7 to allow for easy comparison of chemical composition to DSC thermograms. It must be noted that there was co-elution between hentriacontane (C_{31}) and octacosanol (C_{28}) in some analyses and these numbers have been highlighted in the table.

Table 5.6: The melting points of wheat straw wax compounds

Wheat straw wax compounds	Melting point (K)
Tetradecanoic acid ($C_{14:0}$)[a]	328
Hexdecanoic acid ($C_{16:0}$)[a]	336
Octadecadienoic acid ($C_{18:2}$)[a]	268
Octadecenoic acid ($C_{18:1}$)[a]	286
Octadecanoic acid ($C_{18:0}$)[a]	343
Heptacosane (C_{27})[a]	332
Nonsacosane (C_{29})[a]	336
Hentriacontane (C_{31})[a]	341
Triatriacontane (C_{33})[a]	345
Campesterol[a]	432
Stigmasterol[a]	445
β-sitosterol[a]	405
14,16 Hentriacontanedione (C_{31})[b]	382
16,18 Triatriacontanedione (C_{33})[b]	388
Tetracosanyl hexadecanoate (C_{24}:C_{16})[b]	364
Hexacosanyl hexadecanoate (C_{26}:C_{16})[b]	370
Octacosanyl hexdecanoate (C_{28}:C_{16})[b]	376
Octacosanyl octadecanoate (C_{28}:C_{18})[b]	381
Octacosanyl eicosanoate (C_{28}:C_{20})[b]	386
Octacosanyl docosanoate (C_{28}:C_{22})[b]	391
Octacosanyl tetracosanoate (C_{28}:C_{24})[b]	396
Octacosanyl hexacosanoate (C_{28}:C_{26})[b]	401
Octacosanyl octacosanoate (C_{28}:C_{28})[b]	406
Octacosanol[a]	353
Octacosanal[b]	342

[a] Data collected from MSDS in Sigma-Aldrich UK Limited [b] Data predicted from software ICAS 12 using Merrero and Gani method

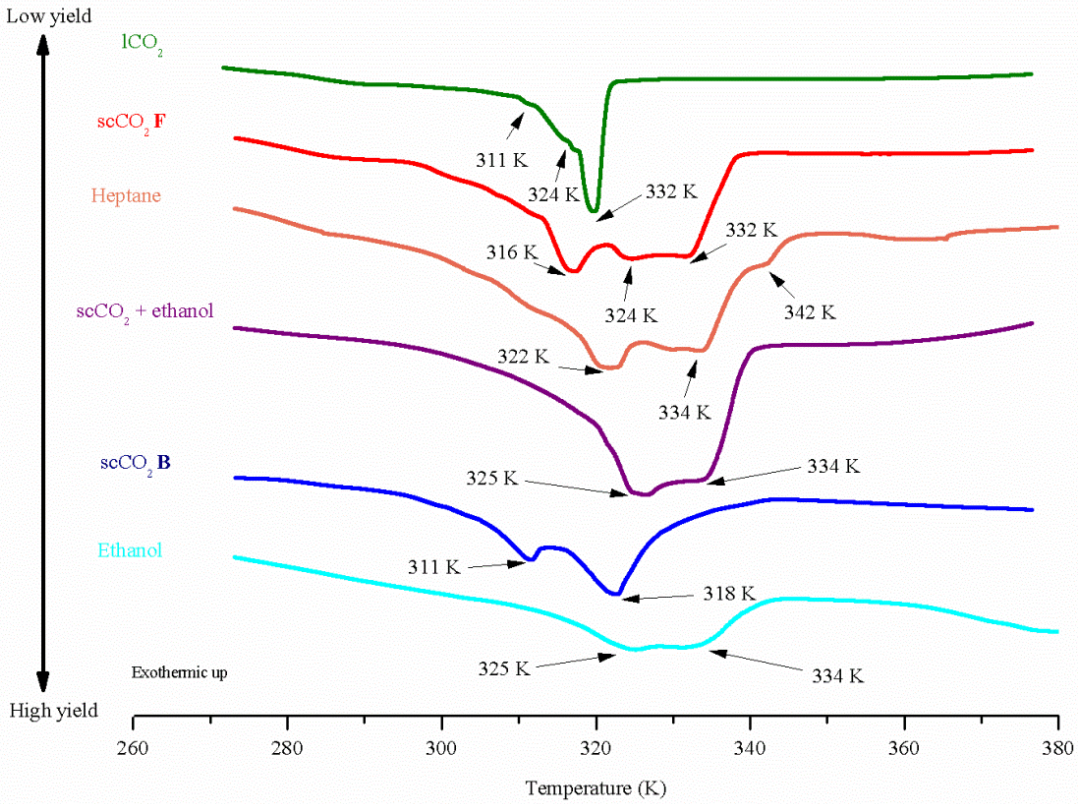


Figure 5.25: DSC thermograms of $scCO_2$ extracts compared with organic solvent extracts (originally in colour)

A number of important similarities can be highlighted from Figures 5.25 and 5.26. First, the lCO_2 and $scCO_2$ **C** extracts both exhibited a sharper endotherm and melted over a smaller range compared to other extracts indicating a high percentage of a single group of compounds within the extracts. Similarly, both have a melting point of around 320 K and 330 K. which is most likely the 30% β-diketones, *n*-alkanes and fatty acids as they appeared to be most predominant. The $scCO_2$ **E** extract consists of roughly equal proportions of the different wax groups and this is apparent in the DSC thermogram as it has a very broad melting range and five distinct melting points. Another interesting point to note is the $scCO_2$ **D** and **F** extracts have almost identical melting curves compared to Soxhlet heptane extract. The Soxhlet ethanol and $scCO_2$ with ethanol extracts can be compared as both have a very broad melting range but do have similar melting points which showed that even the addition of just 10% ethanol

to the $scCO_2$ is enough to manipulate the solvent polarity and its extraction behaviour. The $scCO_2$ extract extracted using the highest CO_2 density contain higher molecular weight compounds that would be more soluble in the $scCO_2$ and this is reflected in the high melting point of 352 K. The $scCO_2$ **B** was extracted using the identical pressure but at higher temperature compared to $scCO_2$ **A** and exhibited a much lower melting range compared to $scCO_2$ **A**. Tt can be explained by the lower CO_2 density ($scCO_2$ **B**) used during extraction so higher molecular weight compounds may still be insoluble in the $scCO_2$. From low to high density CO_2, the melting range increases which showed that larger and higher melting compounds are being extracted as CO_2 density increases.

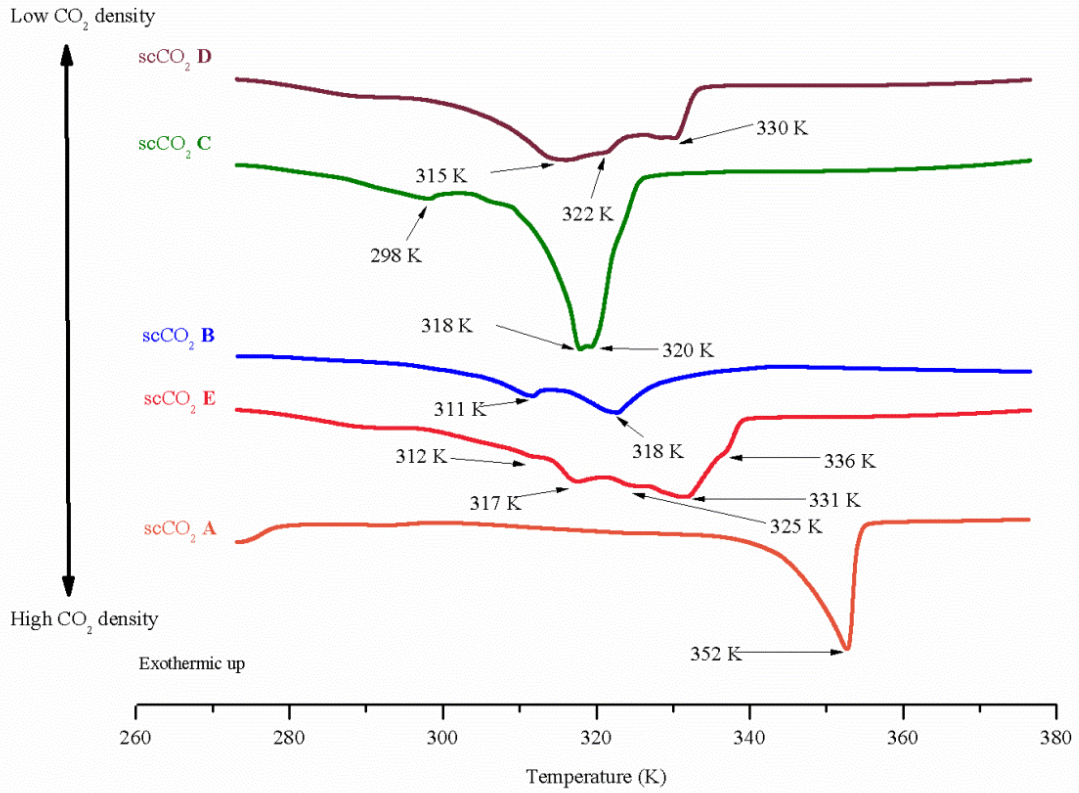


Figure 5.26: DSC thermograms of $scCO_2$ extracts under different temperature and pressure (originally in colour)

Table 5.7: Percentage of wax groups in crude extracts from CO_2 and organic solvent extractions

Extraction conditions	Percentage of wax group in crude extracts								
	Fatty acids	Wax esters	Hydrocarbons	Sterols	β-Diketones	Fatty alcohol	Aldehyde	Ketones	Unknown
lCO_2	7.2	0.7	6.0a	12.1	33.2	3.2	0.6	1.3	35.7
$scCO_2$ **F**	7.6	30.1	8.1	10.8	13.1	7.6	1.1	0.9	20.7
Heptane	5.9	13.0	4.1	4.1	7.4	3.5	0.9	0.7	60.3
CO_2 + Ethanol	3.6	26.0	5.6	3.5	5.0	3.1	0.7	0.5	52.1
$scCO_2$ **B**	5.6	17.6	8.8[a]	6.2	9.3	8.6	0.6	0.6	42.7
Ethanol	5.1	3.2	7.0[a]	5.6	3.0	6.3	0.0	0.4	69.6
$scCO_2$ **A**	11.7	26.6	*9.6*	12.4	18.7	*4.5*	1.3	1.2	14.0
$scCO_2$ **B**	5.6	17.6	*8.8*	6.2	9.3	*8.6*	0.6	0.6	42.7
$scCO_2$ **C**	12.5	1.4	*13.6*	10.2	34.5	*6.4*	1.2	1.2	18.9
$scCO_2$ **D**	16.0	18.4	*3.7*	0.5	0.5	*1.8*	0.7	0.2	58.2
$scCO_2$ **E**	11.7	16.1	*15.6*	11.7	15.0	*13.8*	2.2	0.9	13.1

Table 5.8: Percentage of wax group in the three cereal straw waxes

Straw	Percentage of wax group in crude extract								
	Fatty acids	Wax esters	Hydrocarbons	Sterols	β-Diketones	Fatty alcohols	Aldehydes	Ketones	Unknown
Wheat	8.1	2.4	9.9[a]	21.9	16.0	8.9[a]	0.3	0.0	41.1
Barley	11.5	16.9	19.3[a]	21.0	21.2	10.2[a]	2.2	0.7	7.4
Oat	6.0	14.5	14.6[a]	12.5	11.6	11.9[a]	4.6	0.5	35.1

[a] Co-elution of hentriacontane (C_{31}) and octacosanol (C_{28}) giving the total percentage of hydrocarbons and fatty alcohols

From the DSC thermograms, it can be concluded that physical properties like melting ranges can be tuned by extraction conditions and many extracts can be refined to match the melting ranges of commercial waxes e.g.: the $scCO_2$ **A** extract has a DSC thermogram similar to carnauba wax. It would be beneficial to fractionate some of the extracts to understand how the different wax groups interact with one another in order to correlate chemical composition to melting ranges more easily. As can be observed in Figures 5.24, 5.25 and 5.26, wheat straw waxes do have a real potential to replace some of the existing commercial waxes.

The melting ranges can also be altered by a blend of different waxes or other natural ingredients as well as further downstream processes such as flash chromatography. Figure 5.27 shows the melting ranges of wheat, barley and oat straw waxes from the scale up trial described in Chapter 4. Both wheat and oat straw waxes exhibit minor endothermic events prior to the main melt for the wax which suggested that there are some low melting compounds within the complex mixture. The wheat and barley straw waxes are very similar as there are two main peaks at 315 K, 327 K and 333 K suggesting that the chemical compositions for the two are comparable. Table 5.8 shows the breakdown of the different wax groups within the three straw waxes. As previously, there are co-elution of hentriacontane (C_{31}) and octacosanol (C_{28}) and this has been highlighted in the table. The chemical composition for both wheat and barley wax are similar but do differ in terms of hydrocarbon and wax ester content. Barley straw wax has a much higher hydrocarbon and wax ester content compared to wheat straw wax, both wax groups melt at temperatures between 360 K to 432 K. This higher proportion of hydrocarbons and wax esters in the barley straw wax is reflected in the DSC thermogram as the second melting peaks at 327 K and 334 K are much higher than wheat straw wax which means more energy is needed to create the endotherm indicating a bigger proportion of higher melting wax compounds. This also suggested that the first peak at 315 K may be due to other wax groups such as fatty acids. The thermogram for oat straw wax is distinctly different to the other two cereals as it appears that the peak at 315 K has disappeared. However, it does exhibit the same second broad peak at 334 K and with a shoulder at 327 K. The oat straw wax does have the smallest proportion of free fatty acids in the extract which may lead to the 315 K being extremely small blending into the second peak.

To investigate this further, fractionation of the wax into different wax groups would assist in the correlation between chemical compositions and physical properties. It would be interesting to analyse wax blends at different proportions to observe the influence of chemical composition on thermal properties. As can be seen in Table 5.8, not all the wax compounds in the extracts are identified and quantified so it is important to be aware that there is still a proportion of the extracts that is unknown in terms of chemical compositions which could be responsible for the thermal properties observed. To enhance the arguments from before, it would be beneficial if all the wax compounds were identified so the compounds responsible for certain endotherms can be allocated which can make tuning of thermal properties for specific applications easier.

The wheat straw wax from the scale up trial was saponified using the method described in Section 6.2.7. From the discussion in Section 5.2.4, the saponification proved to be successful as the fatty alcohol fraction does not contain any ester molecules and the fatty acid fraction only consists of free fatty acids. The identification of compounds within each fraction is discussed in Section 5.2.4. Figure 5.28 shows the DSC thermogram of how the saponifcation fractions compared with the original crude wheat straw wax. Wheat straw wax consists of multiple endotherms indicating various groups of compounds within the complex mixture melting. The wheat alcohol and acid fractions together showed a rough representation of the DSC thermogram of the crude wheat straw wax as the main peak shown at 315 K is clearly due to the fatty acids within the mixture. The DSC thermogram of wheat alcohols fraction is more complex as more endotherm peaks can be seen and this could be due to two newly identified 2-ketones in the fraction. The peaks are more defined as at least three lipid groups (fatty acids, wax esters and glycerides) have been removed. The thermogram also highlighted that the peak present at 333 K in the crude wheat straw wax is due to the fatty alcohols, sterols and β-diketones. The large peak at 327 K in the crude wheat straw wax has now been reduced to only a small shoulder in the wheat alcohols fraction which suggests that this could be due to the saponifiable compounds such as glycerides or wax esters. This work has clearly demonstrated that simple saponification can assist in correlating the wax groups with their thermal properties which is valuable information when tuning the physical properties of wax for specific applications. It would be interesting to carry out some blending

experiments with not only the crude waxes but also various fractions in order to create various thermal profiles and generate many different wax products for many applications.

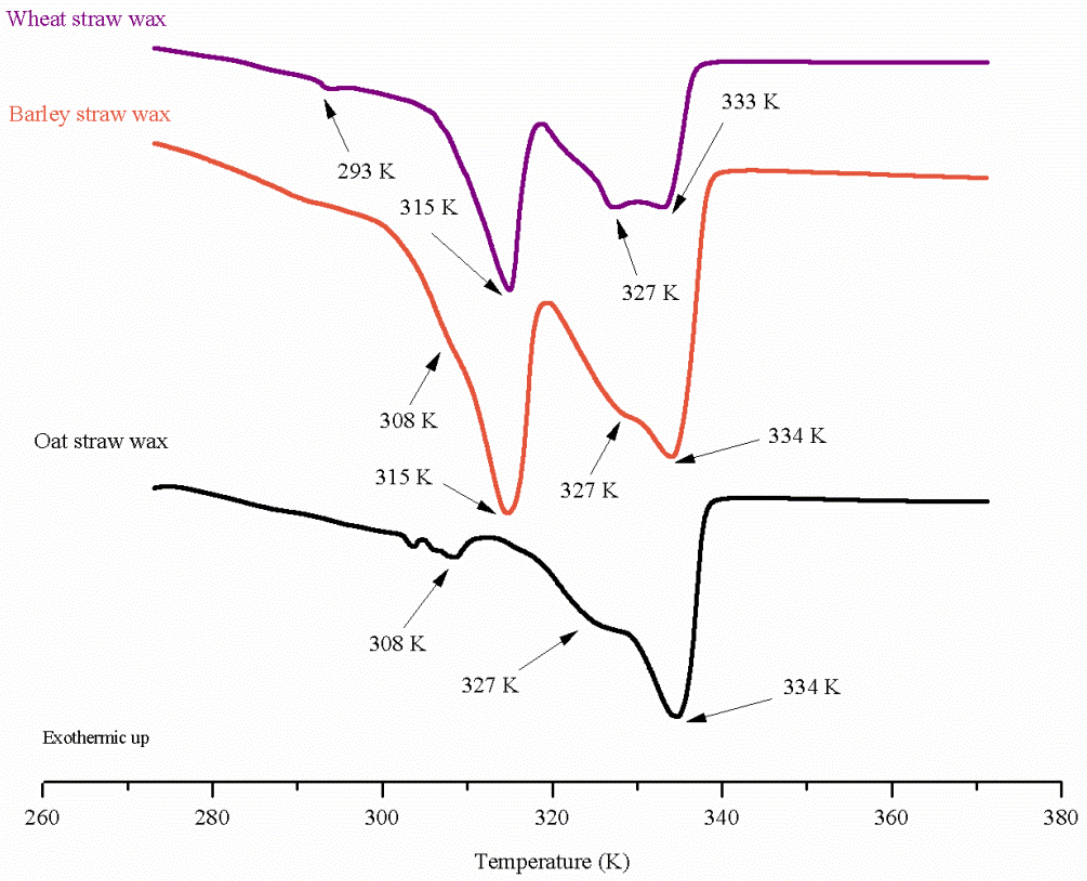


Figure 5.27: DSC thermograms of wheat, barley and oat straw waxes from scale up trials (originally in colour)

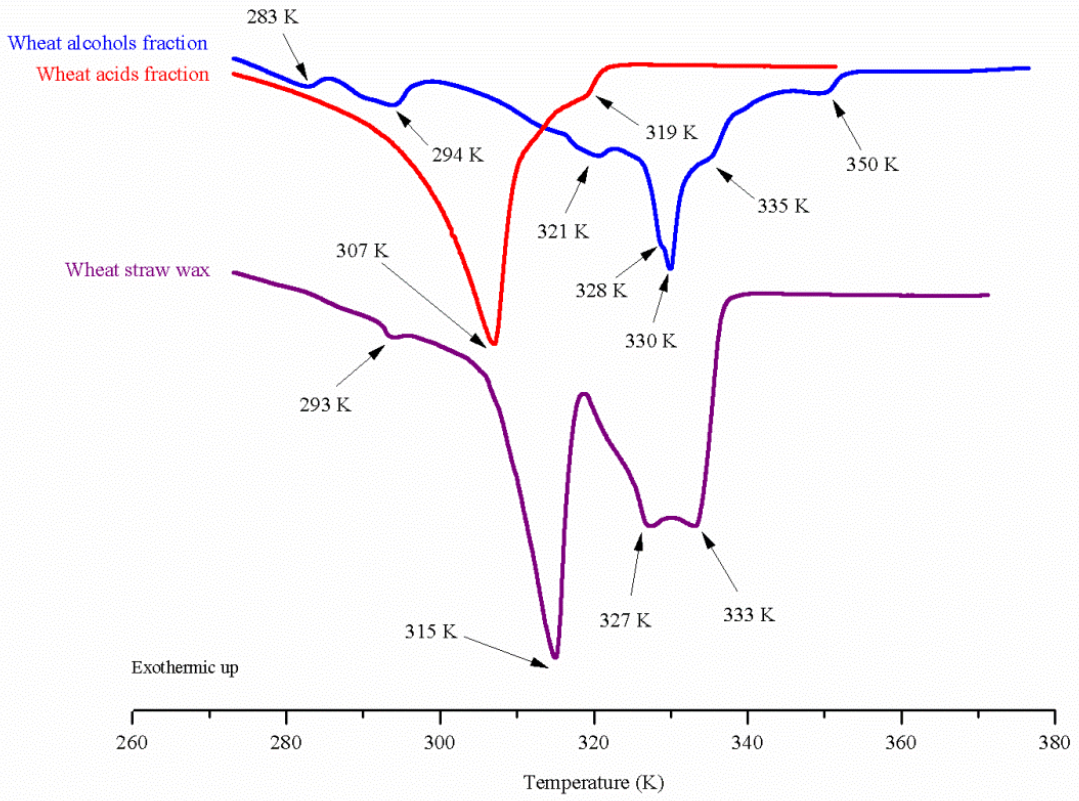


Figure 5.28: DSC thermograms of wheat alcohols and acids fractions compared to wheat straw wax (originally in colour)

From the DSC thermograms, it is apparent that thermal properties of waxes can be manipulated easily by extraction conditions through compositional differences and downstream processing can help to tune the specific thermal profiles.

5.3.2 Acid value, saponification value and drop point

Acid value, saponification value and drop point are simple tests that are commonly performed on lipid samples prior to determining areas of application. On Croda's recommendations, the three cereal straw waxes were assessed with these tests prior to formulation and any application tests. The results would also suggest any necessary downstream processing required targeting specific applications.

Acid value is a common wet technique used to determine the amount of carboxylic acid groups in the sample. In the case of lipids, this would determine the amount of fatty acids in a complex mixture or any di-acids if present. For the cereal straw waxes, only fatty acids were identified and this is a useful and quick method to determine the level of acids in the extracts and compare them with the existing commercial waxes. The acid number was determined using the method described in Section 6.4.3 which represents the mass of potassium hydroxide required to neutralise 1 g of straw wax. Saponification value is another common wet technique used by lipid chemists to determine the amount of potassium hydroxide required to saponify 1 g of wax. In the case of triglycerides, glycerol is also formed in the saponification process as well as the potassium salt. In straw waxes, both glycerides and wax esters are present which will undergo saponification and the saponification number is calculated using the method described in Section 6.4.4. No steryl esters or diesters were found using the GC method which would analyse molecules up to about 6000 KI units. It is not clear whether larger molecules such as di-esters would also undergo saponification. Di-esters such as steryl di-esters, diol di-esters and hydroxyacid diesters have been reported to be present in lanolin and these are very large molecules that cannot be detected using a standard GC method.[321] Table 5.9 shows how the acid and saponification values for the three cereal waxes from the scale up trial compared to four existing commercial waxes. The acid value for commercial waxes tends to be lower though the cereal straw waxes do have similar acid values to beeswax. The acid value can be reduced depending on demand by fractionating the fatty acids out. In a separate experiment described in Section 5.2.4, the wheat straw wax was saponified into two different fractions: fatty alcohols and fatty acids. The fatty alcohol fraction that contains no fatty acids has an acid value of just 1 and the acid value for the fatty acids containing fraction is 165. From this experiment, it can be shown that downstream processing such as saponification can reduce the acid levels. The saponification values of the straw waxes fit well in the current commercial waxes and no modification is required.

Table 5.9: Acid value and saponification value for commercial and cereal straw waxes[113, 116]

Waxes	(mg of KOH per g)	
	Acid value	Saponification value
Lanolin	7 - 15	100 - 110
Beeswax	17 - 36	90 - 149
Candelilla wax	12 - 22	43 - 65
Carnauba wax	2.9 - 9.7	79 - 95
Wheat straw wax	30.8	89.3
Barley straw wax	28.1	95.4
Oat straw wax	34.5	97.2

The drop point of the straw waxes was also measured prior to lip-stick formulation trials which will be carried out by Croda Europe Limited. The drop point of a wax is the temperature at which the solid wax turns into a liquid and drips from the experimental cup described in Section 6.4.5. The temperature suggests the change in solid to liquid phase of a wax and this is especially critical in specific formulations such as lip-sticks. According to Croda's recommendation, for a lip-stick formulation, the wax must have a drop point of around 343 - 358 K. A basic lip-stick formulation typically consists of a combination of waxes for different functionality. The harder waxes such as candelilla wax within the formulation can offer the wax its structure and the softer waxes such as lanolin would give the lip-stick its emollient and the body temperature melting point. Table 5.10 shows the drop points for the cereal straw waxes as well as the fatty alcohols and fatty acids from the wheat saponification. It was found that the drop points for the cereal straw waxes were all at around 337 K. Candelilla and carnauba waxes are commonly used as a structural ingredient in lip-sticks and showed the appropriate drop point for formulation. The wheat alcohols fraction from the saponification showed a promising drop point as the lower melting point acids have been removed. However, other physical properties such as colour must be determined in order to proceed to the formulation phase.

Table 5.10: Drop point for commercial and cereal straw waxes

Waxes	Drop point (K)
Wheat straw wax	337
Barley straw wax	338
Oat straw wax	337
Wheat alcohols fraction	353
Wheat acids fraction	319
Beeswax	334 - 338
Candelilla wax	343 - 348
Carnauba wax	356 - 361

5.3.3 Appearance and aroma

The appearance of the three cereal straw waxes was assessed on both colour and smell. All the waxes exhibit a strong but pleasant grass odour. Typically, waxes such as lanolin are de-odourised using a steam or vacuum de-odouriser.[322, 323] The colour of the straw waxes ranged from yellow to dark green depending on the cereal straw as shown in Figure 5.29. It should be noted that the method of extraction and the conditions used are also very important to the resulting colour as demonstrated in Figure 4.2. In order to compare the colour of the straw waxes to commercial waxes, the Gardner colour method was employed and this is a common method used for measurement of lipid colour. The Gardner colour of the waxes was determined using the method described in Section 6.4.7 and is shown in Table 5.11. The Gardner colour scale is a range from 1 (white) to 18 (dark brown).

The cereal straw waxes appeared to be darker compared to the refined commercial waxes. Wheat straw wax has a Gardner colour of 18 and the other two cereal straw waxes exhibit a Gardner colour of above 18 which is a massive problem as in many applications such as lip-sticks, colour is critical for its performance. However, the unrefined wool grease has a Gardner colour of 16 and is capable of being decolourised to a lighter colour using hydrogen peroxide and the super-refined grade lanolin achieves almost complete decolourisation to Gardener colour 1 or 2 through flash chromatography.[324] Lipids such as waxes can be bleached using a number of methods such as the use of hydrogen peroxide, bleaching clays, strong acids, strong alkalis and

activated carbon.[325, 326, 327, 328] It would be interesting to test these bleaching methods on the straw waxes and observe the decolourisation process.

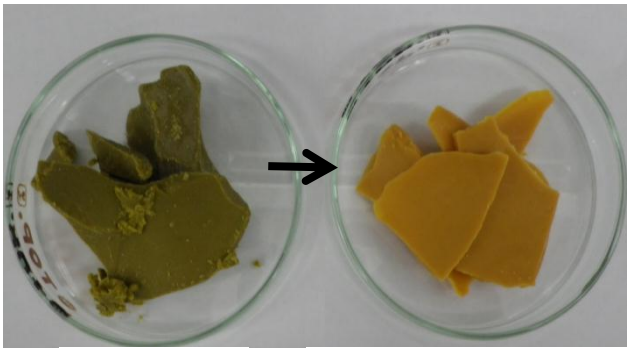
Figure 5.29: Production scale cereal straw waxes (originally in colour)

Table 5.11: Gardner colour for commercial and cereal straw waxes

Waxes	Gardener colour
Lanolin	9
Beeswax	3
Candellila wax	9
Carnauba wax	9
Wheat straw wax	18
Barley straw wax	> 18
Oat straw wax	> 18

Hydrogen peroxide bleaching was carried out on the wheat straw wax using a Croda in-house method (confidential) that is currently used in the decolourisation of lanolin. Unfortunately, no decolourisation occurred and the colour of the wax remained the same. Barley straw wax was super-refined by Croda Japan Limited using the same flash chromatography method (confidential) being carried out on lanolin commercially to produce super-refined lanolin for applications such as nipple creams. Figure 5.30 shows a large improvement in the colour of the barley straw wax as it changed from a Gardner colour of over 18 to 13 with 85% yield.

Further work is needed in order to further improve the decolourisation but from this initial result, it certainly appears to be a promising method of decolourisation for straw waxes



Barley straw Super-refined barley straw

Figure 5.30: Barley straw wax and super-refined barley straw wax (originally in colour)

5.3.4 Water absorption

The water absorption test is a very simple way to determine if the wax has any emulsifying properties for personal care applications. Emulsions are usually formed between two or more usually immiscible components, typically an aqueous and an oily component. Wool wax alcohol is a self-emulsifier that can readily take more than 230% of its own mass in water which makes it a great wax ingredient for skin care applications by forming a very stable water-in-oil emulsion.[329] Following Croda's recommendation, this test would determine whether the wax is suitable for skin care formulations.[324] For this reason, the three wheat straw waxes from the scale up trial and the saponified wheat alcohol fraction were tested for water absorption ability and its potential in this application area. It was found that both wheat straw wax and wheat alcohol fraction are very water resistant as they only took up roughly 20% and 60% of their own mass in water respectively. The low level of water uptake demonstrates that both wheat straw as an unrefined material and the wheat alcohol fraction are not suitable for skin care applications. The water absorption ability did improve when the wheat straw wax was saponified into the alcohol fraction as hydrophobic molecules such as wax esters are being saponified into more hydrophilic molecules such as fatty alcohols. In order to further improve the water absorption ability, other compounds

such as *n*-alkanes present in the wheat straw wax that would have a water repelling effect, need to be fractionated off so that the wax would be more hydrophilic. This would not only improve its water absorbing property, it would also add value to the wax by creating an alkane-rich fraction. The waxes will be tested for home care applications such as furniture wax polishes instead.

5.4 Applications and costs

5.4.1 Potential applications

Cereal straw waxes contain a complex mixture of molecules which can be separated and refined for potentially important applications. From the literature review of existing commercial waxes, it was apparent that waxes are not a single group of compounds but a blend of different wax groups at different concentrations. It is this varied composition that give its unique physical properties that are especially favourable for specific applications. For example one of the biggest wool wax producers, Croda Europe Limited, refined wool grease to lanolin alcohols so it is high in fatty alcohols and sterols to give it its extra emollient and emulsification properties in cosmetics applications. After the identification of the crude cereal waxes, it can be concluded that there are a mixture of both aliphatic and cyclic compounds with good opportunities for many applications. However, it is important to consider both single groups of compounds and a blend of compounds in order to achieve its functionality to fit with existing applications. Table 5.12 summarises the potential applications for the different wax groups identified in cereal straw waxes. The potential applications were discussed in more details in Chapter 2.

One of the biggest global wax applications is candles with over 450,000 tonnes of wax used solely for candle production in the USA.[124] A sample of barley straw wax from the scale up trial was tested for its potential in candles applications in collaboration with Oakbank Products. The congealing point was 329 – 330 K which was too low and unsuitable for use and casting solid pillar candles and in addition the hardness of the wax was found to be unsuitable for casting.[330] The congealing point also showed that the wax has a melting point slightly too high to use in container candles which means that the crude wax has properties in between that required for

container wax and casting wax but from the testing, it was concluded that the barley straw wax was more suited for container wax. The next stage of the trials would be to input additives into the crude wax and carry out performance tests.[330] However, in order to keep the products as natural as possible, bio-derived ingredients would be considered first. It would be interesting to incorporate some wheat alcohols and observe the effects of melting point.

Table 5.12: Potential applications for different wax groups identified in cereal straw waxes

Wax group	Top compounds	Potential applications
Fatty acids	Hexadecanoic acid ($C_{16:0}$), octadecadienoic acid ($C_{18:2}$), octadecenoic acid ($C_{18:1}$), octadecanoic acid ($C_{18:0}$)	Soaps, detergents, lubricating grease/ oils, cleaning compounds/ polishes[167, 168]
Hydrocarbons	Nonacosane (C_{29}), hentriacontane (C_{31})	Paraffine waxes, coatings[170]
Fatty alcohols	Octacosanol (C_{28})	Surfactants, cosmetics[167, 331]
Aldehydes	Octacosanal (C_{28})	Food flavouring[181]
Wax esters	Octacosanyl hexadecanoate (C_{28}:C_{16}), octacosanyl octdecanoate (C_{28}:C_{18})	Hard wax polishes, coatings, cosmetics, plasticisers[182]
Sterols	β-Sitosterol, stigmasterol, campesterol	Nutrient supplements, cosmetics, surfactants[332]
β-Diketones	14, 16-hentriacontanedione (C_{31})	Metal chelators[333]

From the water absorption tests, the waxes were shown to have great water-proofing properties so for this reason, the samples of wheat, barley and oat are currently waiting to be tested by the home care applications team in Croda Europe Limited for applications such as water-proofing furniture polishes and coatings.[324] Also for the same reason, the company has concluded that the crude cereal waxes are unsuitable for skin care creams to substitute current commercial products such as lanolin alcohols.[324]

5.4.2 Economic considerations

The use of CO_2 as an extraction method is well-known for its environmental benefits but this green alternative technology does struggle to replace some of the existing

processes and one of the main reasons is due to its high capital and operating costs. With $scCO_2$ systems running at high pressure, large energy consumption is inevitable and is reflected in high energy costs. This is one of the biggest arguments against using $scCO_2$ as a commercial process for extraction even though now almost all the CO_2 extraction plants are designed to deliver optimum performance and reduced energy consumption. One major concern with extraction of cereal straw waxes is that the bulk density of the raw material is low so extra costs would be added to increase the bulk density of the biomass so increasing the volume of feedstock per batch extraction. However, if the raw materials are too dense, diffusion problems might arise and CO_2 would struggle to penetrate through the cereal straw pellets resulting in low extraction efficiency. The right balance between densification and extraction efficiency must be achieved to result in the most economic extraction process. To date, all the commercial extraction plants are run on batch processes even though continuous extraction would offer a great energy saving so the high throughput batch processes must be taken into account.[334] Brunner showed that by switching batch to continuous processes, the operation costs could be reduced by about 30%.[334] As the current commercial extractors are all batch processes, it is important to design the plant to allow easy loading and unloading of the materials from possibly multiple extractors. The operating costs of a supercritical CO_2 extraction plant are inversely proportional to the size of the plant and these can be broken down to eight different categories as indicated in Figure 5.31. Labour was shown to be the main operating cost as skilled engineers are required to operate high pressure plants.

As well as the consideration of the process itself, a crucial point to be noted is that even though cereal straw is an agricultural by-product and that there is excess quantity with no current commercial uses, the raw materials are not free. Waste generated from one process is essentially a new feedstock for another so fitting this idea into the straw-based bio-refinery, the costs of the net raw materials would be lowered. Extraction can be carried out using virgin cereal straw and any de-waxed straw residues could potentially have a range of applications such as fuels.[19]

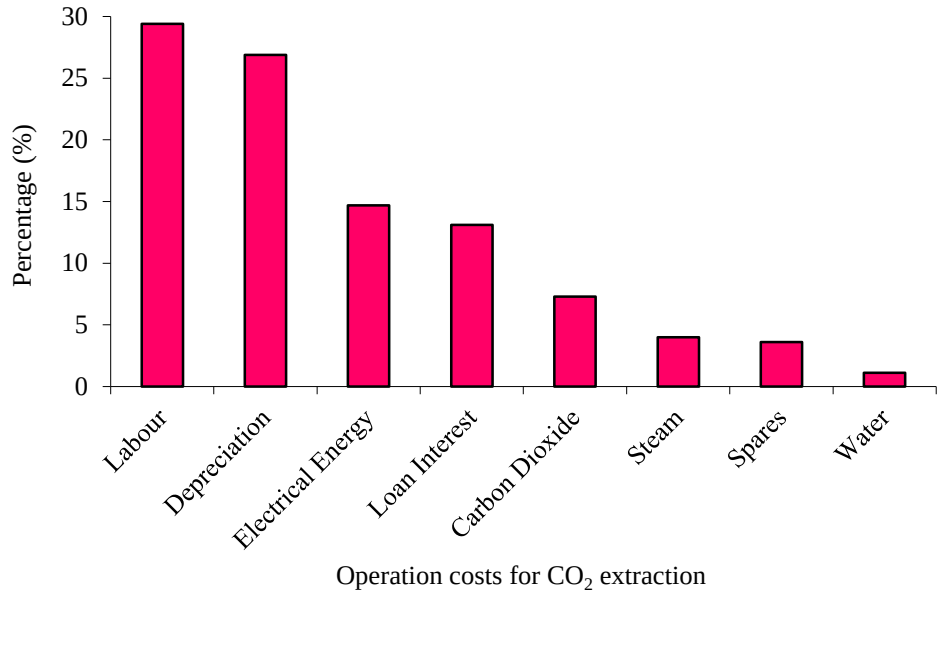


Figure 5.31: The breakdown of operation costs for CO_2 extraction[334] (originally in colour)

An economic assessment for the extraction of cereal straw wax using $scCO_2$ at a commercial scale using prices collected from personal communication in 2012 is shown below. The raw materials and pelletisation costs are current values from Sundown Products Limited and some of the operation costs are based on personal communication with Evonik Industries. Table 3.13 shows the cost of the three cereal straws as well as pelletisation costs carried out in the scale up trial described in this chapter.

Table 5.13: Costs of raw materials and palletisation

Raw materials (Cost/ tonne)	Costs per tonne		Pelletisation	Pelletised straw	
	Low (£)	High (£)	(£)	Low (£)	High (£)
Wheat straw	50	70	45	95	115
Barley Straw	100	100	45	145	145
Oat straw	100	100	45	145	145

Two different costs labelled as “low” and “high” are shown for the raw materials as the costs are different seasonal depending on harvest results. The cost of barley and

oat straw tends to be higher than wheat straw as more wheat is grown compared to barley and oat in the UK. The pelletisation cost indicated is calculated by Equation 5.1 and the cost reflects a combination of both milling and pelletisation based on the scale up trial but with no processing loss built in.

$$Pelletised\ straw\ per\ tonne\ (£) = Cost\ of\ straw\ (£) + Cost\ of\ pelletisation\ (£)$$

Equation 5.1: Cost of pelletised materials

If the straw residues were to be recovered and be used as combustion fuels in power stations, the straw residues can be sold at a cost. Sundown Products Limited estimated the cost of re-sale of extracted straw residues. It has been shown that the calorific value of the extracted straw residues is similar to virgin cereal straw despite the cuticle waxes having been removed. The removal of the cuticle wax does have a negative effect in terms of combustion. During the $scCO_2$ extraction process, the co-extraction of water in the straw occurs which is beneficial for combustion so for this reason, the calorific value remains similar.[19] Table 5.14 shows the estimated readjusted costs for the raw materials and the net costs calculated using Equation 5.2. The cheapest cost was calculated to be £20 per tonne for pelletised wheat straw and this value is used for further calculations in estimation of product costs. However, the percentage of the crude yield of wax as well as the cost of the raw materials must be taken into account when calculating the most economical raw material to use for extraction.

$$\begin{aligned} Net\ cost\ of\ pelletised\ straw\ per\ tonne\ (£) \\ = Initial\ cost\ of\ pelletised\ straw\ (£) \\ - Resale\ price\ of\ straw\ (£) \end{aligned}$$

Equation 5.2: Calculation of net cost of pelletised straw

Table 5.14: Estimated net costs for pelletised raw materials

Power station	Low (£)	High (£)		
Costs of straw (£/t) include repelletisation	75	85		
Raw materials (Cost/ tonne)	Pelletised straw		Net cost of pelletised straw after re-sale	
	Low (£)	High (£)	Low (£)	High (£)
Wheat	95	115	20	30
Barley	145	145	60	70
Oat	145	145	60	70

As discussed before there is a high operational cost when dealing with high pressure extractors and the costs of extraction is scale dependent. Table 5.15 shows a summary of the estimated costs for three different CO_2 scale with the less than 10 tonnes scale values based on the scale up trial carried out in collaboration with Evonik Industries including the costs of CO_2. It is noted that the displayed currency is in Euros (€) as the scale up trial was carried out in Germany. Typically, the cost of food grade CO_2 is approximately £90 per tonne but CO_2 can be easily recompressed, recycled and reused with only less than 0.03% of CO_2 being lost during the decompression stage.[236]

Table 5.15: Costs for CO_2 extraction[236]

CO_2 scale	Cost/kg of straw for extraction (€)	Crude yield (€/ kg of wax)		
		1%	2%	3%
< 10 tonnes (Scale up trial)	3	300	150	100
> 50 tonnes	1	100	50	33.33
10,000 tonnes (own plant)	0.4	40	20	13.33
Note: tonnes in CO_2 scale refer to volume of biomass				

As shown previously the crude yield ranged from 0.8 to 1.5% in the scale up trial and is dependent on the raw materials used so therefore, the costs based on a crude yield of 1 to 3% were calculated. From the optimisation work shown in Chapter 4, it was apparent that higher yields can be achieved compared to what were reported in this chapter. The operating pressure was only 26 MPa so if this can be increased then there would be an increase in crude yield. However, it is important to note that increased pressure would lead to higher energy costs so it is important to find the right balance

between cost and extraction conditions. Other expenditure such as de-watering processing cost, plant maintenance cost, transportation costs etc. are not included in the calculations. Results showed that wheat straw has the lowest wax yield but it is the cheapest source of straw in the UK which suggests pushing wheat straw wax yield to 3% would be very challenging without any plant breeding. The best case scenario is that cheap wheat straw was used as the feedstock with a high 3% crude wax yield in a 10,000 tonnes CO_2 plant, this would result in a production cost of just over £12 per kg of wax. Currently, based on figures from Croda Europe Limited the production cost for the finished product lanolin is about £4 per kg and the most expensive super refined lanolin is about £11 - 12 per kg. There is a large difference in the two prices with straw wax being at least three times more expensive than existing commercial waxes. To make the prices more comparable and for the new straw wax to be able to compete with the existing commercial waxes, the yield must be increased to about 8% which would give an approximately £4 per kg of wax. From this economic assessment, it can be concluded currently; the cost of extraction of wheat straw wax is too high and must be reduced in order to gain more industrial interest as well as further scale up trials.

There are a number of ways that the cost of production of straw wax can be reduced. An obvious way is by increasing the current % crude yield and this may be achieved by plant breeding of cereal crops and controlled growing conditions. As the cost of $scCO_2$ extraction is inversely proportional with size of CO_2 plant, the size of plant may need to be increased if possible. If the cost cannot be reduced then it is important to find a unique high value application for the new product thought research and development.

5.5 Conclusion and future work

As $scCO_2$ can co-extract the water molecules within the biomass, the first intial processing step after the production scale extraction is the removal of co-extracted water. If a plant was to be built specially for the extraction of waxes from biomass, the removal of water should be incorporated into the plant design to increase the efficiency. The use of $scCO_2$ as part of a extraction and fractionation as one step process have been demonstrated successfully but

more work is needed to optimise the fractionation conditions to give a series of wax products with different chemical compositions and physical properties for a variety of applications.

As shown repeatedly that there are still unidentified compounds in the extracts that are too involatile to analyse by simply using GC. Wheat and oat straw waxes were also successfully fractionated by GPC in order to seaprate the high molecular weight compounds for further investigation. It was found that there were a significant number of compounds with high molecular weight (iup to 1500 Da). The molecular weight of these large molecules, believed to be esters from the IR analysis, were determined using MALDI-TOF MS. Further method development is needed in order to accurately analyse these wax esters succesfully each time. This demonstrated that there is an interesting high molecular weight wax fraction. These wax compounds could lead to further new applications and raise the value of the products. Further work is needed for the saponified wax extracts to be analysed on GC-MS for full identification. Other molecules such as phenolics could also be presented in these extracts so further work is needed to fully analyse the complete extracts.

The physical properties of the straw waxes from different $scCO_2$ conditions and scale up trials were explored and this included appearance, melting point, acid value, saponification value, water absortion, drop point and melting points. These physical properties were shown to be highly influenced by the chemical compositions of the wax which are determined by the extraction conditions. These properties were assessed and compared with some commercial waxes in order to understand how these new renewable straw waxes can fit in the current market. The chemical compositions were correlated with some of the physical properties such as melting points to help understand how to tailor the wax properties by extraction conditions. The straw waxes are strongly coloured hard waxes with high melting points and no water absorption ability so therefore it is not ideal for cosmetics applications in its crude form but due to high hydrophobicity, it could be ideal for coatings and polishes applications. Further downstream processing steps e.g.: decolourisation are requried to tune the wax products for specific applications. More work is needed on investigation of poetntial applications of these valuable waxes by carrying out some downstream proceses and formulations tests.

The use of supercritical CO_2 as the extraction method is an expensive technology due to its high operating pressure so an economic assessment was carried out for the extraction of straw waxes based on the scale up trials. The costs of the $scCO_2$ extraction decreases as the size of the extractor increases so the absolute minimum costs was calculated for the use of a

10,000 tonnes extractor. A range of extraction yields and biomass costs were used in order to achieve the minimal cost and it was calculated to be about £12 per kg of straw wax. This calculated value was based on a high yield (3%), the assumption of low biomass costs and no further downstream processing and this was still signifcantly higher than exisiting costs for commercial waxes such as lanolin which can be obtained for £4 per kg. In summary, there is a huge potential in the extraction of straw to gain valuable waxes for the growing wax market. Extraction of cereal straw waxes using $scCO_2$ in industrial scale were proved to be a success but further work is needed on optimisation of conditions and the products resulted showed promising physical properties which can lead to a wide range of industrial applications. The extraction of straw presents an interesting alternaive wax and demonstrates to fit well within the straw bio-refinery concept by adding value to these low cost, high volume agricultural by-products. However, despite the success of the extraction trials and potential applications, the costs of production of cereal straw waxes are significantly higher than the existing commercial waxes and therefore it will be difficult to compete in the current market. In order to commercialise these cereal straw waxes, the only solutions are to minimise the costs further by increasing the extraction yields. As technology develops, the cost of high pressure extractors will decrease and identify unique physical properties and molecules that currently do not exist in the present wax market.

Chapter 6

Experimental Procedures

6. EXPERIMENTAL PROCEDURES

6.1 Materials and reagents

Wheat husk and straw (Claire 07, 08 and Viscount 09) *(Triticum aestivum*) were supplied by Park Farm in Castle Howard. Park Farm is a part of the leading farming company, Velcourt Limited. Wheat straw (Hereward 08, Oakley 08 and Charger 08), barley straw (variety: Carat 08 and Optic 08) *(Hordeum vulgare)* and low erucic acid oilseed rape straw *(Brassica napus)* were supplied by A & A Services, in Cliffe, Selby. Oat Services Limited supplied a mixed variety of oat straw and oat husk. Sunflower husk, straw and capitulum (*Helianthus annuus)* were provided by Auvergne Trituration. The high erucic acid oilseed rape straw *(Brassica napus)* and miscanthus straw (*Miscanthus giganteus*) were supplied by Charles Jackson & Co Limited. Wheat, barley and oat straw (mixed varieties 09) were supplied and pelletised by Sundown Products Ltd.

Organic solvents dichloromethane, heptane, ethanol, diethyl ether, toluene, ethyl acetate, acetone, 2-MeTHF, THF, chloroform and acetonitrile were all analytical grade > 99% and were purchased from Fisher Scientific UK Limited. Pesticides grade hexane and dichloromethane were obtained from Fisher Scientific UK Limited and used for the gel permeation chromatography. The IMS was provided by Croda International Plc. Carbon dioxide (> 99.99%), liquid nitrogen, helium, nitrogen and hydrogen were purchased from the BOC Group. Octacosanol (≥ 99%), stearic acid (~ 95%), oleyl palmitate (~ 99%), hentriacontane (≥ 99%), 4-nitroaniline ((≥ 99%) and ASTM® D5442 C_{12}-C_{60} qualitative retention time mix (containing docosane, dodecane, dotriacontane, eicosane, hexacontane, hexacosane, hexadecane, hexatriacontane, octacosane, octadecane, pentacontane, tetracontane, tetracosane, tetradecane, tetratetracontane and triacontane) were purchased from Sigma-Aldrich UK Limited. Stigmasterol (~ 95%), concentrated nitric acid (67 - 70%), 1 M HCl and 1 M NaOH were obtained from Fisher Scientific UK Ltd. Trans- methyl-β-styrene (~ 97%) was purchased from Acros Organics. Polystyrene calibration standards of molecular weight 500 $gmol^{-1}$, 1000 $gmol^{-1}$, 2000 $gmol^{-1}$ and 3000 $gmol^{-1}$ were obtained from Fluka Limited. Polystyrene calibration standard of molecular weight 2500 $gmol^{-1}$ was purchased from Alfa Aesar. Wool grease, Corona 8®, Medilan®, Medilan Superso JP®, Medilan Ultra So®, Coronet®, Fluilan® and petroleum jelly were provided by Croda Enterprises Limited. The 2 mm borosilicate glass beads, phenolphthalein indicator and

activated carbon Norit® RO 0.8 pellets were purchased from Sigma-Aldrich UK Limited. N,N-Diethyl-4-nitroaniline was obtained from Fluorochem UK.

The cellulose thimbles used for the Soxhlet extractions were bought from Fischer Scientific UK Limited. The PTFE filters used for FexIKA® extraction have a pore volume of 65%, pore size of 10-20 µm and a membrane thickness of 0.2 mm, and were purchased from IKA®. The Bio-Beads S-X3 (200-400 mesh) was purchased from Bio-Rad Laboratories. The metal standards of 1000 ppm used for ICP-AES analysis were all obtained from SCP Science. The MALDI MS grade 2,5-DHB and lithium carbonate (> 99%) were purchased from Sigma-Aldrich UK Limited.

6.2 Pre-treatment, extraction and fractionation procedures

6.2.1 Moisture content of raw materials

Approximately 10 g of biomass was put inside small aluminium dishes and placed in to a 383 K oven (Carbolite Ltd.). The biomass was weighed periodically until constant weight was achieved. The biomass used for extractions all has a moisture level of less than 10%. The moisture level was calculated using Equation 6.1.

$$Moisture\ level\ (\%) = \frac{(Mass\ of\ original\ sample\ -\ Mass\ of\ dried\ sample)}{Mass\ of\ original\ sample} \times 100$$

Equation 6.1: Calculation of moisture level

The moisture level of the saponified mixture described in Section 6.2.8 was determined using Orion AF8 moisture balance. The instrument was programmed to maintain a temperature of 378 K. The weight loss of the sample was continuously monitored with the internal balance in the instrument until a constant weight was achieved and the moisture level indicated.

6.2.2 Pre-treatment of raw materials

All untreated samples were milled using a Glen Creston Limited hammer mill to pass through a 2.5 mm size screen. Unfortunately, the instrument is very old so the model number cannot be retrieved. The biomass was stored in a cool, dry shed prior to milling. Once the biomass is milled, it was stored in paper sacks in a cool, dry place.

The three cereal straws supplied by Sundown Products Limited were milled using an industrial hammer mill with a screen size of 8 mm to fine particle size. It was then conditioned by evenly adding approximately 2 - 5% water. This is to lubricate the straw for pelletisation. The milled straw is then pelletised using a pellet press fitted with a 6 mm ring die. All the milling and pelletisation were carried out using the instruments Swiss Combi 44K and La Meccania 935. The straw pellets were cooled and air-dried prior to packing. The miscanthus straw was densified as briquettes for coal co-firing by Charles Jackson Limited but the details of the procedure were unable to obtain.

Figure 6.1: Alternative pre-treatment processes: milled, chopped, pellets and briquettes (originally in colour)

6.2.3 Soxhlet extraction

Prior to extraction, the raw materials were pre-treated as described in Section 6.2.2. About 13 g of the processed biomass was extracted with 300 mL of selected solvent in a standard Soxhlet extraction apparatus for five hours as shown in Figure 2.1. The exact temperature for the Soxhlet extraction was monitored using a Radleys Discovery Technologies 2006T thermocouple periodically and an average of at least three readings were taken. The recovered extracts were immediately filtered using fluted filter paper to remove any straw residues and concentrated to dryness *in vacuo*. In order to eliminate moisture and traces of residual solvent, extracts were air-dried at room temperature. The extracts were weighed periodically until constant weight was achieved.

6.2.4 FexIKA® extraction

The FexIKA® Vario Control Series Extractor was the chosen extraction method for the screening stage as shown in Figure 2.2. FexIKA® Vario Control Series Extractor is

based on the fluidised bed extraction principle. About 20 g of sample was loaded onto the extraction tube which is fitted with a hydrophobic PTFE filter. The filter was slotted into the solvent resistant connection. The extracting solvent was filled in the basic vessel that contains a magnetic stirrer bar. During a typical run, the solvent is heated and stirred to the set temperature and speed as shown in Table 6.1. The heating and cooling device is controlled by a computer using the software Labworldsoft. Table 6.1 shows the extraction information for each of the extraction solvent used. The recovered extracts were concentrated *in vacuo*. In order to eliminate moisture and traces of residual solvent, extracts were air-dried at room temperature until constant weight was achieved.

Table 6.1: Extraction information for ethanol and hexane

Extraction Solvent	Ethanol		Hexane	
Number of cycles	10		10	
	1st cycle	2nd cycle and after	1st cycle	2nd cycle and after
Rated Temperature heated (K)	393	393	373	373
Boiling time (minute)	9	7	15	10
Rated Temperature Cooling (K)	313	313	313	313
Filtration Time (minute)	5	5	10	10

6.2.5 Supercritical fluid extraction

Supercritical CO_2 extractions were carried out using a Thar SFE 500 system (Thar technologies). Figure 6.2 shows the system that was used for the carbon dioxide extraction study. Approximately 130 g of air-dried milled raw materials can be loaded into the 500 mL extractor. The CO_2 supplied from a cylinder as a liquid and was maintained in this state through cooling unit (273 K) to avoid cavitation in the high pressure pump. The liquefied CO_2 was then sent to the pre-heater and converted into a supercritical fluid prior to entering the extractor. If liquid carbon dioxide extraction was to be performed, a second cooling unit must be adopted to cool the extractor in order for the CO_2 to be maintained as liquid. Temperature (279 K – 373 K), pressure (6.5 - 55 MPa) and flow rate of 40 mLmin ^{1} were selected according for the experiment. The extracts were all collected at 323 K and at atmospheric pressure in separator 1. Co-solvent ethanol was employed in some experiments. The co-solvent flow rate was maintained at 4 $mLmin^{-1}$ (10% of CO_2 flow). The ethanol was released

in the first separator periodically to ensure there was not a build-up of solvent. The extraction lasted four hours and once completed, the system was depressurised over a period of one hour and the separators were washed with a minimal amount of dichloromethane. The dichloromethane was removed *in vacuo* and extracts were air-dried at room temperature until constant weight was achieved.

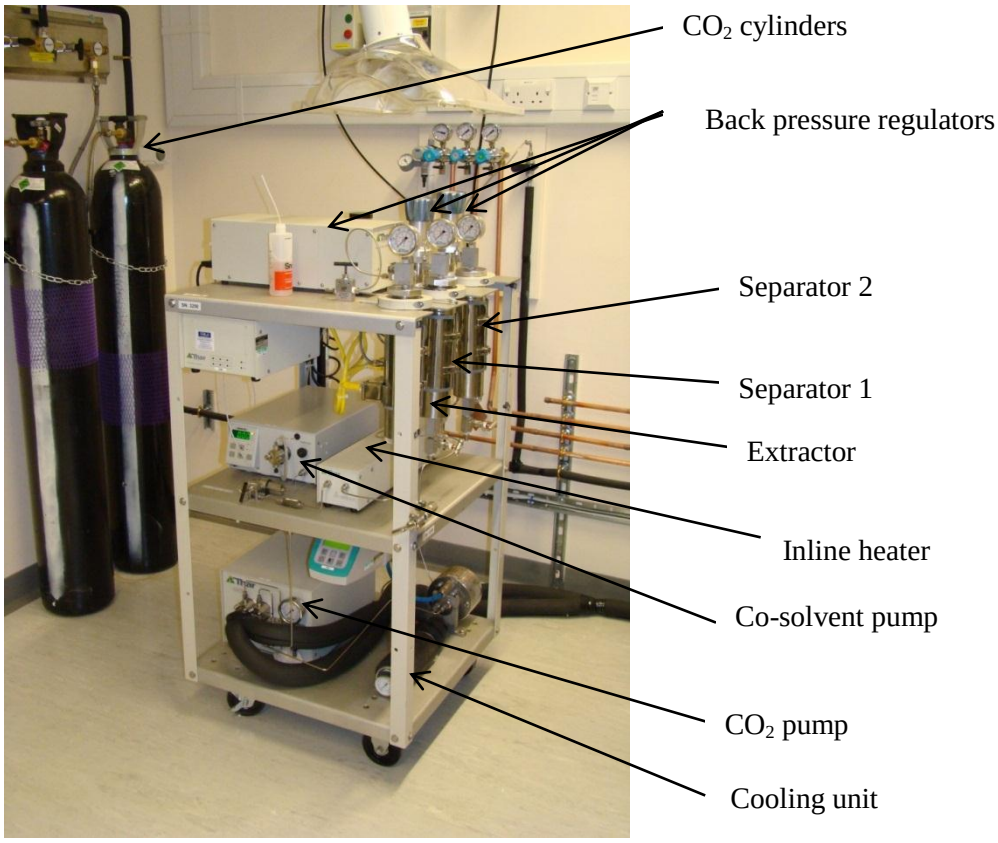


Figure 6.2: Thar SFE 500 system used for carbon dioxide extractions (originally in colour)

Production scale supercritical fluid carbon dioxide extraction was carried out in collaboration with Evonik Industries. The extractions were carried out on an Evonik Industries' CIT plant in Trostberg, Germany. The CIT extraction plant was used for the wax extraction. This plant has 2 x 200 L extractors and has an operating pressure of up to 28 MPa. The wheat straw pellets were extracted without further milling at 323 K and 26 MPa. The separator was set at 323 K and 5.5 MPa. Approximately 50 - 60 kg of CO_2 was used per kg of straw pellets. The only significant problem encountered during the wheat straw extraction was that the extract solidifies after the depressurisation valve and in the separator. After a few batches the separator pipe work was disassembled and extract was found to be coating the inside of the pipe

work which severely restricted the flow. To overcome this problem, a ratio of 0.7- 1.5 parts of rapeseed oil was added as carrier oil. This was injected into the high pressure line between the extractor and separator. As the addition of carrier oil is considered undesirable, the temperature of the extractor and separator was raised to 343 K for other batches so the wax would be melted when separated.

Pure wax extracts were melted using a large stainless steel pot on a hot plate. The wax extracts were stirred using a stainless steel ladle periodically to increase heat transfer and avoid burning. The temperature of melting wax was measured and recorded to be about 348 K. The water from the melted wax was separated off leaving a water-free wax extract. The wax extracts were stored in freezer to avoid oxidation and degradation.

6.2.6 Purification and fractionation of wax extracts

Approximately 0.1 g of solvent extracts was dissolved in 20 mL of DCM and the same volume of de-ionised water was added to the solution. The bi-phasic solutions were inverted a few times and pressure was released periodically. The aqueous wash was repeated two times (2 x 20mL) and the total aqueous solutions were combined and washed with 2 x 20 mL of DCM. The DCM and aqueous washes were combined and the DCM fractions dried *in vacuo*.

Supercritical CO_2 fractionations were carried out using a Thar SFE 500 system (Thar technologies) as shown in Figure 6.4. The waxes from the production scale extraction were loaded onto 2 mm borosilicate glass beads for the fractionation. The waxes were first melted on a 348 K hot plate and were then diluted with an equal volume of dichloromethane. The wax coated glass beads in solvent were evaporated to dryness *in vacuo*. The sample was spinning continuously whilst evaporating the dichloromethane *in vacuo* to ensure the wax was loaded on the glass beads evenly.

The 15 - 17% wax coated glass beads were loaded onto the extractor. The extractor was set at 323 K and 35 MPa; separator 1 was at 308 K and 7.5 MPa and the separator 2 was 323 K and at atmospheric pressure. The CO_2 flow rate remained constant at 4 $mLmin^{-1}$ during the full whole four hours of fractionation time. The system was

depressurised and both of the separators were set to 348 K in order to melt and retrieve the wax fractions.

6.2.7 Saponification

Full saponification of wheat straw wax was carried out using a Croda in-house method QAP02/19. A solution of 340 g of IMS and 60 g of deionised water was added to about 460 g of molten wheat straw wax in a 3-necked flask. A condenser, thermocouple and overhead stirrer were attached to the flask. The solution was warmed to 333 K and stirred gently whilst the 47% w/v of sodium hydroxide was being added gradually. The amount of sodium hydroxide added to the mixture was calculated using Equation 6.2.

$$Amount\ of\ sodium\ hydroxide\ (mL) = \frac{Saponification\ value\ \times\ 500\ \times\ 40\ \times\ 105}{56100\ \times\ 47}$$

Equation 6.2: Calculation for quantity of sodium hydroxide

After the addition, the mixture was continuously refluxed (about 353 K) and stirred gently for approximately eight hours until the reaction was completed. In order to test the reaction for completion, a base value was determined using method described in Section 6.4.9. The base value must be in the range of 0.15 - 25% when completed. If the free base is lower than 0.15%, a further addition of free base is required. The extra sodium hydroxide needed was calculated using Equation 6.3.

$$Amount\ of\ sodium\ hydroxide\ (mL) = \frac{(0.25 - free\ base\ value)\times\ 500}{47}$$

Equation 6.3: Calculation of extra sodium hydroxide for low base value

The saponified mixture was extracted in batches of approximately 40 g. The moisture level was determined using the method described in Section 6.2.1. A mixture of 50 mL IMS and 100 mL deionised water was added to the saponified mixture and warmed to 328 K. The aqueous saponified mixture was extracted using 300 mL warm hexane (about 323 K) in a separating funnel. The solution was swirled gently by hand

and the contents were allowed to come to thermal equilibrium. The funnel was stoppered and inverted gently and pressure was released frequently.

6.3 Chemical compositions analysis procedures

6.3.1 GC analysis

Approximately 25 mg of the extract was dissolved in 1 mL of solvent and was analysed using a capillary column ZB-5HT, 30 m x 0.25 mm I. D. X 0.25 μm film thickness, Phenomenex. A 2 $mgmL^{-1}$ tetradecane in dichloromethane was made and used as an internal standard. This solution was used as the 1 mL solvent to make up the wax samples for quantification analysis. Helium was used as the carrier gas with a flow rate of 2.2 $mLmin^{-1}$ in constant flow mode. The oven was temperature-programmed from 323 K (1 minute hold) to 553 Kat 283 $Kmin^{-1}$ and from 553 K to 613 K (24 minutes) at 278 $Kmin^{-1}$. The injector and the FID were both set at 573 K. All injections were done manually in splitless mode and 1 μL of sample was injected. Capillary columns DB-5HT, 6 m x 0.53 mm I.D. x 0.1 μm film thickness (SGE) and ZB-5 HT, 15 m x 0.25 mm I.D. x 0.25 μm film thickness (Phenomenex) were also employed in the study. Helium was used as the carrier gas, and the flow rate was 2.2 $mLmin^{-1}$ in constant flow mode. However, there was a problem in controlling and maintaining the gas flow with the 6 m column as the required head pressure was lower than could be controlled accurately. The oven was temperature-programmed from 323 K (one minute hold) to 673 K at 283 $Kmin^{-1}$ and the samples were injected at 10:1 split mode manually. A HP 6890 gas chromatography was used for all GC analysis.

Quantification of the wax compounds were carried out using a series of authentic standards. The standards were used as a representative of each wax group (e.g.: stearic acid was used for all fatty acids quantification). The standards used were stigmasterol for sterols, hentriacontane for alkanes, octacosanol for fatty alcohols, stearic acids for fatty acids and stearyl palmitate for wax esters. As there is no authentic standard available for ß-diketones, the Effective Carbon Number (ECN) method was used to quantify this group of compounds.[208] Tetradecane at a concentration of about 2 cm^3mg^{-1} was used as an internal standard for the samples and standards.

6.3.2 GC-MS analysis

Approximately 30 mg of the extracts were dissolved in 1 mL of solvent (dichloromethane or ethanol) and the samples analysed using a capillary column DB-5HT, 30 m x 0.25 mm I.D. x 0.25 μm film thickness; Phenomenex. The oven was temperature-programmed from 333 K (1 minute hold) to 633 K at 281 $Kmin^{-1}$ (10 minutes hold). Exactly 0.5 μl of the sample was injected using 10:1 split mode. The temperature of the injector was 573 K. A Perkin Elmer Clarus 500 Gas chromatograph with an auto sampler was coupled to a Perkin Elmer Clarus 560S mass spectrometer for this analysis.

The mass spectrometer was operated in electron impact (EI) mode at 70 eV, a source temperature of 573 K with the quadruple at 573 K and scanning a mass range of 30 - 1200 amu per second.

6.3.3 Derivatisation for GC analysis

Some wax samples were derivatised in order to increase volatility, improve peak shape and help in identification. Trimethylsilylation was carried out using 200 μL 1% bis-(trimethyl)-trifluoro-acetamide and trimethylchlorosilane and 1mL of toluene were added to about 20 mg of wax sample. The reaction was completed by heating the sample in a 348 K oven for 30 minutes. The sample was allowed to cool prior to GC and GC-MS analysis.

6.3.4 FAME analysis

Fatty acid methyl esters (FAME) analysis was carried out by dissolving about 90 mg of crude extracts in 3 mL of hexane (containing approximately 2 $mgmL^{-1}$ internal standard tetradecane) and followed by the addition of 150 μL of 1 N methanolic sodium hydroxide. The solution was stirred vigorously for 20 minutes and left at room temperature until the organic and aqueous layers were separated. The organic layer was analysed using the GC method as above and FAME was quantified using GCMS and fatty acid as standard.

6.3.5 IR analysis

IR spectra were obtained by analysing on a Bruker Vertex 70 fitted with a Specac Golden Gate ATR. This was controlled by Opus software. The spectrum was scanned from 4000 - 600 cm^{-1}. The number of background scans and sample scans were set at 32 and 16 respectively. The resolution was selected to be 2 cm^{-1}.

6.3.6 GPC analysis

A HP 1090 liquid chromatography with an auto- sampler coupled to a DAD detector were used for the analysis. A Phenogel 5 µ 100 Å column (300 x 4.6 mm) with a guard column (Phenogel 5 µ linear/mixed 30 mm x 4.6 mm) was placed in an oven of 313 K and a detector set at 220 - 480 nm. The mobile phase used was THF. The flow rate was maintained at 0.35 $mLmin^{-1}$ throughout the isoscratic method. This was carried out with the help of Peter Hurst (PhD student). The injection volume was 25 µL. The total run time was 20 minutes. The fractions were collected using a Pharmacia Biotech FRAC-100 sample carousel and programmed to collect the fractions every 30 seconds. The fractions were reanalysed to clarify the peaks collected. The fractions containing the same peaks were combined and analysed using MALDI TOF MS.

The GPC analysis was scaled up using a modified Croda in-house method G17300 which was used as a clean-up method for pesticides analysis. This was carried out under the guidance of Graham Atkinson of Croda International Plc. A Gilson HPLC was used to carry out the wax fractionation. The system used consists of Gilson 306 pump, Gilson 402 syringe pump, Gilson 231 XL sample injector, Gilson 206 fraction collector, Gilson 811C dynamic mixer, Gilson 806 monometric module and Gilson 151 UV-VIS detector. Approximately 5 g of wax was dissolved in 1:1 hexane: dichloromethane and made to volume in a 50 mL volumetric flask. The wax was loaded on via a 5 mL sample loop onto a Bio-Beads SX3 resin (300-400 mesh) which was swollen in 1:1 hexane: dichloromethane then packed in-house into a 600 mm x 25 mm i.d. column.[335] The eluting solvent was 1:1 hexane: dichloromethane and was mixed and pumped at 5 $mLmin^{-1}$. The wavelength for the UV-VIS detector was set at

254 nm with the eluting wax fractions being collected every minute from 10.5 to 20.5 minutes. The mass balance of the wax fractions were calculated.

6.3.7 MALDI-TOF MS analysis and synthesis of LiDHB matrix

A Bruker Solarix FTICR (Fourier Transform Ion Cyclotron Resonance) 9.4T magnet with a smart beam 2 Nd:YAG laser (wavelength 355 nm) was used for MALDI TOF MS analysis under the guidance of Adam Dowle. Laser power used was 20% which equated to 20 micro joules per shot and the laser focus was set to medium. Each data file was an accumulation of 4000 laser shots. The matrix used was 2,5-DHB and lithium DHB. The instrument was calibrated from 900 to 3150 Da using a standard peptide mixture. The sample acquisition was ranged from 500 to 3000 Da yielding a target resolution at m/z 800 of 80,000.

The lithium DHB was synthesised using a method reported by Cvačka et al.[308] The matrix was synthesised by dissolving 0.5 g 2,5-DHB in 10 mL deionised water at 313 K. The mixture was gently stirred until complete dissolution then the stoichiometric amount of lithium carbonate (0.12 g) was added. The pH was checked and maintained pH 6 to prevent oxidation. Inert gas nitrogen was blown over the mixture to reduce the solvent level to about 1 mL. The reduced mixture was crystallised by putting it in a refrigerator of 279 K. White needle-like crystals were formed, filtered and washed with 1 mL of $CHCl_3$. The sample was dried under vacuum to remove the residue water. The matrix was prepared at approximately 10 $mgmL^{-1}$ with 50% acetonitrile, 40% deionised water and 10% of 1% TFA. The wax samples were prepared at about 30 $mgmL^{-1}$ in DCM. About 0.5 µL of sample was spotted on the MALDI plate then 0.5 µL of matrix was added on top of the sample spot and mixed on plate. The plate was left at ambient temperature so that it was completely dried prior to analysis.

The lanolin samples were analysed and the fractions were collected. The samples were initially screened using IR described in Section 6.3.4. The samples which showed ester groups were then analysed using MALDI-TOF MS.

6.4 Physical properties of wax extracts

6.4.1 Decomposition by STA analysis

Simultaneous Thermal Analysis (STA) was carried out using Thermal Sciences STA 625 and the decomposition temperature was determined from the Thermogravimetric Analysis (TGA) trace. Between 5 – 20 mg of sample was placed into the bottom of an aluminium pan prior to analysis. The quantity used was dependent on the nature of the sample. The sample in the aluminium pan was placed in the hanger along with an empty aluminium pan as the reference. The furnace was then heated from room temperature (approximately 298 K) to 898 K at 283 $Kmin^{-1}$ under a constant nitrogen flow of 50 $mLmin^{-1}$.

6.4.2 Thermal transition temperatures by DSC analysis

A TA instruments Q2000 modulated DSC was used to determine the melting point of the wax extracts. About 10 mg of sample was measured into an aluminium hermetic pan and an aluminium hermetic lid was crimped on. A TA instruments refrigerated cooling system 90 was used to cool the sample. The pan containing the sample and an empty reference aluminium pan were both placed into the test cell automatically. A typical sample would be equilibrated at 308 K and heated from 308 K up to 393 K at 283 $Kmin^{-1}$ and held for five minutes, then cooled down to 193 K at 278 $Kmin^{-1}$ and again held for five minutes. After holding the cell at a negative temperature, it was heated from 193 K to 393 K at 278 $Kmin^{-1}$ and held for five minutes.

6.4.3 Acid value

The acid value was calculated using the Croda in-house method G01102. Depending on the predicted acid value of the sample, a range of 2 to 10 g of wax samples was used for the determination. The solvent was prepared by heating equal volumes of IMS and petroleum spirit (373 K – 393 K) to the boiling point and adding 0.5 mL of phenolphthalein solution (1 % w/v in IMS). About 25 mL of the pre-made solvent was used to dissolve the wax sample. This solution was then neutralised with 0.1 M potassium hydroxide whilst keeping it warm to about 343 K. The contents were

titrated using a suitable molarity potassium hydroxide (0.1 M or 0.5 M) until there was a colour change from colourless to pink. If there was precipitation during the titration, this was then warmed on the hot plate. The acid value can then be calculated using Equation 6.4, where T is the volume (mL) of potassium hydroxide required by the sample, M is the molarity of the sodium hydroxide and W is the weight of the sample.

$$Acid\ value = \frac{56.1 \times T \times M}{W}\ mg\ KOH\ per\ g$$

Equation 6.4: Calculation of acid value

6.4.4 Saponification value

The saponification value for the wax samples were calculated using the Croda in-house method G01407. About 2 g of the wax sample was weighed into a conical flask and exactly 25 mL of 0.5 M ethanolic potassium hydroxide solution is added. The sample was refluxed for four hours on a sand bath. When the reflux had completed, approximately 1 mL of phenolphthalein (1% w/v in IMS) was added and swirled around for titration against 0.5 M HCl. The titration was carried out when the solution was warm and the titre value was noted down when the end point colour change from pink to colourless was achieved. For every batch of sample analysed, a blank is made for result validation. The saponification value can be calculated using Equation 6.5 where B is the volume (ml) of HCl used for the blank, T is the volume (mL) of HCl used for the wax sample, M is the molarity of the HCl used for the titrations and W is the weight (g) of the sample.

$$Saponification\ value\ (mg\ of\ KOH\ per\ g) = \frac{56.1 \times (B - T) \times M}{W}$$

Equation 6.5: Calculation of saponification number

6.4.5 Drop point

Drop point was measured using a Croda in-house method G02805. The drop point is the temperature at which the first drop of sample falls from the cup under the specified temperature conditions. This was carried out using a Mettler FP90 thermosystem and Mettler FP83 HT measurement cell. The wax sample was melted completely and was poured into the metal cup in excess. The wax was left to fully set and a spatula was used to scrape off the excess that was overfilling the cup. The wax containing cup was left overnight at room temperature. The cup was loaded onto the instrument the next day. The starting and end temperature were set at 308 K and 368 K respectively with a ramp rate of 273.65 $Kmin^{-1}$. The drop point was noted and monitored by the instrument automatically.

6.4.6 Slip point

The slip point was determined using a Croda in-house method G02900. The wax sample was melted using a hot plate. Two capillary tubes were inserted into the molten wax so that the columns of the sample are at least 1 cm long. The wax sample was then solidified quickly by taking them out of the wax solution and leaving them at room temperature overnight. The two capillary tubes containing the same wax sample were attached to a thermometer. The thermometer and the capillary tubes were clamped in a beaker of water. The water was at room temperature initially and was heated up gradually at about 274 $Kmin^{-1}$ and decreased to 273.65 $Kmin^{-1}$ when close to melting point. The water was stirred by a magnetic stirrer to ensure the water was heated up efficiently. The temperature was noted down when the sample begun to slip up the capillary tube.

6.4.7 Gardner colour

The Gardener colour is the number of the standard reference colour which compares to the test sample when viewed through the Dr Lange 400 spectrometer using a Croda in-house method G01701. The wax sample was melted and poured into a disposable 11 mm round cuvette to a depth of 2 cm. The outside glass of the cuvette was wiped clean andit was important to ensure there were no air bubbles. The wax containing

cuvette was then inserted into the cuvette compartment and the instrument would take a colour measurement ranging from 0 to 18 accurate to one decimal place. The reading was taken as soon as the wax was molten so the reading temperature varied according to the sample.

6.4.8 Water absorption

The water absorption test was carried out by adapting the Croda in-house method G16101. About 0.5 g of wax sample was melted with 9.5 g of petroleum jelly and weighed directly into a mortar. The total weight of the mortar, wax mixture and polythene rod were noted. Deionised water was added dropwise whilst stirring vigorously. More water was added when the wax had fully absorbed all the water. The end point was determined when there was a visible water droplet remaining on top of the wax mixture and cannot be incorporated into the wax mixture. Wool wax alcohol (superhartolan®) would take more than 230% of its own mass in water. The calculation of the percentage of water absorption can be calculated using Equation 6.5 where W is the weight (g) of wax sample, W_2 is the initial weight (g) of wax sample, mortar and polythene rod and W_1 is the final weight (g) of wax sample, mortar and polythene rod.

$$Water\ absorption\ (\%) = \frac{W_2 - W_1}{W} \times 100$$

Equation 6.6: Calculation for water absorption

6.4.9 Base value

The base value was determined using a Croda in-house method G09701. About 20 g of saponified wax sample was weighed out. The exact mass of the sample was calculated by determining the moisture level using procedure described in Section 6.2.8. Approximately 75 mL of ethanol was heated to boil and 0.5 mL of bromophenol blue indicator solution was added. The solvent was then neutralised with 0.1 M HCl whilst keeping the solution at 343 K and the colour change from green to yellow was observed. The neutralised solvent mixture was added to the sample solution and boiled. The solution was titrated with 0.1 M HCl until there was a

definite colour change from green to yellow. The base value was calculated using Equation 6.6, where T is the volume (mL) of HCl required for complete titration, M is the molarity ($gmoleL^{-1}$) of HCl solution and W is the weight (g) of dried sample.

$$Base\ value\ (mg\ KOH\ per\ g) = \frac{56.1 \times T \times M}{W}$$

Equation 6.7: Calculation for base value

Chapter 7

Conclusion and future work

7. CONCLUSION AND FUTURE WORK

The objective of this research was to add value to a low cost, high volume renewable resource through an integrated extraction step to produce potentially high value wax products. Through literature review, the epicuticular waxes on the surface of plants were identified as a source of new potential waxes. Straw is an agricultural by-product of significant volume that is currently being under-utilised and was shown to consist of wax compounds of high interest in many wax applications. Within the concept of a straw bio-refinery, this would be a great opportunity to maximise the use of the biomass by extraction of waxes prior to existing straw uses such as combustion for energy.

Wheat straw is the most abundant cereal crop grown in the UK and through literature review, the chemical compositions of wheat straw wax appeared to have high potential to replace some of the existing commercial waxes. In the first stage of this study, wheat straw wax was extracted by a variety of traditional organic solvents using Soxhlet extraction in order to determine the effect of solvent properties on extraction. This study demonstrated that the extraction yields are highly dependent on the solvent used which suggested different solvent-solute interactions were established during the extraction. A qualitative analysis was carried out on the wheat straw wax from different solvent extractions using GC-MS and showed identical chemical composition profiles. A range of long aliphatic molecules including free fatty acids (C_{14} – C_{18}), fatty alcohol (C_{28}), *n*-alkanes (C_{27} – C_{33}), β-diketones (C_{31} and C_{33}), aldehydes ($C_{28,}$), wax esters (C_{40} – C_{58}) and cyclic sterols were identified. The similar composition suggested that the solvent had very little selectivity on the different groups of wax molecules and the differences between the extracts were the relative abundance of the different compounds. A quantitative analysis was carried out using GC-FID to gain a deeper understanding in the solvent properties with the different wax compounds. Linear Solvation Energy Relationship (LSER) was used to model solvent properties against the extraction of wheat straw wax for the first time. Correlations of crude and various wax group yields with different solvent parameters were carried out. Both crude and wax ester yields showed positive correlation with Kamlet- Taft parameter β (hydrogen bond accepting ability) and molar volume. The model can predict the crude wax and wax ester yields using solvent parameters successfully and this is vital in achieving high wax yields. The waxes were purified using liquid-liquid extraction (aqueous and DCM) and showed that the lipophilic fraction yields were similar. LSER modelling was carried out and

found no trend with the DCM soluble fractions but found a strong correlation of aqueous soluble fractions with solvent parameters β and molar volume. It can be concluded that the lipophilic fraction of the extracts can be extracted using solvents with a wide range of polarities. This model showed the selectivity of the solvents and the model can be used to identify the most selective solvents.

As an alternative to extraction using organic solvents a greener extraction technology using supercritical CO_2 was employed for the extraction of wheat straw wax to give a truly sustainable and environmentally friendly process and effect of temperature and pressure on the extraction were extensively analysed. Both qualitative and quantitative analyses were carried out for the extracts retrieved from different extraction conditions. The chemical composition was identical to the solvent extracts and the change in temperature and pressure of scCO_2 only affected the relative abundance of the wax compounds. The effect of temperature and pressure on the crude and wax groups yield was analysed using first order polynomial modelling. Strong correlations were shown with crude wax, wax esters and β-diketones which suggested there is a linear relationship between temperature and pressure. Aldehydes showed the least correlation which indicated that there is no relationship between temperature and pressure. Further work is required to investigate this non- linear relationship and determine the parameters that dictate the aldehyde yields within the wheat straw wax. The data was also modelled using a density-based Chrastil equation. Both crude and wax esters yield obey the Chrastil equation with a strong correlation which indicated that the extraction yields are strongly influenced by density. However, the β-diketones, which showed a strong linear correlation with temperature and pressure do not correlate linearly with CO_2 density, despite temperature and pressure dictating the CO_2 density. This can be explained by the change in temperature and pressure also altering other CO_2 properties such as polarity, viscosity and diffusivity. More work is needed to understand the relationship between CO_2 properties and the different wax groups so predicted extraction conditions can be calculated to produce tailored wax products for specific applications. Extraction temperature and pressure were optimised using the 2^2 extraction model on both of these parameters. Liquid CO_2 and supercritical CO_2 with a co-solvent (ethanol) were also explored. Liquid CO_2 showed highly selective extraction but failed to extract any wax esters whereas supercritical CO_2 with ethanol showed a high extraction yield but proved very unselective. Whilst using liquid and supercritical CO_2 extraction for wheat straw will leave both the straw residues and wax products completely solvent-free, the introduction of

ethanol as a co-solvent did not add any extra benefit but would add extra solvent elimination and wax purification steps.

Using neat supercritical CO_2 was shown to be a selective and environmentally friendly method to extract straw waxes and was selected to be the technology used for scale up trials. A total of seven different straws (wheat, barley, oat, oilseed rape, sunflower, miscanthus and bagasse) were screened for yield and composition prior to selection of the biomass for scale up trials. Hexane and ethanol Soxhlet extractions were carried out on the seven different straws to give an insight into the polar and non-polar molecules present on the straw surface. Qualitative and quantitative analyses were completed and new compounds were identified. Considering the biomass availability, percentage crude yield and chemical composition profile, wheat, barley and oat straw was shown to be the most promising biomass for the extraction of renewable waxes.

In collaboration with Sundown Products Limited, a total of three tonnes of wheat, barley and oat straw (1 tonne each) were obtained and laboratory scale heptane extraction was carried out to assess the quality of the straws. In the biomass screen, it was apparent that there are many factors such as natural variation that can alter the wax quality and yield so it was important to check for wax quality and quantity by a simple heptane Soxhlet extraction. Following successful laboratory scale heptane Soxhlet extraction and preliminary $scCO_2$ extraction work, the process was scaled up to industrial scale using a 200 L extractor in collaboration with Evonik Industries. Optimised conditions (323 K and 35 MPa) were requested but due to the limitations of the extraction system, the operating pressure was 28 MPa which reduced the CO_2 density. Conditions of 323 – 343 K and 28 MPa were used for all the scale up trials which successfully yielded approximately 60 kg of wax extract. Overall the scale up was successful; there was a small problem with crystallisation of waxes in the pipe work causing blockages. Carrier oil oilseed rape oil was used to flush and clear the pipes to relief the problem and the separator temperature was raised to 343 K. To improve the extraction efficiency, the CO_2 density must be increased for future extraction scale up trials. Extraction times were not investigated in this study so it would be interesting to gather data on percentage of wax extracted in specific extraction times. The wax products retrieved from the extractions contained at least 0.5% co-extracted water so an extra de-watering step had to be carried out. If any extraction plants were to be built solely for the extraction of biomass such as straw, it would be ideal to incorporate a method to de-water the products as

part of the plant design. The waxes were fractionated using laboratory scale $scCO_2$ extractor to demonstrate the feasibility of combining the extraction and fractionation as a one step process. More work is required to optimise the fractionation conditions but if a series of separators were incorporated to give a wide range of separating temperature and pressure into the extractor then the waxes could potentially be fractionated into different high value fractions for a number of different markets including cosmetics, nutraceutical, home care, personal care and agrochemical. From the quantitative analysis, it was apparent that there are still components of the extracts that have not been identified. One reason for this is that the molecular weight of the compounds was too large for GC analysis even using a high temperature GC method. The waxes were separated according to molecular weight by analytical scale GPC. Following the success of analytical scale GPC work, the fractionation was scaled up to pilot scale to achieve enough material for MALDI-TOF MS analysis to gain a deeper understanding of the molecular weight range. Ester molecules of up to 1500 Da were successfully separated and analysed. More work is needed on method development as there were problems with uneven crystallisation of the waxes on the MALDI plate. As only the molecular ion of the compounds were seen, MALDI-TOF MS provided no structural data so further separation needs to be carried out and the wax esters saponified to give greater structural details.

Physical properties (appearance, melting point, acid value, saponification value, drop point and water absorption) were assayed and compared with existing commercial waxes. It was found that the crude cereal waxes were most similar to beeswax. The straw waxes are hard, deeply coloured, and highly hydrophobic and have a strong odour which is not ideal for cosmetic applications in their crude form. Due to the high hydrophobic behaviour, it is thought that they would fit into existing wax applications such as polishes and coatings. Currently, the straw waxes are waiting to be tested for a variety of applications in collaboration with Croda. Wheat straw wax was saponified to fatty alcohols and fatty acids fractions with the aim of modifying its physical properties. As the chemical composition alters, the physical properties such as colour, melting point and water absorption abilities all changed. Further work is needed to link the chemical composition of the wax with its physical properties so that the relevant modifications can be done to obtain tailored wax products.

The work in this thesis has demonstrated the successful use of a sustainable extraction method to obtain high value chemicals from a lost cost, high volume and under-utilised biomass. However, this will not break through to commercialisation without a full economic assessment of the value of the products. The economics of the process to produce the high value straw waxes were considered by incorporating the physical pre-treatment, biomass, extraction and CO_2 costs and showed the extraction process is the most costly. The extraction yields were incorporated into economics calculations which give the value of the straw wax at about £12 per kg. When comparing with the existing commercial finished wax products, the value is extremely high considering beeswax is about £6 per kg and lanolin is about £4 per kg. With the current quoted cost of straw wax, it is impossible for the new product to compete with existing waxes in the market, even though there is a big consumer drive for 100% natural products, unless there are specific unique applications. In conclusion, the extraction yields must be increased to be competitive and this can be achieved either through the use of high pressure extractors or new cereal varieties developed to give higher wax yields. Overall, the extraction of renewable straw waxes have shown to have great potential to replace existing waxes but due to the current high production costs, it would be difficult for the new wax products to move to commercialisation and compete with cheaper waxes.

Chapter 8

References

8. REFERENCES

[1] R.K. Henderson, C. Jimenez-Gonzalez, D.J.C. Constable, S.R. Alston, G.G.A. Inglis, G. Fisher, J. Sherwood, S.P. Binks and A.D. Curzons, *Green Chem.*, 2011, **13**, 854-862

[2] B. Hopwood, M. Mellor and G. O'Brien, *Sustain. Dev.*, 2005, **13**, 38-52

[3] IUCN, UNEP and WWF, *World Conservation Strategy: Living Resource Conservation for Sustainable Development*, Gland, Switzerland, 1980

[4] G.H. Brundtland, *Our Common Future*, WCED, Oxford, 1987

[5] K. Aleklett, M. Höök, K. Jakobsson, M. Lardelli, S. Snowden and B. Söderbergh, *Energ. Policy*, 2010, **38**, 1398-1414

[6] M. Poliakoff, J.M. Fitzpatrick, T.R. Farren and P.T. Anastas, *Science*, 2002, **297**, 807-810

[7] J.E.G. van Dam, B. de Klerk-Engels, P.C. Struik and R. Rabbinge, *Ind Crop Prod*, 2005, **21**, 129-144

[8] J.H. Clark and F.E.I. Deswarte, in *Introduction to Chemicals from biomass*, ed. J.H. Clark and F.E.I. Deswarte, Wiley, Chichester, 2008, ch. 1, 1-18

[9] IEA, *World Energy Outlook 2010 executive summary*, IEA, Paris, 2007, 3-14

[10] IEA, *World Energy Outlook 2010 executive summary*, IEA, Paris, 2010, 3-18

[11] B. Kamm and M. Kamm, *Appl. Microbiol. Biotechnol.*, 2004, **64**, 137–145

[12] C. Francesco, *Energ. Convers. Manage.*, 2010, **51**, 1412-1421

[13] T. Werpy, G.Pedersen, A. Den, J. Bozell, J. Holladay, J. White and A. Manheim, *Top value added chemicals from biomass (Volume I: Results of Screening for Potential Candidates from Sugars and Synthesis Gas)*, US Department of Energy, Oak Ridge, 2004

[14] J.R. Hess, C.T. Wright and K.L. Kenney, *Biofuel. Bioprod. Bioref.*, 2007, **1**, 181-190

[15] F.E.I. Deswarte, J.H. Clark, A.J. Wilson, J.J.E. Hardy, R. Marriott, S.P. Chahal, C. Jackson, G. Heslop, M. Birkett, T.J. Bruce and G. Whiteley, *Biofuels, Bioprod. Bioref.*, 2007, **1**, 245-254

[16] F. Deswarte, Ph.D. thesis, University of York, 2006

[17] J. H. Clark, F. E. I. Deswarte and T. J. Farmer, *Biofuel. Bioprod. Bioref.*, 2009, **3**, 72-90

[18] F.E.I. Deswarte, J.H. Clark, J.J.E. Hardy and P.M. Rose, *Green Chem.*, 2006, **8**, 39–42

[19] V.L. Budarin, P.S. Shuttleworth, J.R. Dodson, A.J. Hunt, B. Lanigan, R. Marriott, K.J. Milkowski, A.J. Wilson, S.W. Breeden, J. Fan, E.H.K. Sin and J.H. Clark, *Energy Environ. Sci.*, 2011, **4**, 471-479

[20] J.H. Clark, *J. Chem. Technol. Biot.*, 2007, **82**, 603-609

[21] P.T. Anastas, J.C. Warner and J. Warner, *Green Chemistry: Theory and Practice*, Oxford University Press, Oxford, 2000,

[22] P.T. Anastas and J.C. Warner, *Green Chemistry: Theory and Practice*, Oxford University Press, Oxford, 1st edn., 1998, 29-34

[23] P. Anastas and N. Eghbali, *Chem. Soc. Rev.*, 2010, **39**, 301-312

[24] J.H. Clark, V. Budarin, F.E.I. Deswarte, J.J.E. Hardy, F.M. Kerton, A.J. Hunt, R. Luque, D.J. Macquarrie, K. Milkowski, A. Rodriguez, O. Samuel, S.J. Tavener, R.J. White and A.J. Wilson, *Green Chem.*, 2006, **8**, 853-860

[25] M. Stacey, *Agr. Progress*, 1976, **51**, 69-75

[26] J. Copeland and D. Turley, *National and regional supply/demand balance for aguiltural straw in Great Britain*, The National Non-Food Crops Centre Report, Central Science Laboratory, Sand Hutton, York, 2008

[27] T.Q. Yuan and R.C. Sun, in *Cereal Straw as a Resource for Sustainable Biomaterials and Biofuels*, ed. R.-C. Sun, Elsevier, Oxford, 1st edn., 2010, ch. 1, 1-5
[28] I.M.Morrison, in *Straw Decay and its Effect on Disposal and Utilization*, ed. E. Grossbard, Wiley, Chichester, 1979, 237-245
[29] Agriculture in the United Kingdom, Defra, Dardni, The Scottish Government: Rural and Environment Research and Analysis Directorate and Welsh Assembly Government: The Department for Rural Affairs and Heritage, 2010
[30] R.C.Sun and T.Tomkinson, in *Encyclopedia of Separation Science*, ed. I.D. Wilson, T.R. Adlard, C.F. Poole, and M. Cook, Academic Press, London, 2000, Ap. 1, 4568-4574
[31] Y.W.Han and A.W. Anderson, *Econ. Bot.*, 1974, **28**, 338-344
[32] S. Kim and B.E. Dale, *Biomass Bioenerg.*, 2004, **26**, 361-375
[33] H.-S. Yang, D.-J. Kim and H.-J. Kim, *Bioresource Technol.*, 2003, **86**, 117-121
[34] Z. Xu, Q. Wang, Z. Jiang, X. Yang and Y. Ji, *Biomass Bioenerg.*, 2007, **31**, 162-167
[35] R.A. Gross and B. Kalra, *Science*, 2002, **297**, 803-807
[36] M. Ahmedna, W.E. Marshall and R.M. Rao, *Bioresources Technol.*, 2000, **71**, 113-123
[37] M.M. Mohamed, *J. Colloid Interf. Sci.*, 2004, **272**, 28-34
[38] J.A. Bird, A.J. Eagle, W.R. Horwath, M.W. Hair, E.E. Zilbert and C. Van-Kessel, *Calif. Agr.* , 2002, **56**, 69-75
[39] D.S. Powlson, D.S. Jenkinson, G. Pruden and A.E. Johnston, *J. Sci. Food Agr.*, 1985, **36**, 26-30
[40] L. Hunter and K.A. Houpt, *J. Anim. Sci.*, 1989, **67**, 1986-1991
[41] D.A. Crutchfield, G.A. Wicks and O.C. Burnside, *Weed Sci.*, 1986, **34**, 110-114
[42] M. Bouhicha, F. Aouissi and S. Kenai, *Cement Cconcrete Comp.*, 2005, **27**, 617-621
[43] Y. Chang and H.J. Hudson, *T. Brit. Mycol. Soc.*, 1967, **50**, 649-666
[44] M. Husseien, A.A. Amer, A. El-Maghraby and N. A.Taha, *Int. J. Environ. Sci. Te.*, 2009, **6**, 123-130
[45] N.C. Everall and D.R. Lees, *Water Res.*, 1997, **31**, 614-620
[46] J.T. Martin, *Annu. Rev. Phytopathol.*, 1964, **2**, 81-100
[47] D. Post-Beittenmiller, *Annu. Rev. Plant Biol.*, 1996, **47**, 405-430
[48] S.K. Wiśniewska, J. Nalaskowski, E. Witka-Jeżewska, J. Hupka and J.D. Miller, *Colloid. Surface. B*, 2003, **29**, 131-142
[49] F. Xu, in *Cereal Straw as a Resource for Sustainable Biomaterials and Biofuels*, ed. R.-C. Sun, Elsevier, Oxford, 1st edn., 2010, ch. 2, 9-43
[50] E.C. Reynhardt and M. Riederer, *J. Phys. D: Appl. Phys*, 1991, **24**, 478-486
[51] I. Basson and E.C. Reynhardt, *J. Phys. D. Appl. Phys.*, 1988, **21**, 1429-1433
[52] B.S. Manheim and T.W. Mulroy, *Phytochemistry*, 1978, **17**, 1799-1800
[53] G. Eglinton, R.J. Hamilton, R.A. Raphael and A.G. Gonzalez, *Nature*, 1962, **193**, 739-742
[54] G.A. Herbin and P.A. Robins, *Phytochemistry*, 1968, **7**, 239-255
[55] G. Audisio, A. Rossini, G. Bianchi and P. Avato, *J. High Res. Chromatog.*, 1987, **10**, 594-597
[56] M. Andrew and R.J.Hamilton, *J. Nat. Prod.*, 1982, **45**, 694-697
[57] R.E. Wilkinson and D.G. Cummins, *Crop Sci.*, **21**, 397-400
[58] G. Bianchi, P. Avato, P. Bertorelli and G. Mariani, *Phytochemistry*, 1978, **17**, 999-1001
[59] N.G. Birgitte, *Phytochemistry*, 1975, **14**, 921-929
[60] G. Bianchi, F. Scole, B. Borghi and M. Corbellini, *Phytochemistry*, 1982, **21**, 639-642
[61] S. Irmak, N.T. Dunford and J. Milligan, *Food Chem.*, 2006, **95**, 312-318

[62] Y. Choi, J. Kim, M. Noh, E. Park and K.-P. Yoo, *Korean J. Chem. Eng.*, 1996, **13**, 216-219
[63] A.J. Matas, M. Sanz, amp, x, J. a and A. Heredia, *International Journal of Biological Macromolecules*, 2003, **33**, 31-35
[64] P.E. Kolattukudy, J.S. Buckner and T.Y.J. Liu, *Arch. Biochem. Biophys.*, 1973, **156**, 613-620
[65] K. Mladenova, B. Stoianova-Ivanova and C.M. Camaggi, *Phytochemistry*, 1977, **16**, 269-272
[66] R. Croteau and I.S. Fagerson, *Phytochemistry*, 1971, **10**, 3239-3245
[67] A.P. Tulloch, in *Chemistry and Biochemistry of Natural Waxes*, ed. P.E. Kolattukudy, Elsevier, Amsterdam, 1976, ch. 7, 235-287
[68] B.R. Baum, A.P. Tulloch and L.G. Bailey, *Can. J. Bot.*, 1989, **67**, 3219-3226
[69] Y.Z. Jiang and T. Wang, *J. Am. Oil Chem. Soc.*, 2005, **82**, 439-444
[70] S. Hubert, *Plant Physiol. Bioch.*, 2004, **42**, 465-476
[71] R.A. Demel and B.D. Kruyff, *Biochim. Biophys. Acta*, 1976, **457**, 109–132
[72] L. Dyas and L.J. Goad, *Phytochemistry*, 1993, **34**, 17-29
[73] D. Takaoka, A. Matsuo and S. Hayashi, *Phytochemistry*, 1987, **26**, 429
[74] G. Holmer, R.L. Ory and C.E. Hoy, *Lipids*, 1973, **8**, 227-283
[75] T. Akihisa, K. Yasukawa, H. Oinuma, Y. Kasahara, S. Yamanouchi, M. Takido, K. Kumaki and T. Tamura, *Phytochemistry*, 1996, **43**, 1255-1260
[76] T. Honda, H.J. Finlay and G.W. Gribble, *J. Nat. Prod.*, 1997, **60**, 1174-1177
[77] N. Banno, T. Akihisa, H. Tokuda, K. Yasukawa, H. Higashihara, M. Ukiya, K. Watanabe, Y. Kimura, J.-I. Hasegawa and H. Nishino, *Biosci. Biotech. Bioch.*, 2004, **68**, 85-90
[78] T. Ohmoto, M. Ikuse and S. Natori, *Phytochemistry*, 1970, **9**, 2137-2148
[79] A.A. Millar and L. Kunst, *Plant J.*, 1997, **12**, 121-131
[80] G. Bianchi, in *Waxes: Chemistry, Molecular Biology and Functions*, ed. R.J. Hamilton, The Oily Press, Dundee, 1995, 6, ch. 2, 91-129
[81] J. Browse and J. Phlrogge, *J. Plant Cell*, 1995, **7**, 957-970
[82] L. Kunst and A.L. Samuels, *Prog. Lipid Res.*, 2003, **42**, 51-80
[83] S.J. Wakil, *Biochemistry*, 1989, **28**, 4523-4530
[84] R.S.V.D. Hoeven and J.C. Steffens, *Plant Physiol. Bioch.*, 2000, **122**, 275-282
[85] L. Samuels, L. Kunst and R. Jetter, *Annu. Rev. Plant Biol.*, 2008, **59**, 683-707
[86] A.Hannoufa, J. McNevin and B. Lemieux, *Phytochemistry*, 1993, **33**, 851-855
[87] R.C. Sun, D. Salisbury and J. Tomkinson, *Bioresour. Technol.*, 2003, **88**, 95-101
[88] Y. Athukorala, G. Mazza and B.D. Oomah, *Eur. J. Lipid Sci.Tech.*, 2009, **111**, 705-714
[89] R.C. Sun and X.F.Sun, *Sep. Sci. Technol.*, 2001, **36**, 3027-3048
[90] Y. Athukorala and G. Mazza, *Ind. Crop. Prod.*, 2010, **31**, 550-556
[91] G. Bianchi and M. Corbellini, *Phytochemistry*, 1977, **16**, 943-945
[92] L.L. Jackson, *Phytochemistry*, 1971, **10**, 487-490
[93] J.D. Mikkelsen and P. von Wettstein-Knowles, *Arch. Biochem. Biophys.*, 1978, **188**, 172-181
[94] P. Wettstein-Knowles, *Planta*, 1972, **106**, 113-130
[95] V. Piironen, D.G. Lindsay, T.A. Miettinen, J. Toivo and A.-M. Lampi, *Journal of the Science of Food and Agriculture*, 2000, **80**, 939-966
[96] P. Benveniste, *Ann. Rev. Plant Physiol.*, 1986, **37**,
[97] A.P. Tulloch and R.O. Weenink, *Can. J. Chemistry*, 1969, **47**, 3119-3126
[98] A.P. Tulloch and L.L. Hoffman, *Phytochemistry*, 1971, **10**, 871-876
[99] A.P. Tulloch and L.L. Hoffman, *Phytochemistry*, 1973, **12**, 2217-2223
[100] A. Tulloch and L. Hoffman, *Lipids*, 1974, **9**, 219-219

[101] G. Bianchi, P. Avato, P. Bertorelli and G. Mariani, *Phytochemistry*, 1978, **17**, 999-1001
[102] G. Bianchi, E. Lupotto and S. Russo, *Cellular and Molecular Life Sciences*, 1979, **35**, 1417-1417
[103] G. Bianchi, E. Lupotto, B. Borghi and M. Corbellini, *Planta*, 1980, **148**, 328-331
[104] P. Avato, G. Bianchi and G. Mariani, *Phytochemistry*, 1984, **23**, 2843-2846
[105] G. Bianchi and M.L. Figini, *J. Agr. Food Chem.*, 1986, **34**, 429-433
[106] *US. Pat.* 4260639, 1981
[107] R. Vollbrecht, *Chem. Ind.-London*, 1982, **6**, 397-399
[108] B. Xiao, X.F. Sun and R.C. Sun, *J. Wood Chem. Technol.*, 2001, **21**, 397-411
[109] X.F. Sun and R.C. Sun, *Wood Fiber Sci.*, 2001, **33**, 386-394
[110] K. Hwang, S. Cuppett, C. Weller and M. Hanna, *Journal of the American Oil Chemists' Society*, 2002, **79**, 521-527
[111] R.C. Sun and J. Tompkinson, *J. Wood Sci.*, 2003, **49**, 0047-0052
[112] W.H. Morrison Iii, R. Holser and D.E. Akin, *Ind. Crop. Prod.*, 2006, **24**, 119-122
[113] R.J. Hamilton, in *Waxes: Chemistry, Molecular Biology and Functions*, ed. R.J. Hamilton, The Oily Press, Dundee, 1995, 6, ch. 7, 258-310
[114] A. Tulloch, *Lipids*, 1970, **5**, 247-258
[115] E.W. Clark, in *The Lanolin Book*, ed. P. Beierdorf, BDF, Hamburg, 1st edn., 1999, ch. 1, 15-50
[116] J. Jacob, in *The Lanolin Book*, ed. P. Beierdorf, BDF, Hamburg, 1st edn., 1999, ch. 2, 55-84
[117] W. Cannon and G. Atkinson, Croda International Plc., personal communication, 2010-2011
[118] E. Taube, *Econ. Bot.*, 1952, **6**, 379-401
[119] W. Hodge and H. Sineath, *Econ. Bot.*, 1956, **10**, 134-154
[120] *US. Pat.* 2113433, 1938
[121] *US. Pat.* 5154919, 1992
[122] *US. Pat.* 2840509, 1958
[123] *US. Pat.* 2967781, 1961
[124] *Global wax industry 2010: Market analysis and opportunities*, Y635A, The Kline Group, 2010
[125] *US. Pat.* 4873078, 1989
[126] Y. Marcus, *The Properties of Solvents*, Wiley, Chichester, 1st edn., 1998, 1-32
[127] D.J. Adams, P.J. Dyson and S.J. Tavener, *Chemistry In Alternative Reaction Media*, Wiley, Chichester, 1st edn., 2004, 1-30
[128] C. Reichardt, *Solvents and solvent Effects in Organic Chemistry*, Wiley-VCH, Weinheim, 3rd edn., 2003, 5-46
[129] J.M. DeSimone, *Science*, 2002, **297**, 799-803
[130] L.G.S. Brooker, G.H. Keyes and D.W. Heseltine, *J. Am. Chem. Soc.*, 1951, **73**, 5350-5356
[131] E.M. Kosower, *J. Am. Chem. Soc.*, 1958, **80**, 3253-3260
[132] C. Reichardt, *Chem. Soc. Rev.*, 1992, **21**, 147-153
[133] R.W. Taft and M.J. Kamlet, *J. Am. Chem. Soc.*, **98**, 2886-2894
[134] M.J. Kamlet and R.W. Taft, *J. Am. Chem. Soc.*, 1976, **98**, 377-383
[135] M.J. Kamlet, J.L. Abboud and R.W. Taft, *J. Am. Chem. Soc.*, 1977, **99**, 6027-6038
[136] M.J. Kamlet, J.L.M. Abboud, M.H. Abraham and R.W. Taft, *J. Org. Chem.*, 1983, **48**, 2877-2887
[137] Y. Marcus, *Chem. Soc. Rev.*, 1993, **22**, 409-416
[138] P.G. Jessop, D.A. Jessop, D. Fu and L. Phan, *Green Chem.*, 2012, **14**, 1245-1259

[139] A.D. Curzons, D.C. Constable and V.L. Cunningham, *Clean Products and Processes*, 1999, **1**, 82-90
[140] C. Jiménez-González, A.D. Curzons, D.J.C. Constable and V.L. Cunningham, *Clean Technol. Envir.*, 2004, **7**, 42-50
[141] L. Lynd, R. Elamder and C. Wyman, *Appl. Biochem. Biotech.*, 1996, **57-58**, 741-761
[142] N. Mosier, C. Wyman, B. Dale, R. Elander, Y.Y. Lee, M. Holtzapple and M. Ladisch, *Bioresource Technol.*, 2005, **96**, 673-686
[143] D. Schell and C. Harwood, *Appl. Biochem. Biotech.*, 1994, **45-46**, 159-168
[144] W.E. Kaar, C.V. Gutierrez and C.M. Kinoshita, *Biomass Bioenerg.*, 1998, **14**, 277-287
[145] G. Bonn, R. Concin and O. Bobleter, *Wood Sci. Technol.*, 1983, **17**, 195-202
[146] A. Zamani, L. Edebo, B. Sjöström and M.J. Taherzadeh, *Biomacromolecules*, 2007, **8**, 3786-3790
[147] S. Eero, *Biomass Bioenerg.*, 1991, **1**, 61-64
[148] T.H. Kim, J.S. Kim, C. Sunwoo and Y.Y. Lee, *Bioresource Technol.*, 2003, **90**, 39-47
[149] A.V. Pethkar and K.M. Paknikar, *J. Biotechnol.*, 1998, **63**, 121-136
[150] W.E. Kaar and M.T. Holtzapple, *Biomass Bioenerg.*, 2000, **18**, 189-199
[151] A.S. Schmidt and A.B. Thomsen, *Bioresource Technol.*, 1998, **64**, 139-151
[152] R.C. Sun and X.F. Sun, *Ind. Crop. Prod.*, 2001, **14**, 51-64
[153] M.J. Fishwick and A.J. Wright, *Phytochemistry*, 1977, **16**, 1507-1510
[154] G. Panfili, A. Fratianni and M. Irano, *J. Agr. Food Chem.*, 2004, **52**, 6373-6377
[155] L. Gomez, E. Rubio and M. Augé, *J. Sci. Food Agr.*, 2002, **82**, 360-369
[156] V. Fabos, G. Koczo, H. Mehdi, L. Boda and I.T. Horvath, *Energy Environ. Sci.*, 2009, **2**, 767-769
[157] K. Alfonsi, J. Colberg, P.J. Dunn, T. Fevig, S. Jennings, T.A. Johnson, H.P. Kleine, C. Knight, M.A. Nagy, D.A. Perry and M. Stefaniak, *Green Chem.*, 2008, **10**, 31-36
[158] L. Crowhurst, P.R. Mawdsley, J.M. Perez-Arlandis, P.A. Salter and T. Welton, *Phys. Chem. Chem. Phys.*, 2003, **5**,
[159] R.P. Swatloski, S.K. Spear, J.D. Holbrey and R.D. Rogers, *J. Am. Chem. Soc.*, 2002, **124**, 4974-4975
[160] H.B. Klinke, B.K. Ahring, A.S. Schmidt and A.B. Thomsen, *Bioresource Technol.*, 2002, **82**, 15-26
[161] E. Kovats, *Helv. Chim. Acta.*, 1958, **41**, 1915-1932
[162] H.V.D. Dool and R.D. Kratz, *J. Chromatogr.*, 1963, **11**, 463-471
[163] U.F.D. Silva, E.L. Borba, J. Semir and A.J. Marsaioli, *Phytochemistry*, 1999, **50**, 31-34
[164] V.S. Mahalwal and M. Ali, *Flavour Fragr. J.*, 2003, **18**, 73-76
[165] M.G.B. Zoghbi, E.H.A. Andrade and J.G.S. Maia, *Flavour Fragr. J.*, 1999, **14**, 411-414
[166] J.A. Pino, R. Marbot, A. Rosado and C. Vázquez, *Flavour Fragr. J.*, 2003, **18**, 271-274
[167] K. Hill, *Pure Appl. Chem.*, 2000, **72**, 1255-1264
[168] N.A. Ruston, *J. Am. Oil Chem. Soc.*, 1952, **29**, 495-498
[169] G.A. Herbin and P.A. Robins, *Phytochemistry*, 1969, **8**, 1985-1998
[170] S.P. Srivastava, J. Handoo, K.M. Agrawal and G.C. Joshi, *J. Phys. Chem. Solids*, 1993, **54**, 639-670
[171] F.C. Abdalla, G.R. Jones, E.D. Morgan and C.d. Cruz-Landim, *Genet. Mol. Res.*, 2003, **2**, 191-199
[172] R.W. Howard and G.J. Blomquist, *Annu. Rev. Entomol.*, 2005, **50**, 371-393
[173] F.R. Dani, *Ann. Zool. Fennici*, 2006, **43**, 500-514
[174] H. Kasinger, J. Denzinger and B. Bauer, *Commun. SIWN* 2008, **4**, 133-139

[175] Y. Zheng and E. White, Retention Data. NIST Mass Spectrometry Data Centre, 2008
[176] J.C. Taylor, L. Rapport and G.B. Lockwood, *Nutrition*, 2003, **19**, 192-195
[177] C.P.F. Marinangeli, P.J.H. Jones, A.N. Kassis and M.N.A. Eskin, *Crit. Rev. Food Sci.*, 2010, **50**, 259-267
[178] J.L. Hargrove, P. Greenspan and D.K. Hartle, *Exp. Biol. Med.*, 2004, **3**, 215–226
[179] A. Pollard, A.C. Chibnall and S.H. Piper, *Biochem. J.* , **27**, 1889
[180] S. Irmak and N.T. Dunford, *J. Agric. Food Chem.*, 2005, **53**, 5583-5586
[181] D.S. Mottram, *Food Chem.*, 1998, **62**, 415-424
[182] E.R. Gunawan, M. Basri, M.B.A. Rahman, A.B. Salleh and R.N.Z.A. Rahman, *Enzyme Microb. Tech.*, 2005, **37**, 739-744
[183] P. von Wettstein-Knowles and A. Netting, *Lipids*, 1976, **11**, 478-484
[184] H. Schaller, *Progr. Lipid Res.*, 2003, **42**, 163-175
[185] J. Galley and B.D. Kruf., *FEBS Lett.*, 1982, **143**, 133-143
[186] N.L. Ruibal-Mendieta, R. Rozenberg, D.L. Delacroix, G. Petitjean, A. Dekeyser, C. Baccelli, C. Marques, N.M. Delzenne, M. Meurens, J.-L. Habib-Jiwan and J. Quetin-Leclercq, *J. Agr. Food Chem.*, 2004, **52**, 4802-4807
[187] L.W. Thomson, A.W. Henry and S. Zalik, *Phytochemistry*, 1986, **25**, 2505-2508
[188] R.E. Ostlund, *Annu. Rev. Nutr.*, 2002, **22**, 533-549
[189] A.M. Lees, H.Y.I. Mok, R.S. Lees, M.A. McCluskey and S.M. Grundy, *Atherosclerosis*, 1977, **28**, 325-338
[190] D.K. Singh, L. Li and T.D. Porter, *J. Pharmacol. Exp. Ther.*, 2006, **318**, 1020-1026
[191] T. Heinemann, G.A. Kullak-Ublick, B. Pietruck and K. von Bergmann, *Eur. J. Clin. Pharmacol.*, 1991, **40**, S59-S63
[192] F.Y. Ntanios and P.J. Jones, *Biochim. Biophys. Acta*, 1998, **1390**, 237-244
[193] *EU Pat.* 1108364, 2001
[194] *EU Pat.* 1121928, 2001
[195] Y.W. Wang, P.J.H. Jones, I. Pischel and C. Fairow, *Lipids*, 2003, **38**, 165-170
[196] J. Snyder, J. King, S. Taylor and A. Neese, *J. Am. Oil Chem. Soc.*, 1999, **76**, 717-721
[197] R.H. Shapiro and C. Djerassi, *J. Am. Chem. Soc.*, 1964, **86**, 2825-2832
[198] Retention data from estimated non-polar retention index NIST Mass Spectrometry library, 2008
[199] L.S. Keiser, I.G. Yazlovetsky and V.N. Retunsky, *J. Anal. Chem.*, 1986, **41**, 1166-1172
[200] A.C. Alberts, T. R. Sharp, D. I. Werner and P.J. Weldon, *J. Chem. Ecol.*, 1992, **18**, 703-712
[201] D.H.S. Horn, Z.H. Kranz and J.A. Lamberton, *Aust. J. Chem.*, 1964, **17**, 464 - 476
[202] Y. Lin, R.D. Brauer, K.E. Laintz and C.M. Wai, *Anal. Chem.*, 1993, **65**, 2549-2551
[203] B. Bhushan, Y.C. Jung, A. Niemietz and K. Koch, *Langmuir*, 2009, **25**, 1659-1666
[204] M. Masur, H.-F. Grützmacher, H. Münster and H. Budzikiewicz, *Org. Mass Spectrom.*, 1987, **22**, 493-500
[205] A.P. Tulloch and L.R. Hogge, *J. Chromatogr.*, 1978, **157**, 291-296
[206] A.P. Tulloch, *Phytochemistry*, 1973, **12**, 2225-2232
[207] G. Bianchi, *Genetica Agraria*, 1985, **39**, 471-486
[208] J.T. Scanlon and D.E. Willis, *J. Chromatogr. Sci.*, 1985, **23**, 333-340
[209] Y.F. Chen, N.T. Dunford, J. Edwards, B. Carver and C. Goad, *J. Sci. Food Agr.*, 2009, **89**, 310-314
[210] W.D. Guenzi and T.M. McCalla, *Agron. J.*, **58**, 303-304
[211] K.-H. Kim, R. Tsao, R. Yang and S.W. Cui, *Food Chem.*, 2006, **95**, 466-473
[212] L. Yu, S. Haley, J. Perret, M. Harris, J. Wilson and M. Qian, *J. Agr. Food Chem.*, 2002, **50**, 1619-1624

[213] M.F. Andreasen, P.A. Kroon, G. Williamson and G.-C. M.-T., *Free Radical Bio. Med.*, 2001, **31**, 304-314
[214] L. Yu, J. Perret, M. Harris, J. Wilson and S. Haley, *J. Agr. Food Chem.* , 2003, **51**, 1566-1570
[215] H.J.C. Dasneves and E.M.M. Gaspar, *Hrc-J. High Res. Chrom.*, 1990, **13**, 550-554
[216] M. Nazzaro-Porro, S. Passi, M. Picardo, A. Breathnach, R. Clayton and G. Zina, *Brit. J. Dermatol.*, 1983, **109**, 45-48
[217] A. Heidbreder, R. Höfer, R. Grützmacher, A. Westfechtel and C.W. Blewett, *FETT-Lipid.*, 1999, **101**, 418-424
[218] *EP Pat.* 0508591, 1992
[219] G. Castaño, R. Mas, L. Fernández, J. Illnait, R. Gámez and E. Alvarez, *Int. J. Clin. Pharmacol. Res.*, 2001, **21**, 43-57
[220] R.D. Hagenmaier, *Proc. Fla. State Hort. Soc.*, 1998, **111**, 251-255
[221] Directive 2003/15/EC of The European Parliament and of the Council of 27 February 2003, 2003
[222] Commission Directive 2010/59/EU of 26 August 2010, 2010
[223] Regulation (EC) No 1223/2009 of The European Parliament and of the Council of 30 November 2009, 2009
[224] Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008, 2008
[225] Directive 2009/32/EC of The European Parliament and of the Council of 23 April 2009, 2009
[226] 1993
[227] J.P. Friedrich, G.R. List and A.R.S. Usda, *J. Agric. Food Chem.*, 1982, **30**, 192-193
[228] H.H. Schumburg and P.S. Spencer, *Brain*, 1976, **99**, 183-192
[229] P.S. Spencer and H.H. Schumburg, *Proc. R. Soc. Med.*, 1977, **70**, 37-38
[230] F.M. Orr, J.P. Heller, J.T. Taber and R.J. Card, *Chemtech*, 1983, **13**, 482-487
[231] Y. Ikushima, N. Saito, M. Arai and K. Arai, *Bull. Chem. Soc. Jpn*, 1991, **64**, 2224-2229
[232] B. Metz, O. Davidson, H.d. Coninck, M. Loos and L.M. (Eds.), *Carbon dioxide capture and storage*, IPCC special report 2005, IPCC, Cambridge, 2005
[233] S. Kim and B.E. Dale, *Biomass Bioenerg.*, 2005, **29**, 426-439
[234] J. Mathews, *Energ. Policy*, 2007, **35**, 4247-4259
[235] A.J. Hunt, E.H.K. Sin, R. Marriott and J.H. Clark, *ChemSusChem.*, 2010, **3**, 306-322
[236] R. Marriott, University of York, personal communication, 2010-2011
[237] Council Directive 93/35/EEC of 14 June 1993 1993
[238] F.M.Kerton, *Alternative Solvents for Green Chemistry*, RSC Publishing, Cambridge, 1st edn., 2009, 60-70
[239] Y. Arai, T.Sako and Y.Takebayashi, *Supercritical Fluids: Molecular Interactions, Physical Properties and New Applications*, Springer, New York, 1st edn., 2002, 1-4
[240] A.A.Clifford, *Fundamentals of Supercritical Fluids*, Oxford University Press, Oxford, 1998, 1-5
[241] P.G.Jessop and W.Leitner, *Chemical Synthesis using Supercritical Fluids*, Wiley-VCH, Weinheim, 1st edn., 1999, 1-5
[242] Natex Prozesstechnologie, http://www.natex.at, November 2009
[243] C.Domingez, E.Jover, J.M.Bayona and P.Erra, *Anal. Chim. Acta.*, 2003, **477**, 233-242
[244] J.P. Friedrich, G.R. List and A.J. Heakin, *J. Am. Oil Chem.Soc.*, 1982, **59**, 288-292
[245] E. Stahl, E. Schuetz and H.K. Mangold, *J. Agr. Food Chem.*, 1980, **28**, 1153-1157
[246] L.H.C.Carlson, R.A.F. Machado, C.B.Spricigo, L.K.Pereira and A.Bolzan, *J. Supercrit. Fluid.*, 2001, **21**, 33-39

[247] A. Garcia, A. deLucas, J. Rincon, A. Alvarez, I. Gracia and M.A. Garcia, *J. Am. Oil Chem. Soc.*, 1996, **73**, 1127-1131
[248] G.Gonzalez, *Anal. Chim. Acta.*, 1998, **360**, 227-241
[249] N.Patouillard and R.Kahleyss, Evonik Industries, personal communication, 2010
[250] A. Brachet, P. Christen, J.-Y. Gauvrit, R. Longeray, P. Lantéri and J.-L. Veuthey, *J. Biochem. Bioph. Meth.*, 2000, **43**, 353-366
[251] L. Ferreira de França, G. Reber, M.A.A. Meireles, N.T. Machado and G. Brunner, *J Supercrit. Fluid.*, 1999, **14**, 247-256
[252] S.Mitra and N.K.Wilson, *J. Chromatogr. sci.*, 1991, **29**, 305-309
[253] I.J. Barnabas, J.R. Dean, W.R. Tomlinson and S.P. Owen, *Anal. Chem.*, 1995, **67**, 2064-2069
[254] F.W. Jones, B.O. Bateup, D.R. Dixon and S.R. Gray, *J. Supercrit. Fluid.*, 1997, **10**, 105-111
[255] R. Alzaga, E. Pascual, P. Erra and J.M. Bayona, *Anal. Chim. Acta*, 1999, **381**, 39-48
[256] M.Cynarowicz-Provost, J.W.King, W.N.Marmer and P.Magidman, *J. Am. Oil Chem. Soc.*, 1994, **71**, 223-226
[257] F. Favati, J. King and M. Mazzanti, *J. Am. Oil Chem. Soc.*, 1991, **68**, 422-427
[258] M. Fattori, N. Bulley and A. Meisen, *J. Am. Oil Chem. Soc.*, 1988, **65**, 968-974
[259] I. Papamichail, V. Louli and K. Magoulas, *J. Supercrit. Fluid.*, 2000, **18**, 213-226
[260] G.Guihard, University of York, Unpublished work, 2011
[261] J. Chrastil, *J. Phys. Chem.*, 1982, **86**, 3016-3021
[262] J.M. Delvalle and J.M. Aguilera, *Ind. Eng. Chem. Res.*, 1988, **27**, 1551-1553
[263] J. Méndez-Santiago and A.S. Teja, *Fluid Phase Equilib.*, 1999, **158-160**, 501-510
[264] K.D. Bartle, A.A. Clifford, S.A. Jafar and G.F. Shilstone, *J. Phys. Chem. Ref. Data*, 1991, **20**, 713-756
[265] Y. Adachi and B.C.Y. Lu, *Fluid Phase Equilibria*, 1983, **14**, 147-156
[266] J.A. Hyatt, *J. Org. Chem.*, 1984, **49**, 5097-5101
[267] F.P. Lucien and N.R. Foster, *J. Supercrit. Fluid.*, 2000, **17**, 111-134
[268] Y. Koga, Y. Iwai, Y. Hata, M. Yamamoto and Y. Arai, *Fluid Phase Equilibr.*, 1996, **125**, 115-128
[269] P. Maheshwari, Z.L. Nikolov, T.M. White and R. Hartel, *J. Am. Oil Chem. Soc.*, 1992, **69**, 1069-1076
[270] T. Funazukuri, S. Hachisu and N. Wakao, *Ind. Eng. Chem. Res.*, 1991, **30**, 1323-1329
[271] D.K. Dandge, J.P. Heller and K.V. Wilson, *Ind. Eng. Chem. Prod. RD*, 1985, **24**, 162-166
[272] T. Chartier, E. Delhomme, J.F. Baumard, P. Marteau, P.Subra and R. Tufeu, *Ind. Eng. Chem. Res.*, 1999, **38**, 1904-1910
[273] Z. Huang, S. Kawi and Y.C. Chiew, *J. Supercrit. Fluid.*, 2004, **30**, 25-39
[274] S. Patel, D.R. Nelson and A.G. Gibbs, *J. Insect Sci.*, 2001, **1**, 1-18
[275] W.H. Gibson, *Chem. Eng. Res. Des.*, 1931, **9a**, 30-35
[276] *US Pat.* 2009522, 1935
[277] G. Nuissier, P. Bourgeois, M. Grignon-Dubois, P. Pardon and M.H. Lescure, *Phytochemistry*, 2002, **61**, 721-726
[278] Y. Sun and J. Cheng, *Bioresource Technol.*, 2002, **83**, 1-11
[279] I. Lewandowski, J.C. Clifton-Brown, J.M.O. Scurlock and W. Huisman, *Biomass Bioenerg.*, 2000, **19**, 209-227
[280] G. Antolin, F.V. Tinaut, Y. Briceno, V. Castano, C. Perez and A.I. Ramirez, *Bioresource Technol.*, 2002, **83**, 111-114
[281] R. Purdy, *J. Am. Oil Chem. Soc.*, 1986, **63**, 1062-1066
[282] T. Itoh, T. Tamura and T. Matsumoto, *J. Am. Oil Chem. Soc.*, 1973, **50**, 122-125
[283] P. Wettstein-Knowles and A. Netting, *Carlsberg Res. Commun.*, 1976, **41**, 225-235

[284] G. Verardo, E. Pagani, P. Geatti and P. Martinuzzi, *Anal. Bioanal. Chem.*, 2003, **376**, 659-667
[285] P.J. Holloway, C.E. Jeffree and E.A. Baker, *Phytochemistry*, 1976, **15**, 1768-1770
[286] R.A. Franich, L.G. Wells and P.T. Holland, *Phytochemistry*, 1978, **17**, 1617-1623
[287] P.E. Kolattukudy and T.-Y.J. Liu, *Biochem. Bioph. Res. Co.*, 1970, **41**, 1369-1374
[288] P. Khristova, N. Yossifov, S. Gabir, I. Glavche and Z. Osman, *Cellul. Chem. Technol.*, 1998, **32**, 327-337
[289] *EP Pat.* 0508591, 1961
[290] T.C.S. Kanya, L.J. Rao and M.C.S. Sastry, *Food Chem.*, 2007, **101**, 1552-1557
[291] D.E. Purcell, G.J. Leonard, M.G. O'Shea and S. Kokot, *Chemometr. Intell. Lab.*, 2005, **76**, 135-147
[292] M.I. Whitecross and D.J. Armstrong, *Aust. J. Bot.*, 1972, **20**, 87-95
[293] T. Shepard, G.W. Robertson, D.W. Griffiths, A.N.E. Birch and G. Duncan, *Phytochemistry*, 1995, **40**, 407-417
[294] M. Kock, *J. Am. Oil Chem. Soc.*, 1983, **60**, 198-202
[295] Z. Shen, M.V. Palmer, S.S.T. Ting and R.J. Fairclough, *J. Agr. Food Chem.*, 1996, **44**, 3033-3039
[296] K.A. Evelein, R.G. Moore and R.A. Heidemann, *Ind. Eng. Chem.*, 1976, **15**, 423-428
[297] V. Hentschel, K. Kranl, J. Hollmann, M.G. Lindhauer, V. Böhm and R. Bitsch, *J. Agr. Food Chem.*, 2002, **50**, 6663-6668
[298] M.T. Escribano-Bailón, C. Santos-Buelga and J.C. Rivas-Gonzalo, *J. Chromatogr. A*, 2004, **1054**, 129-141
[299] N. Nicolaid and H.C. Fu, *Lipids*, 1970, **5**, 299-307
[300] F.J. Ramirez, P. Luque, A. Heredia and M.J. Bukovac, *Biopolymers*, 1992, **32**, 1425-1429
[301] S. Merk, A. Blume and M. Riederer, *Planta*, 1998, **204**, 44-53
[302] H. Schulz and M. Baranska, *Vib. Spectrosc.*, 2007, **43**, 13-25
[303] Y. Ozaki, R. Cho, K. Ikegawa, S. Muraishi and K. Kawauchi, *Appl. Spectrosc.*, 1992, 1503-1507
[304] J. Dubois, F.R. vandeVoort, J. Sedman, A.A. Ismail and H.R. Ramaswamy, *J. Am.Oil Chem. Soc.*, 1996, **73**, 787-794
[305] R.G. Sinclair, A.F. McKay, G.S. Myers and R.N. Jones, *J. Am. Chem. Soc.*, 1952, **74**, 2578-2585
[306] M.D. Guillen and N. Cabo, *J. Am. Oil Chem. Soc.*, 1997, **74**, 1281-1286
[307] J.R. Dodson, Ph.D. thesis, University of York, 2011
[308] V. Vrkoslav, R. Míková and J. Cvačka, *J. Mass Spectrom.*, 2009, **44**, 101-110
[309] G. Kühn, S. Weidner, U. Just and G. Hohner, *J. Chromatogr. A*, 1996, **732**, 111-117
[310] J. Cvačka and A. Svatoš, *Rapid Commun. Mass Sp.*, 2003, **17**, 2203-2207
[311] J. Cvačka, P. Jiroš, J. Šobotník, R. Hanus and A. Svatoš, *J. Chem. Ecol.*, 2006, **32**, 409-434
[312] R. Congiu, D. Falconieri, B. Marongiu, A. Piras and S. Porcedda, *Flavour Fragr. J.*, 2002, **17**, 239-244
[313] N. Aligiannis, E. Kalpoutzakis, I.B. Chinou, S. Mitaku, E. Gikas and A. Tsarbopoulos, *J. Agric. Food Chem.*, 2001, **49**, 811-815
[314] K.E. Espelie, B.B. Dean and P.E. Kolattukudy, *Plant Physiol.*, 1979, **64**, 1089-1093
[315] K.E. Espelie and P.E. Kolattukudy, *Plant Sci. Lett.*, 1979, **15**, 225-230
[316] *US Pat.* 5460808, 1995
[317] *US Pat.* 5948393, 1999
[318] K. Nakasone, K. Shiokawa, Y. Urabe and N. Nemoto, *J. Phys. Chem. B*, 2000, **104**, 7483-7489
[319] D.H. Bonsor and D. Bloor, *J. Mater. Sci.*, 1977, **12**, 1559-1564

[320] A.G. Gibbs, *Comp. Biochem. Phys. B*, 1995 **112**, 667-672
[321] M.E.Stewart and D.T.Downing, *Adv. Lipid Res.*, 1991, **24**, 263-302
[322] *US Pat.* 2736667, 1956
[323] *US Pat.* 2498201, 1947
[324] G. Atkinson, Croda International Plc., personal communication, 2011
[325] *US Pat.* 2795595, 1957
[326] L. Tobares, C. Guzmán and D. Maestri, *Eur. J. Lipid Sci.Tech.*, 2003, **105**, 749-753
[327] *US Pat.* 1777766, 1930
[328] *US Pat.* 4154750, 1979
[329] *US Pat.* 3489690, 1970
[330] I. Cuthbertson, Oakbank Products, personal communication, 2010
[331] U. Schuchardt, R. Sercheli and R.M. Vargas, *J. Braz. Chem. Soc.*, 1998, **9**, 199-210
[332] B.M. Folmer, *Adv. Colloid Interface Sci.*, 2003, **103**, 99-119
[333] B.J. Hall and J.S. Brodbelt, *J. Am. Soc. Mass Spectrom.*, 1999, **10**, 402-413
[334] G. Brunner, *J. Food Eng.*, 2005, **67**, 21-33
[335] F.W. Jones, *J. Agric. Food Chem.*, 1996, **44**, 3197-3201