Supercritical CO_2 extraction of waxes as part of a holistic biorefinery

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Abstract

The scope of this project was to utilise supercritical carbon dioxide ($scCO_2$) extraction technology as a pre-treatment step in a holistic C_4 biorefinery in order to extract added value metabolites from C_4 agro-residues. The lipid profile of three different types of C_4 biomass agro-residues namely miscanthus, maize and sugarcane was studied. Wax extractions with organic solvents and $scCO_2$ were conducted on different parts of the plant. A diverse range of hydrophobic molecules were identified including long-chain fatty acids, *n*-policosanols, fatty aldehydes, *n*-alkanes, wax esters, sterols, steroid ketones and triterpenoids. The extracts exhibited significant differences in melting temperatures, highlighting the possibility for utilisation in different applications. The advantages of $scCO_2$ extraction over conventional organic extraction are clearly demonstrated in this work. Herein, the first reported fractionation of maize stover wax gave rise to three waxy fractions. Fraction A consisted of high molecular weight compounds while Fraction B was predominantly phytosterols. Fraction C had the lowest melting profile and the wax was tested as a renewable antifoaming agent. Fraction C successfully reduced foam and had no negative effects on the detergent performance.

The extraction of wax from C_4 biomass only utilises around 1% of the total biomass. In order to have a systemic view of a C_4 biomass processing scenario where $scCO_2$ extraction is integrated into a biorefinery, the effects of $scCO_2$ extraction on the downstream processing of maize stover and miscanthus were studied. In fermentation of maize stover for surfactant production, results show that there was a higher glucose consumption (19%) and greater growth (18%) for the $scCO_2$-extracted maize stover when compared to non-treated maize stover. In fermentation experiments for ethanol production a 40% increase in overall ethanol production for the $scCO_2$-extracted maize stover was obtained. Saccharification results on miscanthus leaves showed a 22% increase in sugar release for $scCO_2$ extracts.

Finally, this work cannot be developed further unless an economic evaluation of the manufacturing process is done. This is the first time an economic assessment for the $scCO_2$ extraction of waxes from biomass has been carried out. The cost for miscanthus and maize stover wax extraction was found to be €148/kg of wax and €88.19/kg of wax respectively. If the biomass was pelletised, leaves were solely taken into consideration (4 times the wax yield) and the biomass was combusted following the extraction, then the cost of the wax could be reduced to as much as €10.43/kg of wax for miscanthus and €10.47/kg of wax for maize.

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Declaration

Some of the results of this thesis were obtained by, or in collaboration with other workers, who are fully acknowledged in the text. All other results are the work of the author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

Thomas Michael Attard

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Collaborator	Work conducted
Processum	Hydrolysis and fermentation data (Chapter 3).
Ecover	Wax lab-foaming experiments and fermentation data (Chapter 3).
Dr. Richard Gammons	Miscanthus saccharification data (Chapter 4).

Chapter 1

Introduction

1 Introduction

1.1 Aim of the Work

The preliminary scope of this work was to study the metabolite profile of three different types of C_4 biomass waste: namely miscanthus, maize and sugarcane, in order to identify and extract high-quality compounds using an environmentally benign solvent which would potentially add value to this waste biomass.

This work can be principally divided into four main themes:

Extraction of lipids from various types of C_4 biomass: The waxy constituents from sugarcane, miscanthus and maize were extracted using conventional organic solvents as well as supercritical carbon dioxide. The lipophilic extractives from different parts of the plant were characterised, quantified and potential applications identified. Comparisons between the conventional organic solvent-extracted and supercritical-extracted waxes were also made.

Optimisation of supercritical extraction and fractionation of waxy constituents: The supercritical extraction of waxes was optimised by means of a 2x2 factorial experimental design whereby temperature and pressure were varied to see the effect this has on the % yield of wax extracted. Furthermore, supercritical fractionation of waxes from maize stover was carried out on a semi-pilot scale using a number of fractional separators in order to obtain waxy fractions having different textures, melting points and lipid profiles. Application-testing on one of the maize stover wax fractions, as a defoaming agent, was also carried out.

Economic assessment of the supercritical extraction of waxes: In order to see whether supercritical extraction is an economically viable process, it is important to estimate the extraction costs of waxes from C_4 biomass. Therefore, an economic study was carried out whereby the costs associated with the industrial supercritical extraction of waxes from miscanthus and maize stover were estimated.

Effects of supercritical extraction on downstream processing of biomass in a holistic biorefinery: Supercritical extraction of waxes from C_4 plants should be the first stage of an integrated biorefinery. Therefore, the effects of supercritical extraction on the downstream processing of the biomass were investigated.

1.2 Green Chemistry

For a number of decades, sustainable development has been a vital worldwide issue. In 1987, the Brundtland Commission (United Nations Commission on Environment and Development) defined sustainable development as:

> '*...to make development sustainable to ensure that it meets the needs of the present without compromising the ability of future generations to meet their own needs.* '[1]

From a chemical and energy point of view, two key aspects arise from sustainable development:

(i) How quickly can fossil fuels be consumed by the current generation?

(ii) How much waste can be introduced into the environment on a sustainable basis?

There is a natural capacity that the Earth has to deal with pollution and waste generated by society and unsustainability is achieved when this capacity is exceeded.[1, 2] The amount of fossil feedstocks available is drastically declining. Throughout the 20th century, there was a massive surge in the economies of developed countries, accelerated by the abundance of cheap petroleum feedstocks that provided energy and resources to a number of industries. Fossil fuels were consumed at alarmingly high rates without sparing a thought for future generations. Recently, a boom in the manufacturing industries in developing countries, especially in Asia, has further increased the stress and reliance on the ever-declining fossil fuels.[3]

In order to address this issue, the term *Green Chemistry* was coined by the United States Environmental Protection Agency (EPA) in the beginning of the early 1990s:

> '*To promote innovative chemical technologies that reduce or eliminate the use or generation of hazardous substances in the design, manufacture and use of chemical products.* '[4]

Green Chemistry is not a new form of chemistry but a philosophy, whereby chemistry and engineering should be practiced in a sustainable way. In 1998, Paul Anastas and John Warner set up the 12 principles of Green Chemistry shown below (Figure 1-1):

1. It is better to prevent waste than to treat or clean up waste after it is formed.
2. Synthetic methods should be designed to maximise the incorporation of all materials used in the process into the final product.
3. 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. Chemical products should be designed to preserve efficacy of function while reducing toxicity.
5. The use of auxiliary substances (e.g. solvents, separation agents, etc.) should be made unnecessary wherever possible and, innocuous when used.
6. 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. A raw material of feedstock should be renewable rather than depleting wherever technically and economically practicable.
8. Unnecessary derivatisation (blocking group, protection/deprotection, temporary modification of physical/chemical processes) should be avoided whenever possible.
9. Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
10. 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. 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. Substances and the form of a substance used in a chemical process should be chosen so as to minimise the potential for chemical accidents, including releases, explosions, and fires.

Figure 1-1 Twelve principles of Green Chemistry.[5]

Of particular relevance to the work carried out in this thesis is principle no. 7. The use of renewable feedstocks will reduce the heavy reliance of obtaining chemicals from petroleum feedstocks. In the 19th and 20th centuries, natural product extraction was extensively carried out for obtaining useful chemicals. However, between 1960 and 1980, there was a rapid decline in this type of extraction mainly due to:

(i) Expensive and time-consuming processes to extract, fractionate and purify compounds.

(ii) Problems in scale-up from laboratory processes.

(iii) Natural variation in compounds and harvest yields.

(iv) Cheaper alternatives in petrochemical feedstocks.[6]

However, since then there have been considerable breakthroughs in extraction technologies, such as the advancement of selective extraction techniques (such as supercritical fluid extraction), which has led to a decrease in the volumes of solvent required, shorter extraction times and a higher selectivity.[6] This, combined with the ever-increasing costs of petroleum feedstocks, has led to a renewed interest in natural product extraction.

1.3 SUNLIBB project

The work carried out in this thesis on miscanthus, maize and sugarcane forms part of the SUNLIBB project. The SUNLIBB project, which stands for Sustainable Liquid Biofuels from Biomass Biorefining, was initiated on the 1st of October 2010 as part of the European Seventh Framework Programme (FP7) within the Energy theme: Second Generation Biofuels – EU Brazil Coordinated Call.[7] The project consists of a wide range of research including feedstock improvement, innovations in pre-treatment and saccharification and generation of added value products (Figure 1-2). The main focus is on C_4 grasses (maize, miscanthus and sugarcane).

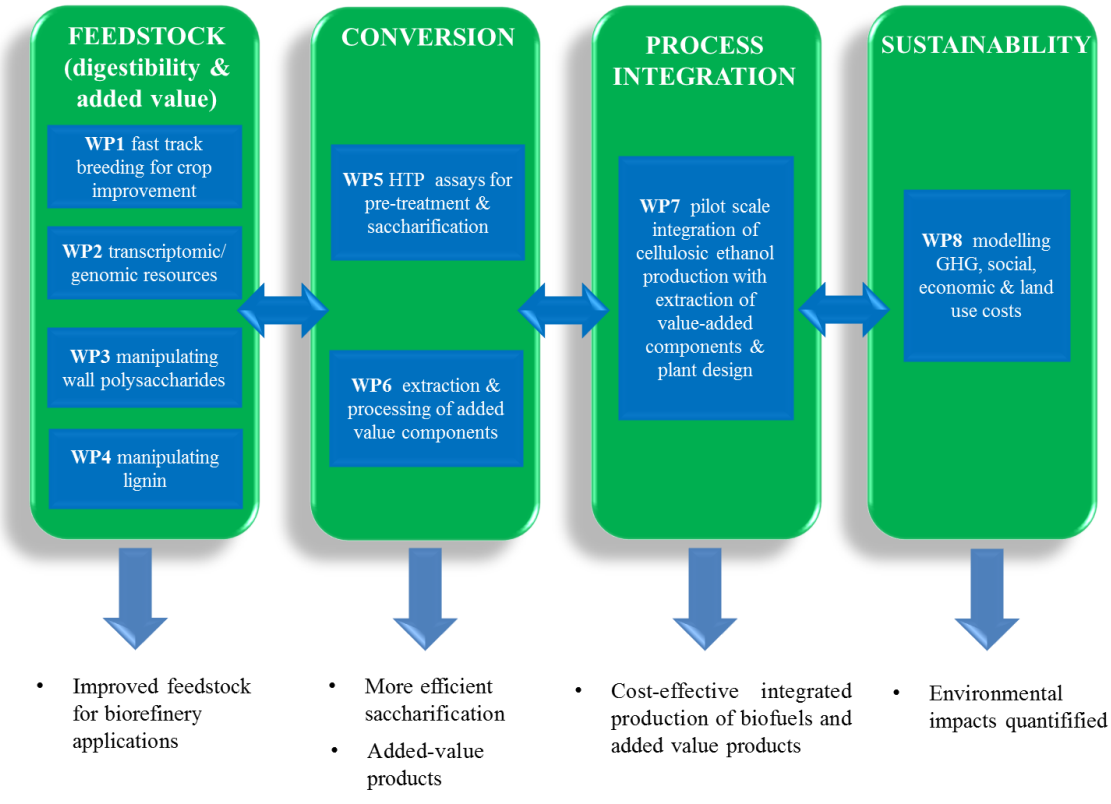


Figure 1-2 Project Overview.

The main objectives of the project include:

- To enable the cost-effective production of ethanol by improving the feedstock quality of lignocellulose.[8]
- Upgrading residues and by-products and generating other value streams from the feedstock in order to add value to the overall conversion process in biomass.
- Improving the conversion process by which sugar is produced.
- Capturing maximum value from lignocellulosic biomass by developing integrated processes in which a range of product streams are integrated in addition to bioethanol.
- Ensuring that the sustainability requirements of the newly developed processes are achieved by cutting greenhouse gas emissions, reducing other types of air pollution, having the lowest impact possible on biodiversity and local environments and building sustainable rural industries.[8]

The present work forms part of work package 6 (WP6) (Figure 1-2), which deals with generating added value from biomass. There are a range of added value products that have been identified in C_4 grasses which have potential markets and the aim of WP6 is to determine extraction and processing methods required to extract and synthesise lignin derivatives, waxes and poly/oligosaccharides from C_4 grasses.[9]

Particular focus was given on identifying added value products from the hydrophobic component of the C_4 grasses and therefore this work dealt with extracting waxes and lipid products from maize, miscanthus and sugarcane as well as identifying possible commercial applications.

1.4 Miscanthus

Miscanthus is a perennial grass that belongs to the Poaceae family. It is tall, rhizomatous and exhibits C_4 photosynthesis.[10] The genus *Miscanthus* is native to the tropics and subtropics, but a variety of species are spread throughout wide climatic regions in East Asia.[10] Miscanthus is able to adapt remarkably well to changes in environmental conditions and it is therefore perfectly capable of growing under a range of North American and European climatic conditions.[11, 12] It was first introduced to Europe from Japan in 1930 for ornamental purposes, and a number of species have now

established themselves in Europe.[12] The worldwide distribution of *Miscanthus sinensis* is shown in Figure 1-3.

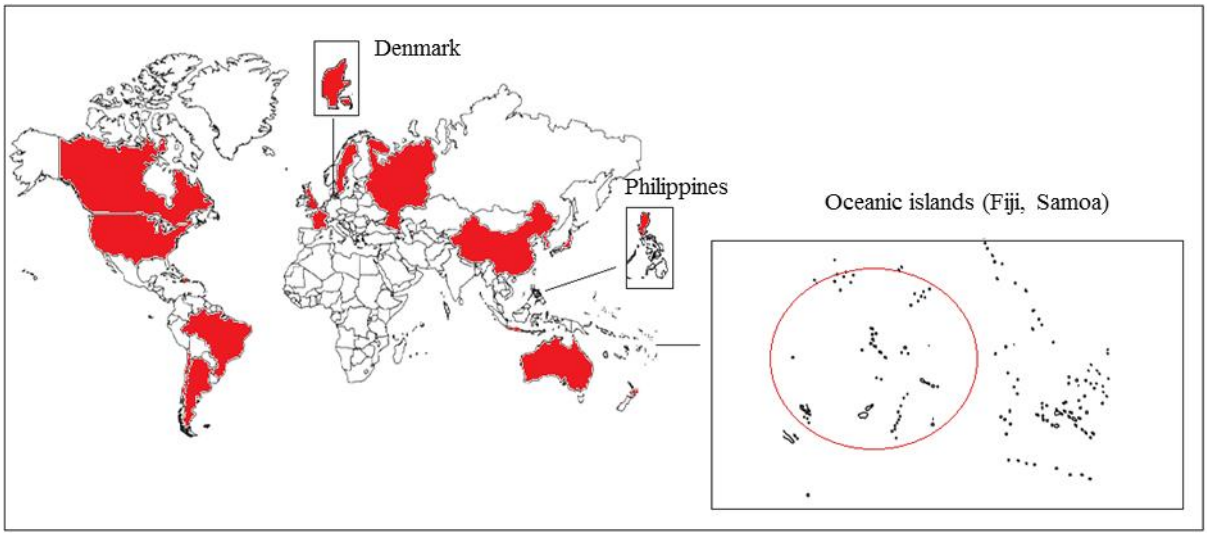


Figure 1-3 Worldwide distribution of *Miscanthus sinensis*.

Since miscanthus follows the C_4 photosynthetic pathway, it shows high photosynthetic activity and has a high rate of carbon dioxide fixation, high radiation and high water use efficiencies, which results in rapid growth and excellent productivity.[10,13-15] It can grow to substantial heights (tropical and subtropical genotypes typically grow 3 – 4 m in height when grown in Europe) and can produce large amounts of biomass under low input levels, around 20 – 30 tonnes dry weight per hectare annually.[13]

The most cultivated species of miscanthus in Europe is *Miscanthus giganteus (M. giganteus)*, which is a sterile triploid hybrid between a diploid *Miscanthus sinensis* (*M. sinensis*) and a tetraploid *Miscanthus sacchariflorus* (*M. sacchariflorus*).[10, 13, 16] It displays very vigorous growth and is considered to be environmentally friendly as its production requires low input levels. It is also ideal to cultivate, as authorities in the UK (where the crop is most widely grown) have reported no pests or diseases which attack this crop.[12, 17]

Different species of miscanthus have different types of rhizomes, but typically there are three main categories of rhizomes:

(i) Rhizomes which are broad creeping and thick stemmed such as those found in *M. sacchariflorus*.

(ii) Rhizomes which are tuft-forming and thin stemmed, characteristic of *M. sinensis*.[18]

(iii) Hybrids of the two, such as *M. giganteus*, have rhizomes with characteristics that are intermediate between the two.[18]

A number of projects were established in Europe in order to evaluate *M. giganteus* as a potential source of biomass due to the emergence of promising preliminary results.[12, 19] In 1989, the European JOULE programme was implemented, in which field trials were carried out in northern Europe including Denmark, Germany, Ireland and the UK.[12] A larger research project entitled Miscanthus Productivity Network AIR 1-CT92-0294 was established in 1993, whereby southern European countries were included in the field trials including Greece, Italy and Spain.[19] In addition, a number of separate nationally funded projects were established in Austria, Denmark, Germany, Netherlands and Switzerland, where research was carried out on the growth and establishment of miscanthus, management practices, harvesting techniques and handling.[12]

The results obtained from the projects indicated both promising results as well as limitations. Key results included:

(i) High yields of dry matter – around 30 tonnes ha^{-1} $year^{-1}$ in the southern European plantations.[12]

(ii) Low input levels – low fertiliser and pesticide usage is required.[19]

(iii) Experiments which showed the possibility of utilising *M. giganteus* as a solid biofuel and construction material (pressed particle-boards).[20]

(iv) Identifying the possibility of producing chemicals from *M. giganteus* such as activated carbon, adhesives, ethanol, methylcellulose and carboxymethylcellulose by organosolv fractionation of its main constituents, i.e. cellulose, hemicellulose and lignin.[21-26]

All of the above show promise for utilising *M. giganteus* within a biorefinery framework, in which *M. giganteus* is used as a feedstock to generate chemicals, fuels and other materials.[27] However, there are some major limitations, in relation to its production, that have been outlined namely:

(i) Since *M. giganteus* is a triploid genotype, it is sterile and does not generate fertile seeds and must therefore be grown by vegetative propagation. This results in significant establishment costs, around 3000 – 6000 euro per hectare.[28]

(ii) In different areas in northern Europe, during the first year following plantation, poor overwintering resulted in substantial losses of *M. giganteus.*[12]

1.5 Maize

Maize (*Zea mays)* is a C_4 crop, belonging to the Poaceae family, which has its origins in the sub-tropics, most likely the highlands of Mexico. Its cultivation spread southwards to Chile and northwards to the south of Canada.[29] The cultivation of maize in Europe spans 400 years. However, its importance in north-western Europe as a grain and forage group has only become evident in the last 2 decades.[30] In northern France and the Netherlands, it has established itself as a major crop. It is grown to a lesser extent in England and Denmark.[30]

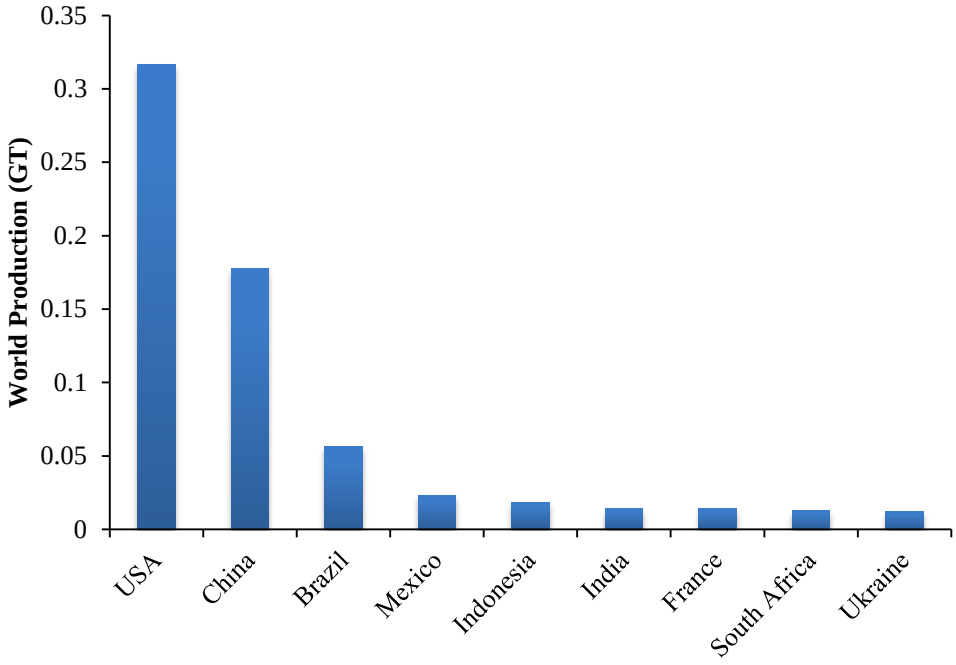


Figure 1-4 Maize production (GT) in the top ten highest producing countries.

Globally, maize is the third most abundant crop in terms of production and, together with wheat (*Triticum aestivum L.*) and rice (*Oryza sativa L.*), supplies around two-thirds of the total energy in human diets.[31] Figure 1-4 illustrates the ten highest producing countries of maize in 2010. There has been a significant increase in maize production over the last five to six decades, in which the maize grain yield quadrupled.[32-35] This increase in grain yield has occurred as a result of appropriate selection of modern hybrids. These showed increased productivity at increasingly higher planting densities, which therefore led to increased total biomass production.[33-35] For a number of decades, there has been an increase in the planting density in maize at a rate of around 1000 plants ha^{-1} $year^{-1}$ and this is most likely to increase in the near future.[33]

Once the maize grain is harvested, the residue that is left behind is known as the corn stover (or maize stover), which is composed of the stalk, leaf, cob and husk tissues.[36] Pordesimo *et al.* investigated the chemical composition of two commercial hybrids at grain physiological maturity and when grain moisture was 30.6%.[37] They concluded that the corn mass distribution of dry matter was found to be 45.9% grain, 27.5% stalk, 11.4% leaf, 8.2% cob and 7% husk. Therefore over half of the stover biomass is attributed to the maize stalk (50.9%), followed by the leaves (21.0%), cobs (15.2%) and husk (12.9%).[37] The majority of the stalk biomass is situated in the rind tissue, which is a mixture of vascular bundles that are densely packed and embedded in a matrix containing schlerenchymatous cells.[36] They also noted that the maximum dry matter yield in the stover occurred at physiological maturity, which is approximately 118 days after plantation. After physiological maturity, there was a noticeable loss of plant material due to weathering which resulted in a decrease in the dry matter, particularly in the leaves.[37]

After the maize grain is harvested, the corn stover is usually incorporated back into the soil where decomposition results in the release of nutrients.[36] It has been suggested that the abundance of corn stover could make it an ideal strategic feedstock for the manufacture of bio-based products as well as for bioenergy.[38, 40] However, important factors that need to be taken into consideration are the harvesting and processing of corn stover with minimal losses in order to improve its quality as an industrial feedstock or fuel.[37] A key issue is collecting the crop residue and how much should be harvested, if any at all.[41] Some argue that removing the corn stover would not only remove vital nutrients but also increase the risk of soil erosion by wind and rain. Furthermore, removal of corn stover would result in reduced carbon sequestration leading to an increase in carbon dioxide levels in the atmosphere.[41]

One the other hand, one study indicated that the removal of corn stover is beneficial as around 85% of the stover left in the soil rotted, releasing considerable amounts of carbon dioxide into the atmosphere while the remaining 15% contributed to soil organic matter.[42] Therefore, it was argued that removal of the corn stover actually decreases the greenhouse gas emissions from maize.[42]

Furthermore, studies indicated that under no till conditions, two-thirds of the stover in certain corn-growing regions can be harvested on a sustainable basis without affecting the soil adversely.[43-45]

1.6 Sugarcane

Sugarcane (*Saccharum officinarum*) is a tall, perennial, C_4 grass belonging to the Poaceae family.[46] The plant originated from South and South-East Asia, but, is now widespread throughout various tropical and sub-tropical countries.[46, 47] It is approximately 2 inches thick and generally grows from 2 – 6 m in height. The crop is cultivated in around 200 countries and a number of different horticultural varieties exist, differing in length and stem colour.[46]

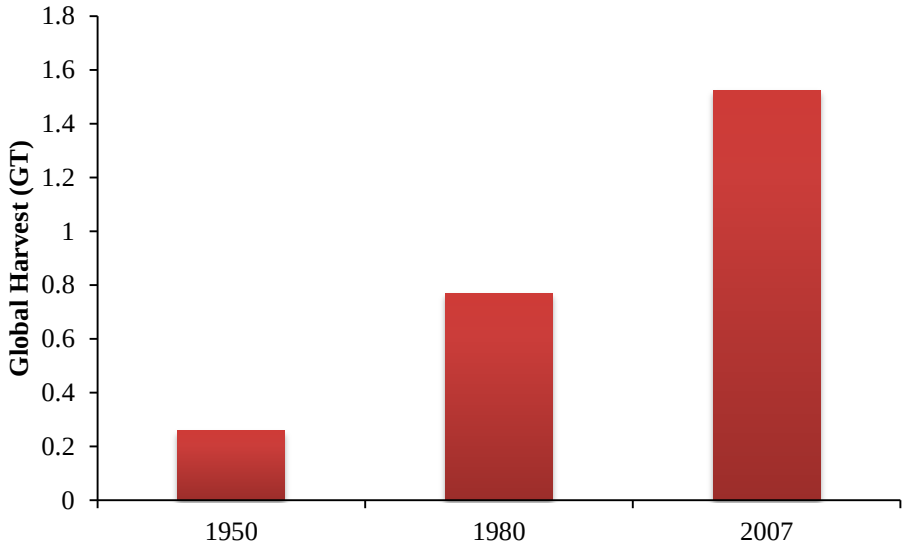


Figure 1-5 Global sugarcane harvest (GT) from 1950 – 2007.

According to the world crop statistics that have been gathered by the Food and Agricultural Organisation of the United Nations (FAO), the global harvest of sugarcane has drastically changed over the past 6 decades; from 260 million tonnes in 1950, to 770 million tonnes in 1980, to 1525 million tonnes in 2007 (Figure 1-5). Therefore, there has been nearly a six fold increase in the global sugarcane harvest from 1950 to 2007.[47] Figure 1-6 illustrates the ten highest producing countries of maize in 2010. The top three countries (Brazil, India and China) contributed around 67% of the total sugarcane production in 2010; Brazil alone contributed approximately 43%. The global sugarcane production and expansion is therefore dominated by Brazil. From 2000 to 2007, 75% of sugarcane area increases was solely attributed to Brazil.[47]

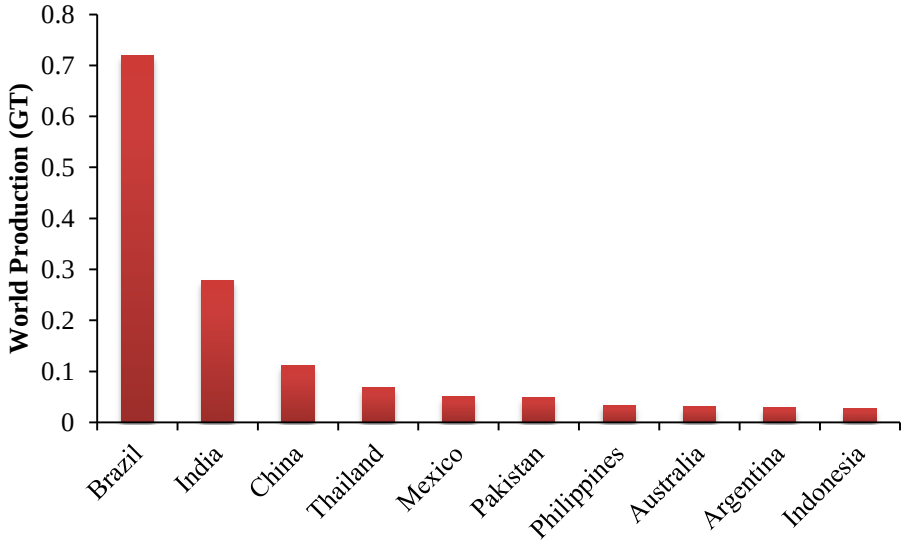


Figure 1-6 Sugarcane production (GT) in the top ten highest producing countries.

Sugarcane is processed in conventional sugar mills, which involves crushing the sugarcane to extract the juices, followed by heating of the juices to form syrup and crystallising out the sugar from the syrup.[47] After conventional milling, a fibrous residue remains, which is referred to as sugarcane bagasse.[48] This is a lignocellulosic material which is collected in large amounts following sugarcane processing. In a typical sugar mill, the processing of one metric tonne of sugarcane gives rise to around 270 kg of bagasse (with 50% moisture) which therefore gives approximately 135 kg of dry matter.[49, 50]

Sugar and ethanol plants normally use around 50% of this dry matter to generate heat and power. The rest is normally stockpiled by sugar mills, which poses an environmental problem to both the sugar mills and surrounding districts, since stockpiling for long periods of time could increase the risk of spontaneous combustion.[51] There have been various reports which highlight the use of sugarcane bagasse in a variety of applications ranging from animal feed to the production of various industrial enzymes (cellulases, lipases etc.), chemicals, pulp and paper.[51]

1.7 Types of Extraction techniques

1.7.1 Soxhlet extraction

Soxhlet extraction is one of the conventional techniques for extracting metabolites from biomass. A porous cellulose thimble is loaded with the plant material and placed in a Soxhlet extractor, which is directly connected to a solvent reservoir. The solvent of choice is heated to its boiling point and condenses into the Soxhlet extractor, which

slowly fills up with the solvent. While filling up, lipids are extracted by the solvent from the plant material. Once the Soxhlet extractor reaches the overflow point, the solvent containing the lipids is flushed back into the solvent reservoir by means of a siphon, completing one cycle. This process is repeated until no more lipids are extracted by the solvent.

There are numerous advantages when using this technique. Unlike other conventional leaching techniques, in Soxhlet extractions, the biomass continuously comes into contact with fresh solvent which displaces the extraction equilibrium.[52] Therefore, the highest yields of metabolites can be achieved, making it more efficient when compared to other conventional extraction techniques. Furthermore, the heat supplied to the solvent reservoir reaches the Soxhlet extractor and therefore considerably high extraction temperatures are maintained throughout the entire system. Simultaneous extractions can be carried out by having a number of Soxhlet extractors in parallel. which increase biomass throughput.[52]

However, there are a number of disadvantages associated with Soxhlet extractions. First of all, it is a time-consuming process, with relatively large extraction times needed. Large volumes of solvent are required for the extraction (leading to problems in waste disposal) and the high temperatures required to heat the solvent to its boiling point increases the risk of thermal decomposition of thermolabile molecules.[52, 53] After the extraction the solvent has to be evaporated to concentrate the products, which is an energy consuming process. Finally, the extraction of specific metabolites is dependent on the selectivity of the solvent. A large number of solvents have relatively poor selectivity which makes the extraction of specific metabolites rather difficult.[52, 53] Typical solvents that are used in Soxhlet extractions are hexane, DCM, chloroform, methanol, ethanol and acetone. A number of these solvents are associated with toxic and environmental problems and are also non-renewable.[54]

1.7.2 Supercritical Fluid Extraction

1.7.2.1 Supercritical Fluids

A supercritical fluid refers to a substance that has its pressure and temperature above their critical point values (P_c, T_c).[55-57] The physical properties of a substance change when varying the temperature and pressure. This phenomenon can be explained with the aid of a phase diagram, which is essentially a plot of vapour pressure vs temperature (Figure 1-7).[58]

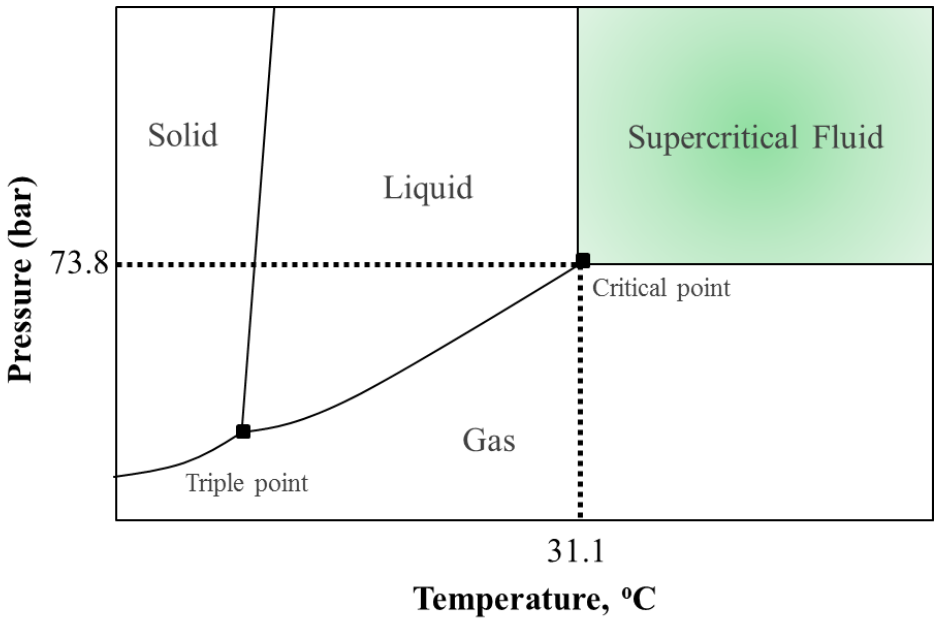


Figure 1-7 Phase diagram for carbon dioxide.[58]

All stable compounds have a triple point and critical point.[59] The triple point represents the temperature and pressure at which the three phases are in equilibrium with one another and can therefore coexist. The line found lying between the liquid and gas region represents the gas-liquid coexistence curve.[60] When moving along the gas-liquid coexistence curve, towards the critical point, the density of the liquid phase decreases due to thermal expansion, while the density of the gas phase increases due to the increase in pressure.[60] At the critical point, the density of the liquid phase becomes equivalent to the density of the gas phase resulting in identical properties and thus it is no longer possible to distinguish between the two phases.[58, 60] The critical point represents the maximum temperature and pressure that can be applied where a substance can be found as a liquid and a gas in equilibrium with each other. Increasing the temperature and pressure beyond the critical point terminates the gas/liquid equilibrium, where the substance no longer behaves as a liquid or a gas.[58] The above phase diagram is for carbon dioxide, in which the critical point is found at 73.8 bar and 31.1 °C.[56]

1.7.2.2 Supercritical fluids: Physical Properties

The range in which the most interesting supercritical fluid (SCF) applications occur is $1< T/T_c <1.1$ and $1< P/P_c <2$. When in this range, SCFs are in a single condensed phase and have properties between those of a gas and a liquid.[55] Slight changes in temperature and pressure could lead to a significant variation in these physical properties.[56]

It has been demonstrated by a variety of different authors that the *"logarithm of solubility is approximately linearly dependent on the solvent density"*.[61-63]A number of

experiments involving hydrocarbons demonstrated that there is an exponential variation in hydrocarbon solubility with a change in density of the supercritical fluid (supercritical CO_2, ethane or ethylene).[64-67] Slight adjustments to the temperature and pressure lead to a significant change in solvent density which in turn causes variations in the density-dependent solvent properties such as the partition coefficient, solubility parameter and dielectric constant.[68]

In addition, apart from solubility, other factors which influence the solvent power of supercritical fluids are diffusivity and viscosity.[69] Table 1-1 summarises the densities, viscosities and diffusion coefficients of a solid, liquid and supercritical fluid. From Table 1-1 it can be seen that the viscosity of a supercritical fluid is generally an order of magnitude lower than the viscosity of a liquid while the diffusivity is an order of magnitude higher.[69, 70] Furthermore, supercritical fluids such as carbon dioxide (CO_2) have negligible vapour pressure. Therefore, this leads to enhanced heat and mass transfer.[71]

Table 1-1 Thermophysical properties of the three fluid states.

Fluid State	Density (kg m^{-3})	Viscosity (N s m^{-2})	Diffusion Coefficient (m^2 s^{-1})
Liquid	800 – 1200	$10^{-3} – 10^{-2}$	$10^{-8} – 10^{-9}$
Supercritical fluid	250 – 800	$10^{-4} – 10^{-3}$	$10^{-7} – 10^{-8}$
Gas	1 – 100	$10^{-5} – 10^{-4}$	$10^{-4} – 10^{-5}$

It was suggested by Dobbs and Johnston that there is a phenomenon referred to as an entrainer effect, whereby there is a greater solubility enhancement with the presence of solutes in the supercritical phase that act as co-solvents.[72] They measured the solubilities for binary, ternary and quartenary systems, consisting of supercritical carbon dioxide, a co-solvent and combinations of solid phases. It was suggested that in ternary systems, involving two solutes and the supercritical phase, there is a proportional increase in the solubility of one solute in the system relative to the solubility of the second solute. Therefore, an entraining effect occurs (as a result of the more soluble solute) which leads to the enhancement of the less soluble solute. This indicates that the

addition of compounds in supercritical fluids results in a change in the solvent properties.[72]

Furthermore, Dobbs *et al.* noted that the addition of small amounts of various co-solvents led to a marked improvement in the selectivity of a non-polar supercritical fluid solvent for polar vs non polar solids.[72, 73] The addition of several mole percent of various co-solvents led to enhanced solubility of certain solids in supercritical carbon dioxide. There was an increase in the solubility of 2-aminobenzoic acid by 620% with only 3.5 mol. % methanol.[73]

1.7.2.3 Supercritical carbon dioxide

The most commonly used supercritical fluid is carbon dioxide (CO_2). CO_2 is an ideal supercritical solvent for a number of applications ranging from extraction processes to pharmaceutical applications.[74] Due to the relatively low critical temperature of CO_2, the benefits of near-critical operation can be exploited at temperatures below 35 ^{o}C. In addition, CO_2 is non-flammable, has minimal toxicity and is widely available. It is relatively inexpensive, recyclable and is an unregulated solvent. One slight disadvantage is the relatively high critical pressure of carbon dioxide (73.8 bar). However, operating at such pressures has become fairly routine in industrial-scale extraction processes in which supercritical carbon dioxide ($scCO_2$) is used, such as in the extraction of hops and decaffeination of coffee.[74, 75]

Traditionally, the extraction of lipids molecules was carried out using conventional organic solvents such as hexane.[76] The polarity of CO_2 is very similar to that of hexane and toluene.[74] A convenient method for measuring polarity is by the addition of a probe molecule such as Reichardt's dye [2,6-diphenyl-4-(2,4,6-triphenylpyridinio) phenolate]. Reichardt's dye is a zwitterionic compound and therefore displays solvatochromic effects, one of the largest of any known organic compound.[77] This solvatochromic effect occurs as a result of the interaction between the ground state of the dye and the solvent.[78] The empirical scales of solvent polarity associated with this dye are the $E_T(30)$ and E_T^N scales:

$$E_T(30)(kcal\ mol^{-1}) = \frac{28951}{\lambda_{max}^{abs}(nm)}$$

$$E_T^N = \frac{[(E_T\ solvent - 30.7)]}{32.4}$$

It was proposed by Kamlet and Taft that the interactions involving the phenoxide oxygen with the solvent is approximately two-thirds of the shift of maximum absorption

wavelength of Reichardt's dye. The E_T^N value has been defined by Reichardt and Harbusch-Gornert as being a dimensionless figure where $E_T^N = 0$ for tetramethylsilane (extremely nonpolar solvent) and $E_T^N = 1$ for water (extremely polar solvent.)[78]

Table 1-2 E_T^N values for a variety of different solvents.

Solvent	E_T^N values
Tetramethylsilane (TMS)	0
Hexane	0.009
Supercritical carbon dioxide	0.012 – 0.034
Toluene	0.099
DCM	0.309
Methanol	0.762
Water	1

Therefore, it is a general rule of thumb that low molar mass compounds having appreciable vapour pressures that dissolve in hexane should also be soluble in $scCO_2$.

It has been shown that hexane and toluene are two excellent solvents for the extraction of plant waxes.[54] $ScCO_2$ was found to be an ideal solvent for the extraction of plant lipids. The advantage of using $scCO_2$ as a solvent is that the extraction of non-polar compounds can be made selective by fine-tuning the solvent power, which is done by varying the temperature and pressure.[75, 79-83] Furthermore, the extraction yields can be improved by adding polar modifiers (e.g. methanol, ethanol) which increase the solvent polarity. However, this results in a decrease in selectivity towards plant lipids, as a higher proportion of polar compounds are extracted. In the case of ethanol, the maximum concentration where the modifier is completely miscible with $scCO_2$ is 20%.[53, 84]

1.8 Lipids

It is difficult to find a strict definition for 'a lipid' because it is a term that is often used broadly. A loose definition for 'lipids' is any biological compound that exhibits a hydrophobic character in nature and often has a high solubility in a number of conventional organic solvents such as hydrocarbons, chloroform, alcohols, ethers and aromatics.[76, 85] There are a wide range of molecules which cover these chemical properties such as fatty acids and their derivatives, bile acids, sterols, phospholipids, sphingolipids, terpenes and carotenoids.[76] In his review, Christie considers lipids to be fatty acids and their derivatives as well as compounds that resemble these molecules functionally or biosynthetically. This includes molecules such as sterols but omits other compounds such as carotenoids, terpenes and steroidal hormones.[76] In their review, Fahy *et al.* define lipids as small molecules that are hydrophobic or amphipathic in nature, and are synthesised entirely or partly by carbocation-based condensation of isoprene units (sterols etc.) or carbanion-based condensation of thioesters (fatty acids, polyketides etc.)[86]

1.9 Waxes

The term 'waxes', when adhering to the strict chemical definition, refers to the ester products formed from the esterification of long-chain fatty acids with long-chain primary alcohols.[87] However, often the term 'plant wax' is used collectively to describe the complex mixture of surface lipids covering the aerial tissues of herbaceous plants.[88]

Early morphological work, reviewed by Martin & Juniper and Baker, led to the first description of the terminology defining plant wax, in which the 'cuticular membrane' was used to describe the entire waxy coating which lined the outer surface of the plant epidermal cells.[84,85] It comprises three distinct regions namely: the exterior epicuticular wax, the cuticle proper and the interior intracuticular wax (Figure 1-8).[88] The cuticle proper is made up of a biopolyester consisting of hydroxy and epoxy fatty acids, collectively known as cutin, which covers the epidermal cells forming an electrodense layer.[88-90] The intracuticular wax, which is embedded in the cutin, contains amorphous mixtures of lipids that link the cuticle to the cell wall matrix.[88, 90] The epicuticular wax refers to the complex mixture of surface lipids, comprising of cyclic and aliphatic long-chain molecules, which cover the cuticle proper forming a smooth film exterior or

crystalloids.[87, 88] It consists of a large variety of chemicals which may be subdivided on the basis of their structure, functional group type and their homologue distribution.[87]

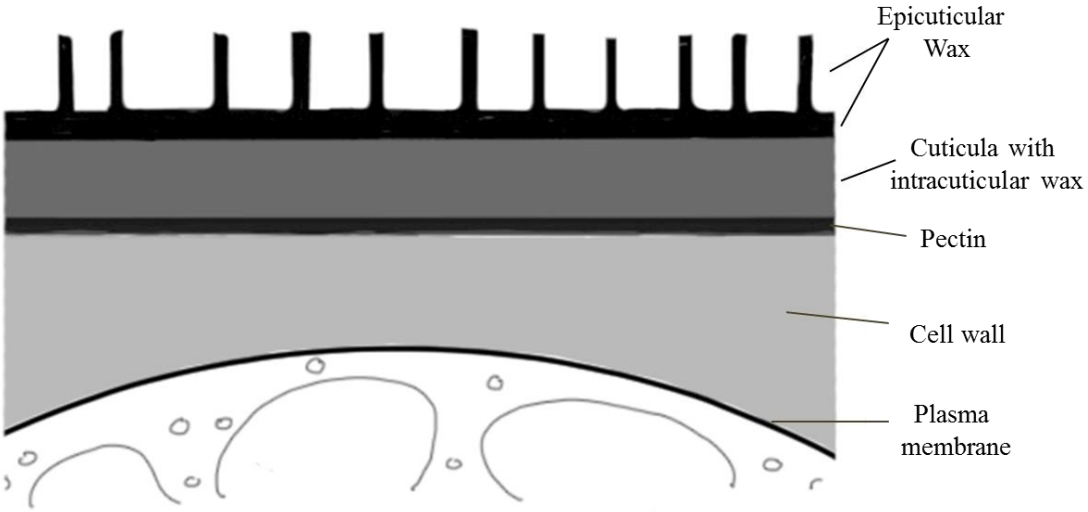
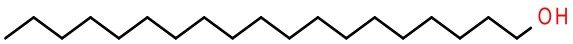
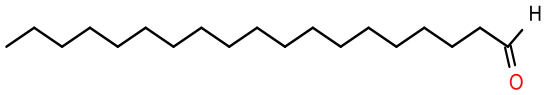
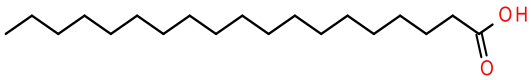
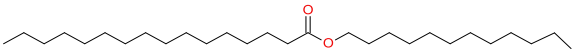
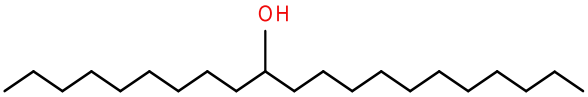
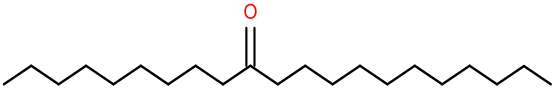
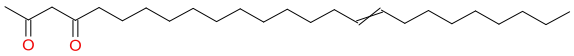


Figure 1-8 Depiction of the plant cuticle.[88]

The most common long-chain aliphatic compounds include hydrocarbons, primary alcohols, aldehydes, fatty acids and wax esters.[87] Long-chain aliphatic compounds that are less common include ketones, β-diketones and secondary alcohols.[87] Cyclic compounds that are found in the plant cuticular wax include sterols, flavonoids and terpenoids. These are summarised in Table 1-3 and Table 1-4.[88]

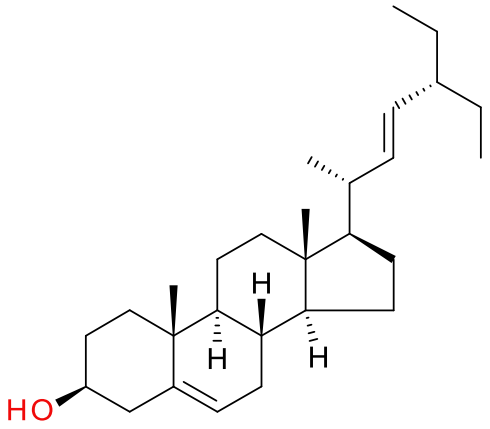
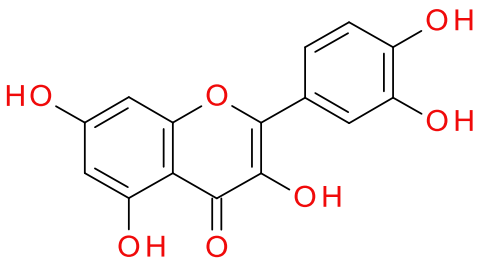
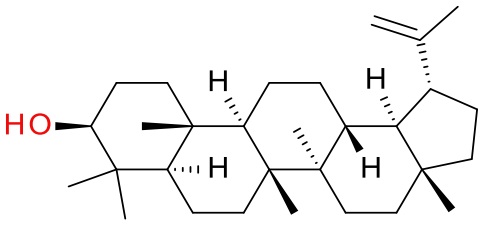
In the majority of plant waxes, the hydrocarbon fraction consists of a number of *n*-alkanes varying in chain length from C_{25} to C_{35}. The predominant *n*-alkanes are those which contain an odd number of carbon atoms. In most cases, the predominant chain length (usually 90% or more of the total hydrocarbon fraction) is C_{29} or C_{31}.[91] However, this is a generalisation and there are a number of exceptions in relation to the number of carbon atoms and the predominant chain length. There have been some studies in which alkanes having chain lengths less than C_{25} have been reported.[91] In addition, in the majority of microalgae, the predominant chain length is $C_{17.}$[91] There have also been reports in which alkanes of chain length C_{62} have been identified in cane grass wax.[91]

Table 1-3 Various types of long-chain aliphatic compounds found in plant waxes.

Aliphatic Compounds	Structure	Chain length	Preferred no. of C. atoms
n-alkanes		C_{19} -C_{37}	odd
Primary alcohols		C_{12} – C_{36}	even
Aldehydes		C_{14} – C_{34}	even
Fatty acids		C_{12} – C_{36}	even
Wax esters		C_{30} – C_{60}	even
Secondary alcohols		C_{21} – C_{33}	odd
Ketones		C_{25} – C_{33}	odd
β-diketones		C_{27} – C_{35}	odd

On the other hand, in the case of primary alcohols, aldehydes and fatty acids, the predominant compounds are those which possess an even number of carbon atoms.[87] In a number of plant waxes, the chain length of ketones is related to the hydrocarbon chain length. Two examples are nonacosan-15-one and hentriacontan-16-one, which are normally present in plant waxes where the predominant hydrocarbons are nonacosane and hentriacontane respectively.[92-95] In addition, in some plant tissues, mixtures of ketones occur where mixtures of alkanes are present.[96, 97] However, in the case of β-diketones, the chain length of the compound is not related to the hydrocarbon chain length of the same tissue. Tritriacontan-16,18-one appears to be the most common β-diketone.[98, 99] Occasionally, the type of secondary alcohol present is closely related to the type of ketone found in the plant wax.[100]

Table 1-4 Major cyclic compounds found in plant waxes.

Cyclic Compounds	Structure
Sterols	
Flavonoids	
Terpenoids	

Wax esters are generally comprised of *n*-alkanoic acids and *n*-alkan-1-ols and often possess an even number of carbon atoms. In plant waxes, the presence of double bonds and branches in wax esters is rare, unlike the situation in microbial and animal waxes. Usually, the fatty acid and fatty alcohol portions of the ester correspond to the free fatty acid and free fatty alcohol in the plant wax.[101]

1.9.1 Biosynthesis of long-chain aliphatic compounds: Brief overview

The generation of long-chain aliphatic compounds is a complex process involving the coordinated activities of a variety of enzymes that are thought to be arranged into

multienzyme complexes.[88] Long-chain aliphatic compounds are produced via two main steps:

(i) The synthesis of saturated very long chain fatty acid molecules (VLCFA)

(ii) Production of aliphatic wax constituents from VLCFAs.[88] All of the reactions involved in the synthesis of aliphatic compounds occur in the epidermis.[102]

1.9.1.1 VLCFA Production

VLCFAs are the precursor molecules from which all other aliphatic components of the cuticular wax are synthesised.[88] The formation of VLCFAs occurs in the epidermal cells by two main steps. The first involves the synthesis of C_{16} and C_{18} fatty acids in the stroma of plastids using various soluble enzymes which collectively from the fatty acid synthase complex (FAS).[88, 103, 104] The second step involves the elongation of the C_{16} and C_{18} fatty acids to form the VLCFAs.[88, 105] This is carried out by a multienzyme fatty acid elongase (FAE) complex situated on the endoplasmic reticulum.[88, 105]

1.9.1.2 Production of aliphatic wax constituents from VLCFAs.

As stated previously, the other aliphatic wax components are derived from the VLCFAs. This occurs via two biosynthetic pathways:

(i) The acyl reduction pathway: which leads to the formation of primary alcohols and wax esters.

(ii) The decarbonylation pathway which gives rise to aldehydes, alkanes, secondary alcohols and ketones.[88, 105]

Figure 1-9 summarises the synthesis of long-chain aliphatic components in plants.

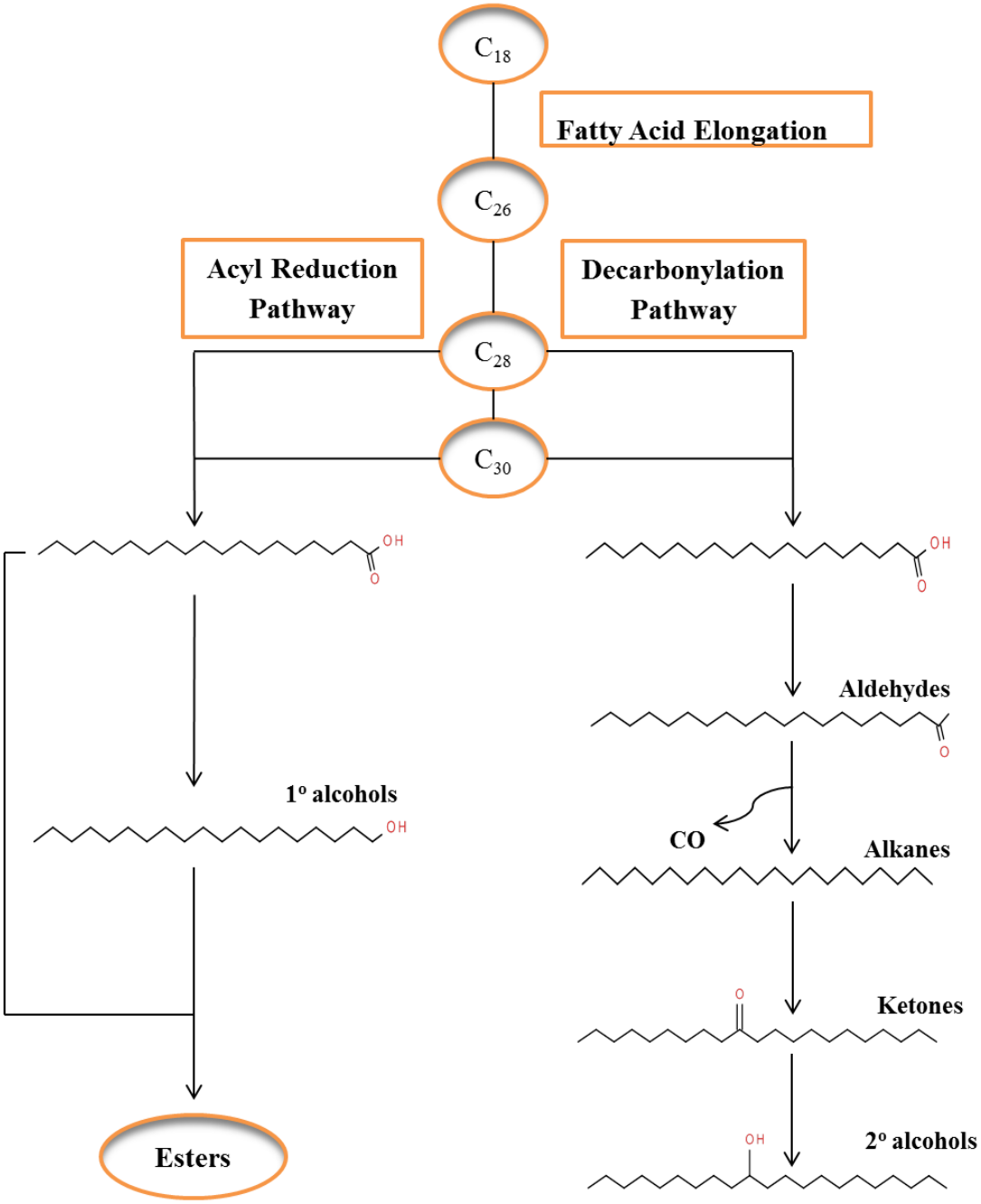


Figure 1-9 Schematic summarising the synthesis of long-chain aliphatic components in plants.

1.10 Chemicals from miscanthus

To the author's knowledge, there are very few publications regarding the chemical composition of epicuticular waxes in miscanthus. Villaverde *et al.* investigated the chemical composition of the lipophilic fraction of the bark and core of *Miscanthus giganteus*.[106] Waxes from the bark and core were extracted in a Soxhlet with dichloromethane as the solvent.[106]

Results indicated that both the core and bark were found to be mainly composed of sterols, aromatic compounds and fatty acids. The amount of aromatic compounds found in the bark and the core were found to be 521 and 829 mg/kg respectively. The most common compounds were found to be vanillic acid, vanillin, syringaldehyde, *p*-hydroxybenzaldehyde and *p*-coumaric acid. The aromatic compounds that were found are summarised in Table 1-5[106]

Table 1-5 Major aromatic compounds found in the bark and core of *M. Giganteus*.

Aromatic compound	**Bark (mg/kg)**	**Core (mg/kg)**
benzoic acid	3	6
p-hydroxybenzaldehyde	64	115
Resorcinol	3	3
Vanillin	93	105
4-hydroxybenzoic acid	7	14
Syringaldehyde	22	28
vanillic acid	108	115
4-hydroxy-3-methoxycinnamaldehyde	17	24
3-vanillyl propanol	3	5
Syringic acid	18	41
1-guaiacyl-2-hydroxyethanone	21	34
p-coumaric acid	50	160
3,5-dimethoxy-4-hydroxy cinnamaldehyde	7	14
guaiacyl glyoxylic acid	39	55

2-hydroxy-1-syringyl ethanone	5	13
ferulic acid	2	8
aromatic non-identified	54	83
Vanillylethanediol	5	6

A number of fatty acids were found in the bark and core of *M. giganteus* which constituted around 393 and 453 mg/kg of the bark and core respectively. Aliphatic saturated acids ranged from C_{16} to C_{30}. In addition, two unsaturated (C_{16} and C_{18}) acids and four α-hydroxy fatty acids were also present. In the bark, the dominant acids were found to be C_{28} acid and C_{26} acid, followed by C_{18} and C_{30} acids. On the other hand, in the core samples the most abundant compound was found to be C_{16} acid followed by similar amounts of C_{22}, C_{24}, C_{23} acids and two α-hydroxy acids. A variety of odd-chain fatty acids were identified in the bark and core ranging in chain length from C_{17} to C_{29}. Table 1-6 summarises the number of fatty acids identified in the bark and core of *M. giganteus*.[106]

Table 1-6 Major fatty acid compounds found in the bark and core of *M. Giganteus.*

Fatty acids	Bark (mg/kg)	Core (mg/kg)
Hexanoic acid	5	5
Heptanoic acid	3	3
Octanoic acid	5	4
Nonaoic acid	8	10
Decanoic acid	3	5
Dodecanoic acid	4	4
Tridecanoic acid	3	9
Tetradecanoic acid	7	13

Pentadecanoic acid	3	8
Hexadecanoic acid	89	133
Heptadecanoic acid	4	10
Octadecanoic acid	19	26
Nonadecanoic acid	1	3
Eicosanoic acid	11	6
Heneicosanoic acid	2	7
Docosanoic acid	8	31
Tricosanoic acid	8	29
Tetracosanoic acid	13	29
Pentacosanoic acid	4	6
Hexacosanoic acid	12	4
Heptacosanoic acid	7	1
Octacosanoic acid	109	14
Nonacosanoic acid	5	0
Triacontanoic acid	18	3
Hexadecenoic acid	1	1
cis-9-octadecenoic acid	15	16
2-hydroxyeicosanoic acid	3	7
2-hydroxydocosanoic acid	7	28
2-hydroxytricosanoic acid	4	2
2-hydroxytetracosanoic acid	12	36

In the bark, sterols were the third most common compounds, representing about 275 mg/kg. In contrast, in the core, sterols were the most common compounds accounting for 949 mg/kg. The most dominant sterol was β-sitosterol followed by stigmasterol and campesterol. These three sterols comprised 64.0% and 66.3% of the bark and core respectively. There were also a number of oxidised sterol derivatives present such as stigma-3,5-dien-7-one. These are summarised in Table 1-7 below.[106]

Table 1-7 Major sterols found in the bark and core of *M. Giganteus*.

Compound	Bark (mg/kg)	Core (mg/kg)
Campesterol	33	84
Stigmasterol	45	137
B-sitosterol	98	408
Stigma-3,5-dien-7-one	15	41
Stigma-4-*en*-3-one	29	64
Stigmast-6-*en*-3,5-diol	10	19
7-hydroxy-β-sitosterol	7	29
7-oxo-β-sitosterol	38	167

The least abundant family of aliphatic compounds found in the bark and core of *M. giganteus* were found to be the long-chain fatty alcohols, comprising 93 and 32 mg/kg respectively. Octacosanol was found to be the major alcohol in the bark. These are summarised in Table 1-8.[106]

Table 1-8 Major fatty alcohols found in the bark and core of *M. Giganteus.*

Compound	Bark (mg/kg)	Core (mg/kg)
Heptacosanol	3	1
Pentancosan-1,2-diol	3	4
Octacosanol	81	25

Additional compounds, which were found in small amounts were octacosanal, heptacosane and pentadecan-2-one (Table 1-9).[106]

Table 1-9 Minor compounds found in the bark and core of *M. Giganteus.*

Compound	Bark (mg/kg)	Core (mg/kg)
Octacosanal	58	0
Heptacosane	11	2
Pentadecan-2-one	19	38

1.11 Chemicals from maize

There have been several studies that looked into the composition of epicuticular waxes in maize (*Zea mays)*, for both wild type and mutant varieties.[107-109] For the purpose of this study, results concerning the wild type variety shall be discussed. Bianchi *et al.* investigated the % composition of waxes in seedlings and mature plants.[107] From the results obtained it was concluded that there was a difference in wax composition between the adult plant and the seedlings. In the latter, it was observed that the main class of compounds were alcohols (63%) followed by aldehydes (20%) and esters (16%).[108] On the other hand, the wax composition of leaves of the mature plant indicated that esters became the dominant class of compounds (42%) followed by alkanes (17%).[107, 109]

Table 1-10 % Composition of epicuticular waxes in maize.

Composition	Maize Seedlings (%)	Maize Mature Plant (%)
Alkanes	1	17
Alcohols	63	14
Aldehydes	20	9
Acids	Trace	14
Esters	16	42
Sterols	-	4

These are summarised in Table 1-10. Furthermore, it was deduced that there is a reduction in wax production in maize on ageing.[110]

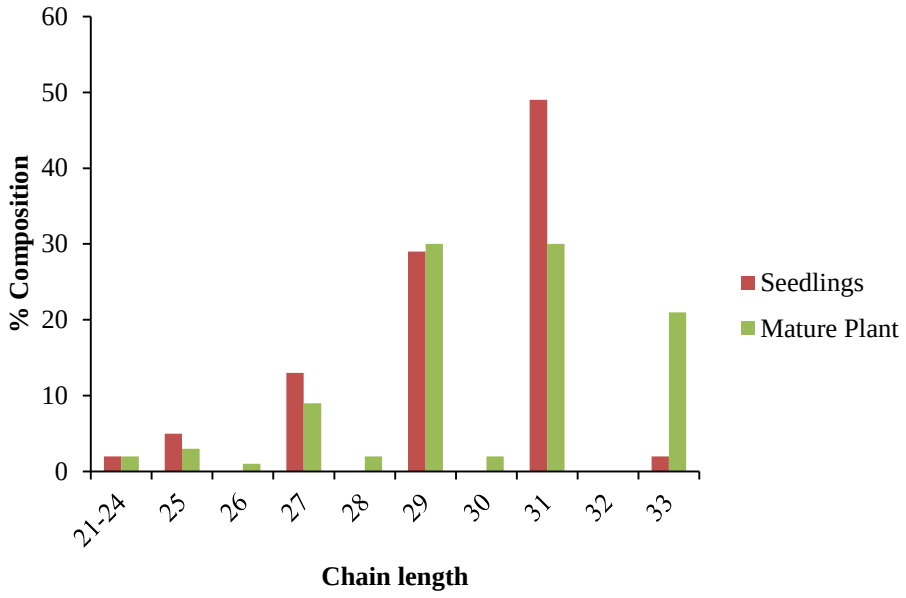


Figure 1-10 % Composition of alkanes in maize.

Figure 1-10 illustrates the % composition of alkanes of varying chain lengths in maize for seedlings and adult plants. In the seedlings, C_{29} and C_{31} alkanes are the dominant alkanes, with the latter making up 49% of the total alkanes.[109] In the mature plants, the dominant alkanes are once again C_{29} (30%) and C_{31} (30%), however the proportions of

each are more evenly distributed. In addition, there are much higher quantities of the C_{33} alkane (21%) when compared to the seedlings (2%).[109]

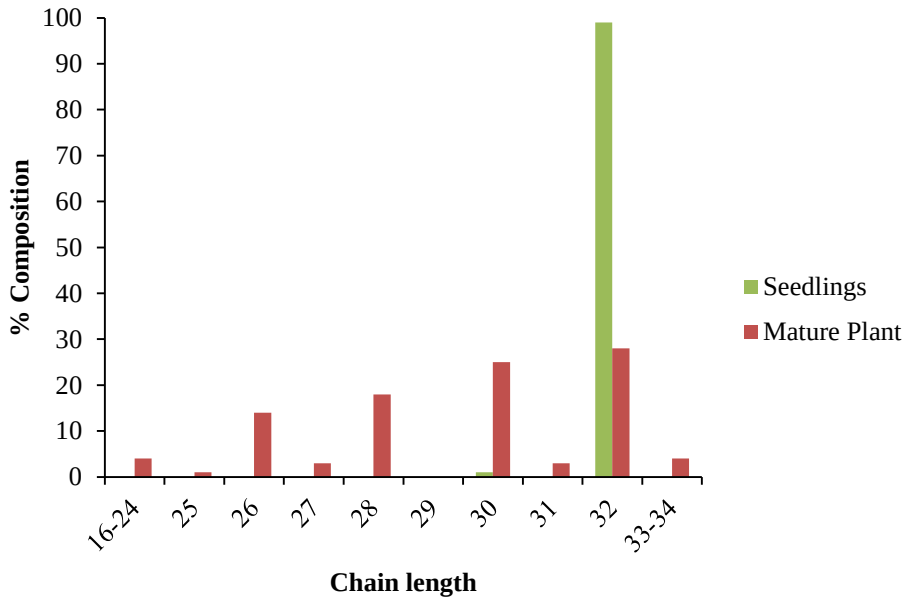


Figure 1-11 % composition of alcohols in maize.

The % composition of alcohols in maize for seedlings and adult plants is summarised in Figure 1-11. The most important homologue in the seedlings is the C_{32} alcohol, which constitutes 99% of the total alcohols.[107, 109] In mature plants there is a much wider variety of alcohols, varying in chain length from C_{16} to C_{34}. C_{32} remains the most common chain length (28%), followed close by C_{30} (25%) and C_{28} (18%). Therefore, when comparing the homologue composition of alcohol fractions of mature plants with the seedlings, it can be seen that there is a clear chain length shortening.[109]

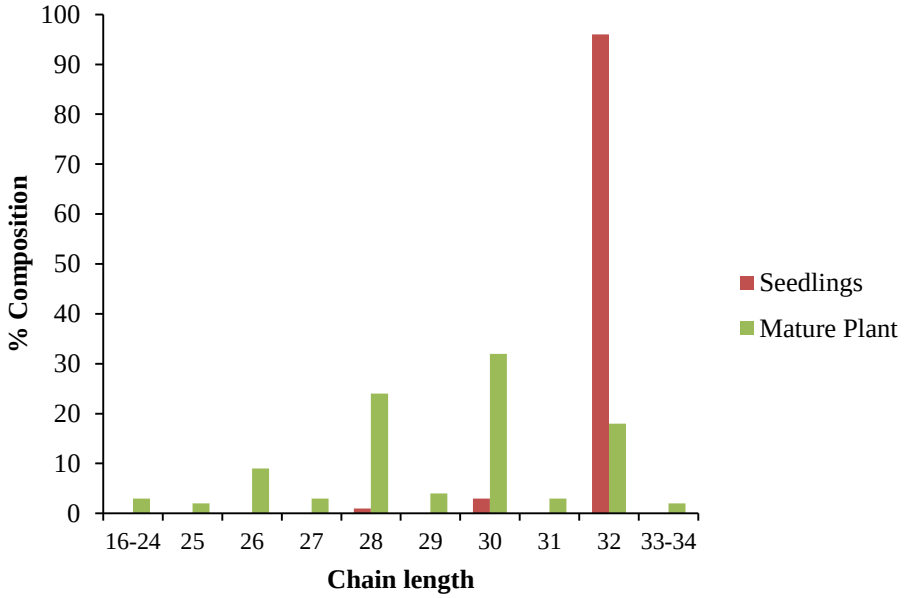


Figure 1-12 % composition of aldehydes in maize.

The % composition of aldehydes in maize (Figure 1-12) shows a very similar trend to the % composition of alcohols. In seedlings, C_{32} is the most dominant chain length (96%).[107, 109] Once again, a clear chain length shortening is observed in the mature plants. The most common chain length in adult plants is the C_{30} aldehyde (32%).[109]

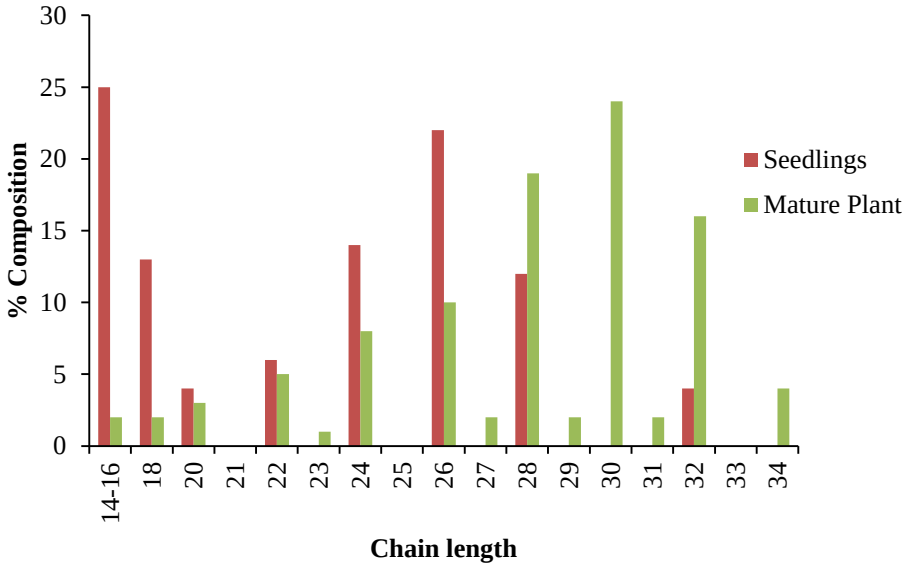


Figure 1-13 % composition of acids in maize.

Figure 1-13 indicates the % composition of acids in maize. In the seedlings, acids having a short chain length, C_{14} – C_{16}, dominate (25%) followed closely by C_{26} (22%). A chain lengthening trend is observed on moving from seedlings to mature plants, in

which a higher proportion of long-chain acids are found. The most common homologue is the C_{30} acid (24%) followed by the C_{28} acid (19%) and C_{32} acid (16%).[109]

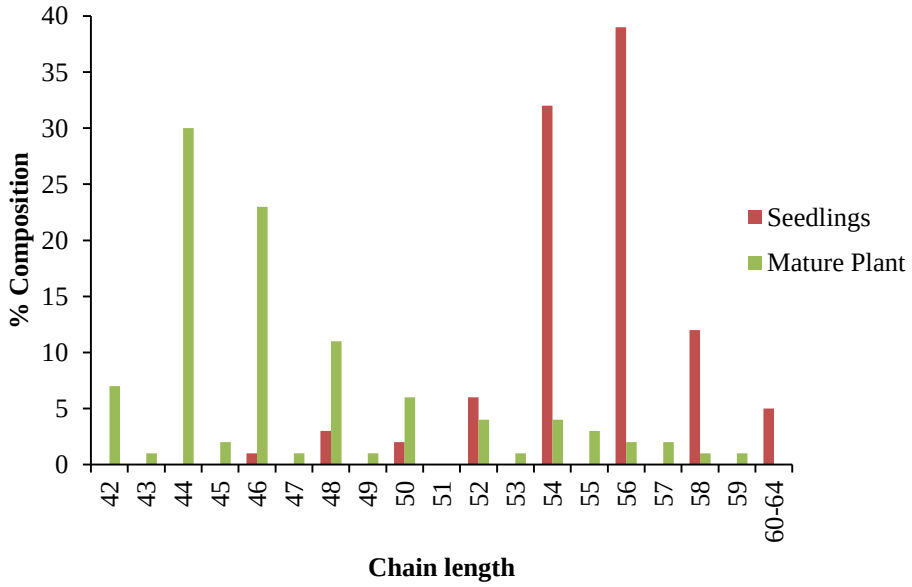


Figure 1-14 % composition of esters in maize.

From Figure 1-14, it can be seen that the seedlings demonstrate the highest specificity for the production of long chain homologues C_{54}, C_{56} and C_{58}, which together constitute 83% of the total ester portion. On the other hand, in adult plants, there is a preference for shorter chain esters, where C_{44} and C_{46} esters make up 53% of the total ester fraction. There is also an appreciable amount of odd-chain esters.[108, 109]

In the seedlings, there are generally one or two isomeric esters for the majority of ester chains. The dominant alcohol component for C_{48} – C_{60} esters is C_{32}.[110] In contrast, in adult plants there is a wide range of isomeric esters for each chain length. In addition, unlike what was observed in seedlings, there is no observable pattern between isomeric esters and alcohol chain length. In some cases it can be noted that the acid component brings about partial dominance of certain ester isomers, such as C_{20} and C_{22} acids in the C_{46}, C_{48}, C_{50} and C_{52} ester chains.[110] Besides linear esters, there were also small amounts of triterpenol esters present in the waxes of mature plants.[109]

1.10 Chemicals from sugarcane.

There are few publications which deal with the chemical composition of epicuticular waxes of sugarcane. The majority of work carried out focuses on the extraction of lignin from sugarcane and sugarcane bagasse.[111, 112]

Asikin *et al.* investigated the *n*-policosanol, long-chain aldehyde and wax ester content in the rinds, pith and whole stalk of a variety of sugarcane cultivars.[113] Soxhlet extractions were carried out using a mixture of hexane and methanol (20:1 *v/*v). They found that in the sugarcane wax, 55 – 60% was long-chain aldehydes and steryl esters, 32 – 40% was *n*-policosanols and small quantities of long-chain fatty acids and plant sterols. However, no information was provided on the chain lengths identified as well as the abundances of each chain length.[113]

The cyclohexane extraction of wastes obtained from sugarcane rum factories (distillation and fermentation of sugarcane juices) showed that long-chain alkanes (C_{19} – C_{33}) and wax esters (though no information of the type and quantity of each chain length was provided) were the main components in the wax extracts.[114] Minor compounds found were long-chain fatty acids, plant sterols, fatty acid methyl and ethyl esters as well as triterpene methyl esters.[114]

Lucas *et al.* investigated the extraction of long chain *n*-alcohols from sugarcane crude wax using scCO_2.[115] Sugarcane crude wax is a by-product of sugarcane production which is obtained from sugarcane filter mud by extracting with heptane. They carried out the supercritical extractions using a variety of temperatures (50 – 100 ^{o}C) and pressures (300 – 350 bar) and varied the ratio of KOH used during the saponification stage in order to optimise extraction yields.[115]

Results indicated that the optimal conditions for extraction were P = 350 bar, T = 100 ^{o}C and 20% KOH/crude wax.[122]

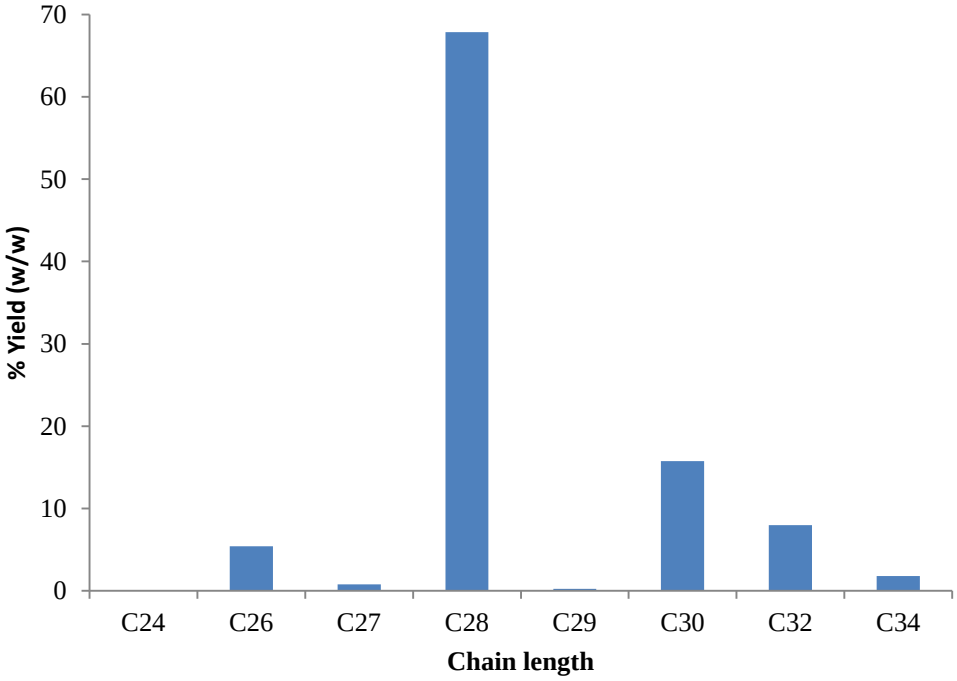


Figure 1-15 % yield of long-chain n-alcohols in sugarcane crude wax.

Figure 1-15 indicates the alcohol composition in sugarcane crude wax. The dominant fatty alcohol in the crude wax is octacosanol, accounting for approximately 67% (w/w) of the total alcohols, followed by triacontanol (≈ 16%), dotriacontanol (≈ 8%) and hexacosanol (≈ 5.5%). The remaining alcohols, i.e. tetratriacontanol, nonacosanol and heptacosanol are minor constituents.[115]

Prado *et al.* looked into developing a scale-up supercritical fluid extraction process for Brazilian raw materials, including sugarcane residue.[116] Once again, the work focussed on the crude wax obtained from the sugarcane filter mud. The extraction was carried out at 350 bar and 60 ^{o}C. The chemical composition (% *w/w*) of the sugarcane residue extracts is summarised in Table 1-11 and Figure 1-16. The three main compounds which were extracted and identified were octacosanol, stigmasterol and β-sitosterol.[116]

Table 1-11 Chemical composition (% *w/w*) of the sugarcane residue.

Time (min)	Separator	Octacosanol	Stigmasterol	β-sitosterol	Total
30	S_1	8.30	0.70	0.94	9.94
	S_2	1.51	0	0.27	1.78
60	S_1	4.78	0.74	1.04	6.55
	S_2	1.55	0.29	0.27	2.11
120	S_1	2.87	0.57	0.74	4.18
	S_2	1.22	0.24	0.26	1.72
180	S_1	2.48	0.45	0.54	3.46
	S_2	2.48	0.40	0.48	3.36

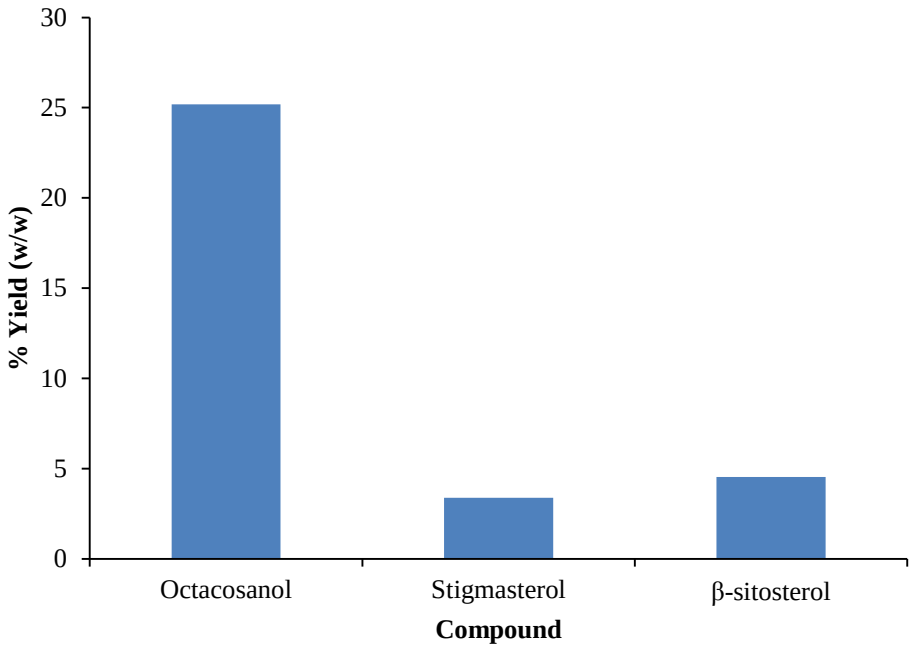


Figure 1-16 Chemical composition of sugarcane residue extracts.

1.12 Biorefinery Concept

This work will not only focus on the $scCO_2$ extraction of waxes from biomass but also on how the supercritical extraction of waxes can be incorporated within a holistic biorefinery. Essentially, a biorefinery is the conversion of biomass into various types of products including biomaterials, chemicals and energy. The aim of a biorefinery is to utilise all of the biomass wherever possible, maximising biomass throughput and generating low amounts of waste.[117] There are three different types of biorefineries:

(i) Phase I biorefinery: where only a single biomass is utilised to generate one single product using one process. There are a number of phase I biorefineries in Europe producing biodiesel from vegetable oil.

(ii) Phase II biorefineries: Phase II biorefineries again utilise a single feedstock; however, a number of products are obtained from this feedstock using a variety of processes. An example of a phase II biorefinery is Roquette in France, where over 600 carbohydrate derivatives are produced from cereal grains such as sweeteners, native and modified starches, polyols and bioethanol.

(iii) Phase III biorefinery: In this type of biorefinery, a multitude of feedstocks are utilised to produce a variety of chemical and energy products using various processes. This makes a phase III biorefinery the most advanced type of biorefinery. There are numerous advantages of a phase III biorefinery, including security of feedstock availability, various ways to make a profit and maximise returns (due to having various feedstocks) and the ability to change according to the market demands (again due to having various feedstocks). Currently, no phase III biorefineries exist; however extensive research is being conducted by the United States (US) and European Union (EU) and five types of phase III biorefinery systems have been brought forward: Whole crop biorefinery, lignocellulosic feedstock biorefinery, two-platform concept biorefinery, green biorefinery and marine biorefinery.[117]

The work carried out in this thesis will look at how the supercritical extraction of waxes can be incorporated in a phase II biorefinery for maize stover and miscanthus as shown in Figure 1-17.

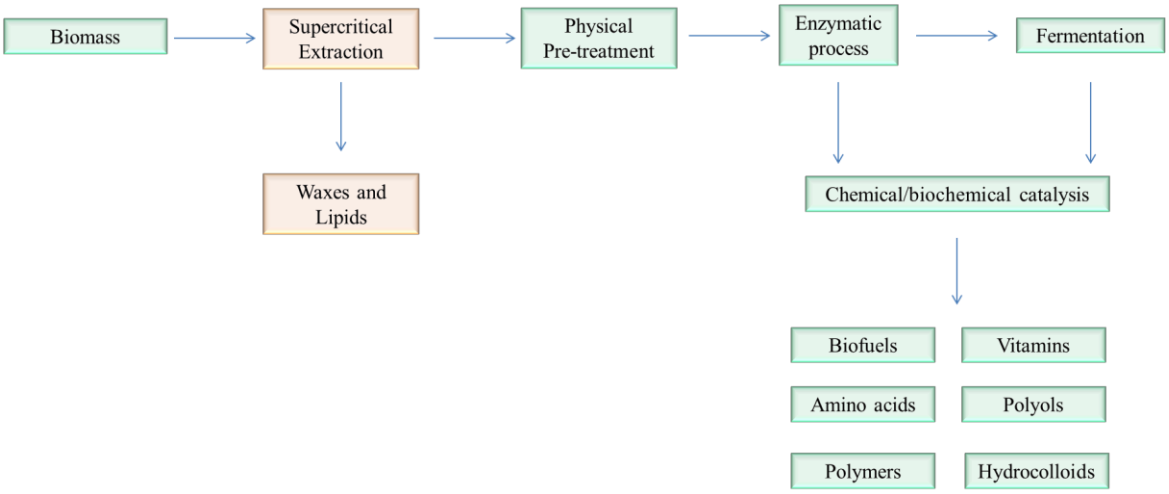


Figure 1-17 Incorporation of supercritical extraction of waxes within a biorefinery.

1.13 Introduction to work in this thesis

The above literature review has given an indication of the types of molecules that can be found in miscanthus, maize and sugarcane. However, in the case of maize, the lipid profile was carried out on the plant at different growth stages, rather than on the maize stover waste (post-harvesting). The extractive profile of the miscanthus stem was solely analysed; no information was provided on the extractive products that can be obtained from the leaves (a significant amount of miscanthus leaf waste is generated). Furthermore, no $scCO_2$ extractions on maize or miscanthus have been reported. A full characterisation of the lipids from different parts of the sugarcane plant (waste residues) has not been carried out. No fractionation of lipophilic products from C_4 plants is reported and the integration of natural product extraction from C_4 plants in a holistic biorefinery has not been previously considered. The economics of wax extraction using $scCO_2$ has not been previously investigated.

The work carried out in this thesis can be split into five main parts:

Firstly, natural wax extraction was carried out on different types of sugarcane waste (rind, leaf and bagasse) using $scCO_2$ and conventional Soxhlet with hexane. A full characterisation of the lipid extracts from each waste was carried out and the different solvent techniques (renewable vs non-renewable) were compared (Chapter 2).

Secondly, the $scCO_2$ extraction of valuable waxy compounds from maize stover as a pre-treatment technology in a holistic maize stover biorefinery has been investigated. $ScCO_2$ fractionation of the waxes was carried out and applications testing on one of the wax fractions was done. Optimisation of the $scCO_2$ process was also carried out. The

effect of $scCO_2$ extraction on the downstream processing of the maize stover (in a biorefinery) was also investigated (Chapter 3).

Thirdly, a full characterisation of heptane and $scCO_2$ extracts from different parts of *miscanthus giganteus* and *miscanthus sinensis* plants (leaves and stems) was carried out. The effect of $scCO_2$ extraction on the downstream processing of miscanthus was also investigated (Chapter 4).

Fourthly, the costs associated with $scCO_2$ extraction of waxes from miscanthus and maize stover on an industrial scale were assessed (Chapter 5).

Finally, the extraction of waxes from a C_3 biomass waste residue (hemp) from a hemp processing facility in North Yorkshire was carried out using heptane and $scCO_2$. The lipid profiles were characterised and optimisation of the $scCO_2$ process was conducted (Chapter 6).

Chapter 2

Sugarcane waste residues as a valuable source of hydrophobic molecules

2 Chapter 2

2.1 Introduction

Natural waxes are in increasingly high demand due to the diminishing supply of petroleum waxes, together with the preference for greener products by consumers. Currently, natural waxes contribute to only 4% of the total global wax production,[118] and there is a growing interest in obtaining them from biomass. In addition, waxes from agri-residues are preferable, so as not to compete with the agricultural industry or with food production.[118]

Waste from sugarcane (*saccharum officinarum L.*) is one possible source of natural waxes. Sugarcane is a C_4 plant that has experienced nearly a six fold increase in the global harvest from 1950 to 2007 (global total production of 1.5 billion tonnes).[47] The harvesting and processing of sugarcane produces a significant amount of residues including waste leaves, rind and bagasse. While the leaves are disposed of during sugarcane harvesting, the rind (the hard outer layer of the plant stalk) and the bagasse waste are obtained during sugarcane milling for sugar or ethanol production. Recently, traditional manual harvesting methods which require pre-burning of the sugarcane fields have come under environmental pressure resulting in an increase in mechanised green harvesting.[119] This has led to an appreciable increase in the quantities of sugarcane leaf trash produced.[119]

Sugarcane bagasse refers to the lignocellulosic residue remaining from conventional milling of sugarcane and is collected in large amounts after sugarcane processing.[47, 48] In a typical sugar mill, the processing of one metric ton of sugarcane gives rise to around 270 kg of bagasse (with 50% moisture), which equates to approximately 135 kg of dry matter.[49, 50]

Sugar and ethanol plants normally use approximately 50% of the residual bagasse to generate heat and power. The rest is normally stockpiled on site, posing an environmental problem to both the mills and the surrounding districts. This is because long-period storage could increase the risk of spontaneous combustion.[51] There have been various reports highlighting the use of sugarcane bagasse in a variety of applications, ranging from animal feed to the production of various industrial enzymes (cellulases, lipases etc.), chemicals, pulp and paper.[51, 120, 121]

Wax extraction from biomass is normally carried out using conventional solvents such as dichloromethane (DCM), chloroform, hexane and toluene. The use of these solvents is becoming restricted because of their various toxicological and environmental problems.[54] An excellent alternative solvent for the extraction of natural products is supercritical carbon dioxide (scCO2), since carbon dioxide (CO_2) is non-flammable, non-toxic and widely available.[74] Furthermore it is recyclable and doesn't require regulation.[74]

Herein, the supercritical extraction of waxes from sugarcane leaves, rind and bagasse was investigated. The hydrophobic molecules present in each sugarcane waste were characterised, quantified and their potential applications were included, since these molecules are added-value products.

2.2 Supercritical extraction wax yields

$ScCO_2$ extraction on the rind leaves and bagasse was carried out using a pressure of 350 bar and at 50 ^{o}C. The yields of lipophilic extractives are summarised in Figure 2-1. The highest yield of wax was obtained from the leaves which accounted for 1.60% of the dry biomass, followed by the rind (0.8%) and the stem (0.53%). The leaves have a much higher surface area to volume ratio and as such are more prone to loss of water and more susceptible to mechanical damage from various environmental factors. Therefore, larger quantities of wax are required in order to prevent loss of water by transpiration, minimise leaching losses and prevent mechanical damage due to environmental conditions. The leaf surface wax is particularly important for minimising the amount of water lost through evaporation, especially when the stomatal pores of the plant are closed in response to reduced turgor.

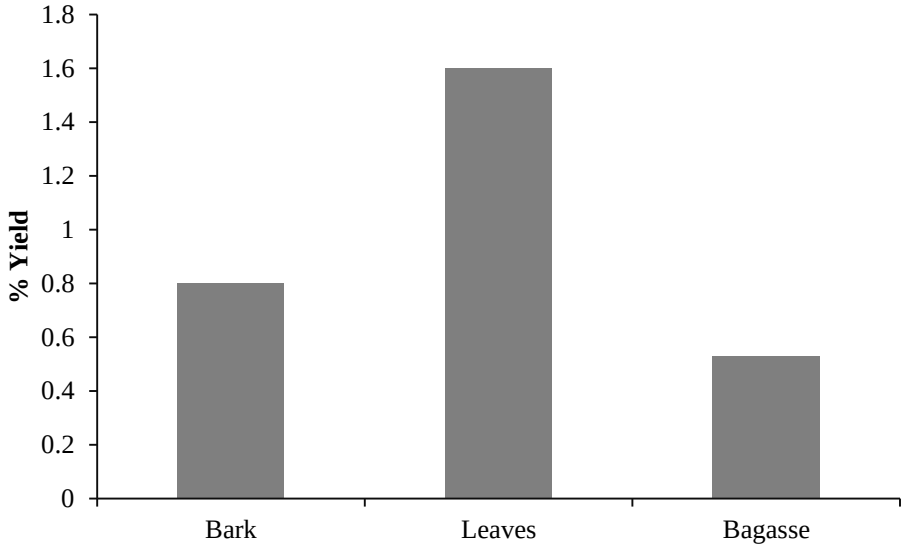


Figure 2-1 % Extraction yields of wax from sugarcane rind, leaves and bagasse.

2.3 Characterisation and quantification of lipids in different sugarcane waste

The primary analytical tools that were used for analysing the lipid components were gas chromatography (GC) and mass spectrometry (MS), with both field-ionisation mass spectrometry (FI-MS) and electron-ionisation mass spectrometry (EI-MS). The compounds were identified by interpretation of the mass spectra, whereby the molecular weights of the compounds were obtained from the FI-MS spectra while the fragmentation patterns from the EI-MS spectra were analysed to confirm the structure of the compound under investigation. Other methods implemented for identifying molecules include comparing the GC retention times of the unknown compounds with those of standards and directly comparing the EI-mass spectra of the unknown compounds with a NIST library (v. 2.2) and standards. Other analytical tools such as ^{13}C NMR were also utilised for the analysis of certain compounds.

2.3.1 Long chain Hydrocarbons (*n*-alkanes)

The long-chain *n*-alkanes were identified by means of gas chromatography coupled with mass spectrometry and by calculating Kovats indices. The Kovats index, originally developed by Kovats in 1958, is a retention index which is used for qualitative identification.[122] This retention index system is based on the *n*-alkanes series as the standard reference molecules since : (i) a wide range of boiling points are covered by *n*-alkanes (ii) Practically any column can be used to separate *n*-alkanes (iii) *n*-alkanes are readily obtained. (iv) *n*-alkanes show high chemical stability.[122]

For all temperatures and for any column used, the Kovats retention index for *n*-alkanes is defined as 100 times the number of carbon atoms making up the hydrocarbon chain. Thus, as an example, a KI value of 800 is assigned for octane. Therefore:

$$I = 100n$$

Where I = retention index

n = number of carbon atoms making up the hydrocarbon chain.

The Kovats index makes use of the linear relationship between the adjusted retention time and the retention index of a series of *n*-paraffins (number of C atoms x 100).[122]

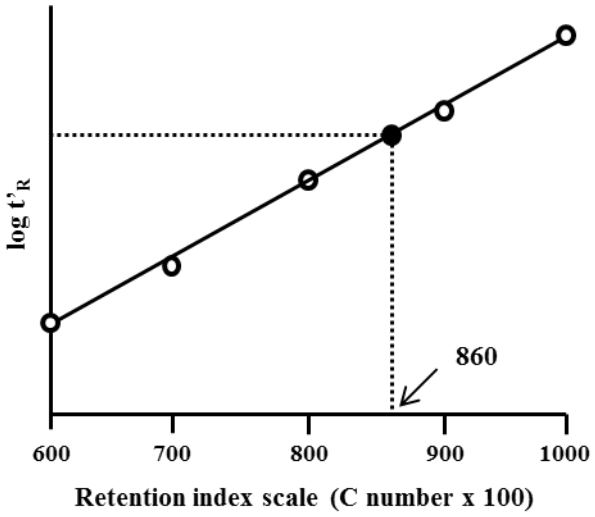


Figure 2-2 Linear relationship between the absolute retention time and the retention index.[122]

For an unknown compound that lies between a pair of linear *n*-alkanes, the retention index can either be calculated graphically or by using the following equation:

$$I = 100i\ \frac{\log V_{(g)(x)} - \log V_{(g)(n)}}{\log V_{(g)(n+i)} - \log V_{(g)(x)}}$$

Where $V_{(g)}$= specific retention volume

n = number of carbon atoms in the compound.

i = difference in numbers of carbon atoms of the reference materials.

In this equation, Kovats utilises the logarithms of the retention volumes and illustrated that the retention index (I) is linearly dependent on temperature.

The above equation was simplified by Van den dool and Kratz[123], who derived a more generalised equation for the retention index such that:

$$I = 100i\ \frac{X - M_{(n)}}{M_{(n+i)} - M_{(n)}} + 100n$$

Where i = difference in numbers of carbon atoms of the reference materials.

X = unknown compound retention time.

$M_{(n)}$ = reference material with *n* carbon atoms.

$M_{(n+i)}$ = reference material with *(n+i)* carbon atoms.

n = number of carbon atoms in the compound.[123]

Therefore calculating the KI values is a simple and efficient method for determining the chain-length of *n*-alkanes in a waxy sample.

In the EI-mass spectrum of long-chain alkanes (Figure 2-3), the molecular ion peak was visible for the majority of chain lengths but for higher-chained alkanes identified this was either absent or present in low intensities. The fragmentation patterns in the EI-mass spectra of long-chain alkanes consist of clusters of peaks, in which the corresponding peaks of each cluster are 14 mass units apart (CH_2). In each cluster, the most intense peak denotes a C_nH_{2n+1} fragment and is therefore found at *m/z* = 14_{n+1}. Hence the most intense peaks of each cluster are observed at *m/z* 43, 57, 71, 85, 99, 113 and 127. These are accompanied by an unsaturated ion series consisting of C_nH_{2n-1} fragments (*m/z* 41, 55, 69, 83, 97, 111 and 125). Since alkanes consisting of more than 8 carbon atoms have very similar EI-mass spectra, the molecular ion peak is required in order to identify the compound. Table 2-1 indicates *n*-alkanes that have been identified in the extractives along with their molecular weights and calculated KIs.

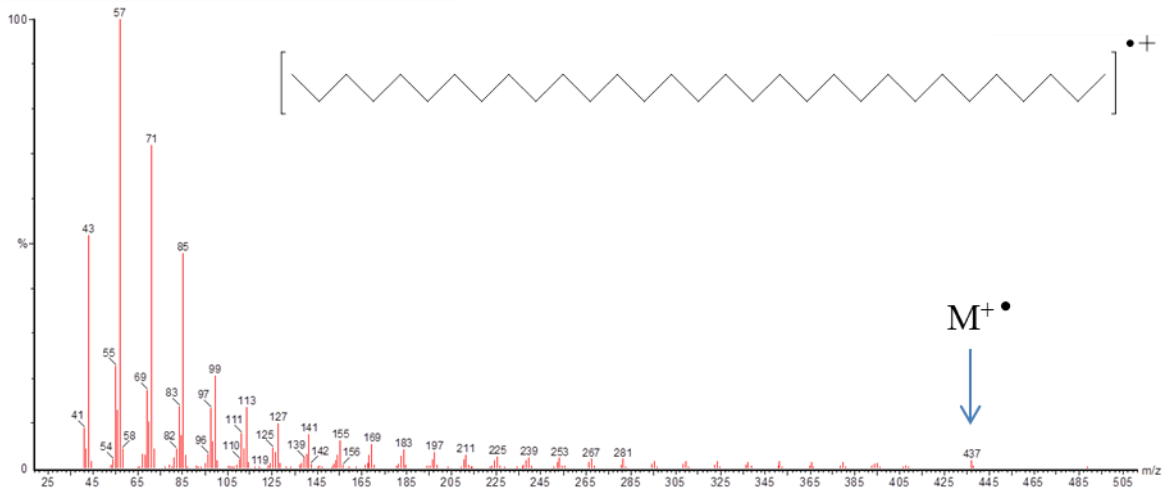


Figure 2-3 Mass fragmentation patter of Hentriacontane (C_{31} alkane)

Table 2-1 List of identified alkanes with their molecular weights and KI

Alkane	Molecular Weight	KI
Tricosane	324.6	2300
Pentacosane	352.7	2500
Heptacosane	380.7	2700
Nonacosane	408.8	2900
Hentriacontane	436.8	3100
Tritriacontane	464.9	3300
Pentatriacontane	492.9	3500

Table 2-2 Quantification of *n*-alkanes in the rind, leaf and bagasse extracts in µg/g of dry plant

***n*-hydrocarbons**	**Rind (µg/g of dry plant)**	**Leaves (µg/g of dry plant)**	**Bagasse (µg/g of dry plant)**
Tricosane	3.6 ±0.3	8.2 ±0.1	1 ±0.0004
Pentacosane	7.4 ±0.2	5 ±2.7	4.1 ±0.0001
Heptacosane	113.1 ±3.3	20.2 ±0.1	41.1 ±0.3
Octacosane	2 ±0.7	-	1.8 ±0.03
Nonacosane	41.4 ±8.2	45.5 ±0.5	22.5 ±0.3
Hentriacontane	315.8 ±23.3	167.2 ±2.4	33.6 ±1.7
Triatriacontane	-	303.6 ±13	32.4 ±0.5
Pentatriacontane	-	44.6 ±1.9	-
Total hydrocarbons	**483.3 ±36**	**594.3 ±20.7**	**136.5 ±2.8**

The rind extracts had the lowest range of hydrocarbons, with chain lengths of C_{23} to C_{31} detected. In the bagasse extract, in addition to the hydrocarbons found in the rind, tritriacontane (C_{33}) was detected while the leaf wax also contained pentatriacontane (C_{35}). The dominant hydrocarbon in the rind and bagasse waxes was found to be hentriacontane (315.8 ±23.3 ug/g of plant and 33.6 ±1.7 ug/g of dry plant respectively)

while triatriacontane (C_{33}) was the most abundant hydrocarbon in the leaves (303.6 ±13 ug/g of dry plant). The leaves had the highest amount of hydrocarbons with 594.3 ±20.7 µg/g of dry plant followed by the rind (483.3 ±36 µg/g of dry plant).

Long-chain hydrocarbons have been shown to display semiochemical properties, where they play a role in plant-insect interactions.[124] Semiochemicals refer to biochemical molecules that act as "messages" for insects and other organisms. There are two groups of semiochemicals; allelochemics and pheromones.[125] The former are involved in interactions between different species while the latter are involved in same-species interactions.[125]

Work has been carried out looking into the 'pseudocopulatory' behaviour of male bees, *Andrena nigroaena*, towards the flowers of *Ophrys sphegodes*.[124] Results have shown that this orchid synthesises a variety of chemical compounds which are present in the sex pheromone of *Andrena nigroaena* in similar abundances. Gas chromatography-electroantennographic data indicates that a total of 14 compounds are present in the orchids, which are found in the attractive odour sample of female bees which cause an electroantennographic response in the antennae of males. GC-MS data indicates that these compounds are saturated and unsaturated long-chain hydrocarbons have chain lengths which vary from C_{21} to C_{29}.[124] Work by Han demonstrated that an alkane fraction isolated from wheat straw wax can successfully be used as natural aphid insecticides.[126] Cereal aphids are insect pests that attack crops resulting in the reduction of both yield and quality of cereals.[127] They have been serious pests since at least the 18th century and the removal of aphids was regarded as being of economic importance in the 1950s.[127]

2.3.2 Long-chain fatty acids

The long-chain saturated fatty acids were identified using gas chromatography coupled with mass spectrometry. In the EI-mass spectra of long-chain acids (Figure 2-4), there are usually two series of peaks which arise as a result of cleavage at each C-C bond, whereby the charge remains either on the oxygen-containing fragment (*m/z* = 45, 59, 73, 87 etc.) or the alkyl fragment (*m/z* = 29, 43, 57, 71, 85 etc.).

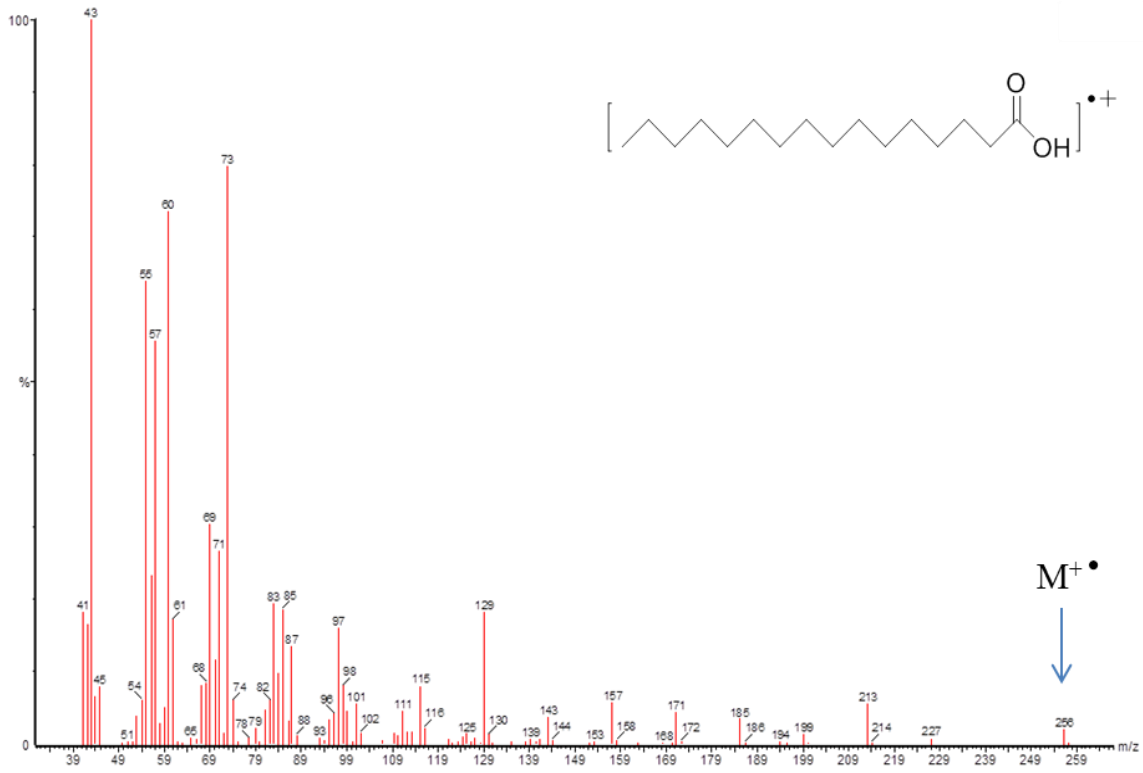
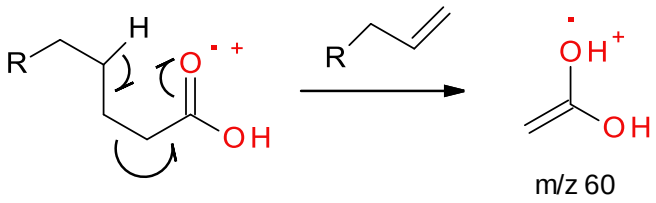


Figure 2-4 Mass spectrum of hexadecanoic acid (C_{16}) from the leaf extract.

The most characteristic peak is found at (*m/z* = 60) which occurs as a result of the McLafferty rearrangement, which is the most common fragmentation for long-chain fatty acids. The McLafferty rearrangement refers to γ-hydrogen transfer to a double-bonded atom, through a six-membered transition state, with β-bond cleavage.[128] Therefore, the McLafferty rearrangement occurs only with compounds that: (i) Contain a heteroatom (e.g. oxygen) (ii) Possess a π system (normally a double bond) (iii) Contain hydrogen atoms that are located γ to the heteroatom (iv) Have enough flexibility to allow close proximity of the γ-hydrogen to the heteroatom (the distance between the γ-hydrogen and the heteroatom must be less than 1.8×10^{-10} m). The acceptor group must also be in plane with the Cγ-H bond. The McLafferty rearrangement results in the formation of a stable enol radical cation and loss of a stable neutral molecule; in the case of acids, an alkene is lost.[128]



Scheme 2-1: McLafferty rearrangement.

Fractionation of the wax extracted from sugarcane leaves resulted in a fraction containing a number of saturated fatty acids as can be seen in Figure 2-5.

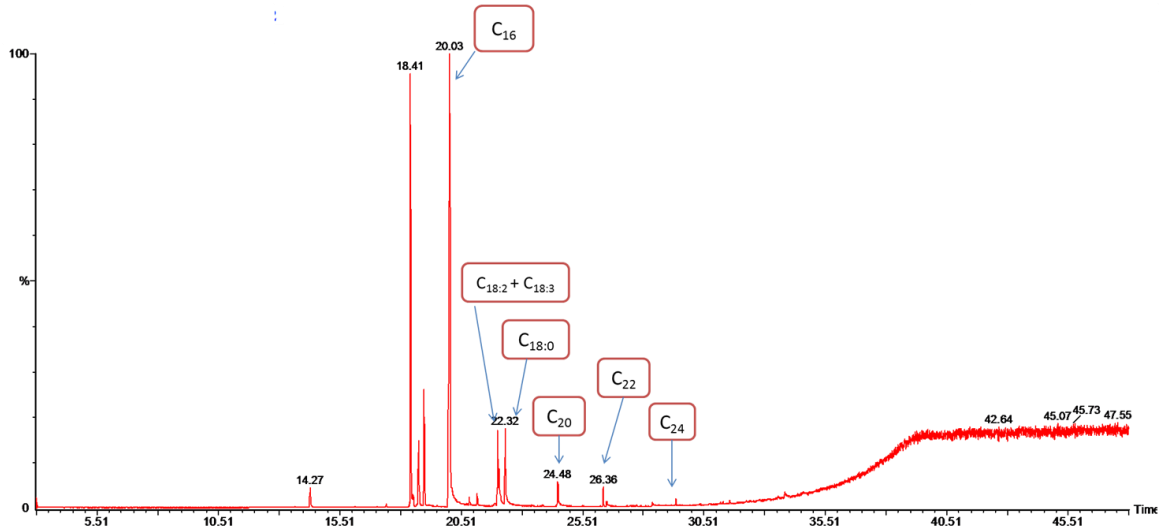


Figure 2-5 Fraction of leaf extract containing various saturated fatty acids.

Silylation of the polar carbonyl group allowed for the presence and identification of long-chain fatty acids having chain lengths of C_{26} or higher (these were not detected in the underivatised extracts) as well as determination of the low-molecular fatty acids (C_6 to C_{12}) and odd-chain fatty acids. Furthermore, silylation yielded the molecular ion of the TMS-ester derivative which enabled assigning molecular weights to the higher-chain fatty acids. Figure 2-6 is an EI-mass spectrum of a silylated C_{26} fatty acid from the leaf extract. The most abundant high-mass ion in the spectra of silylated fatty acids corresponds to the loss of a methyl group $[M-15]^+$ from the trimethysilyl group. In the silylated C_{26} acid this ion is found at m/z = 454.

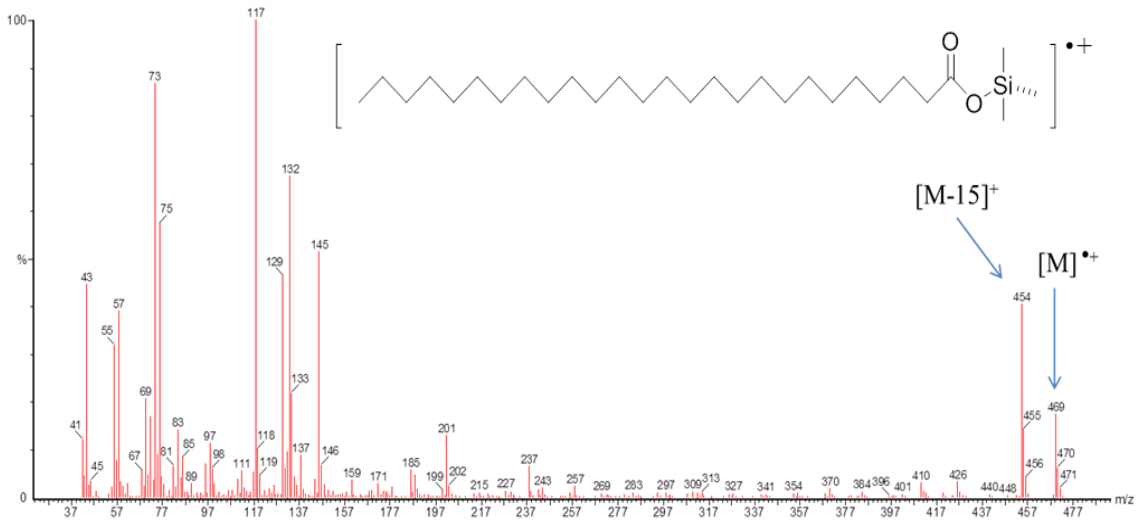


Figure 2-6 Mass fragmentation pattern of a silylated C26 acid from the leaf extract.

In the rind, only even-chained saturated fatty acids were detected having chain lengths ranging from C_6 to C_{24}. In the leaf and bagasse extracts a much wider distribution of chain lengths were identified ranging from C_6 to C_{30} and a large variety of odd-chain fatty acids were detected (C_7 to C_{25}) albeit in smaller quantities.

Table 2-3 indicates the type and quantity of saturated fatty acids found in the different wax extracts. Palmitic acid (C_{16}) was the dominant fatty acid in all extracts, where a high quantity was found in the leaf wax (866.8 ±4.2 µg of plant). Relatively high quantities of small chain fatty acids were found in the rind extract compared to the leaf and bagasse extracts, with C_6 and C_8 fatty acids found in concentrations of 17.2 ±1.6 and 25.1 ±1 µg/g of dry plant respectively. The leaf wax demonstrated the highest quantity of saturated fatty acids studied, with a total concentration of 1515 ±11.7 µg/g of dry plant, while the rind had the smallest amount (212.9 ±24.2 µg/g of dry plant).

Table 2-3 Type and quantity of saturated fatty acids in the rind, leaf and bagasse extracts in µg/g of dry plant.

Sat. fatty acids	Rind (µg/g of dry plant)	Leaves (µg/g of dry plant)	Bagasse (µg/g of dry plant)
Hexanoic acid	17.2 ±1.6	1.4 ±0.1	3.3 ±0.04
Heptanoic acid	-	1.3 ±0.05	0.3 ±0.03
Octanoic acid	25.1 ±1	2.8 ±0.1	1.2 ±0.05
Nonanoic acid	-	2.7 ±0.2	1.6 ±0.03
Decanoic acid	0.8±0.1	7.7	0.5
Dodecanoic acid	4.5 ±0.3	112.4 ±2.4	3.1
Tetradecanoic acid	3.5 ±0.4	104.5 ±0.3	3 ±0.04
Pentadecanoic acid	2.2 ±0.2	13.1	1.9 ±0.03
Hexadecanoic acid	126.6 ±15.3	866.8 ±4.2	99.9 ±1.1
Heptadecanoic acid	-	29.5 ±0.1	1.8 ±0.04
Octadecanoic acid	15.9 ±1.7	94.1±0.7	13.8 ±1.4
Nonanoic acid	-	9.1 ±0.3	0.4
Eicosanoic acid	14 ±1.2	119.9 ±1.1	18.1 ±0.3
Heneicosanoic acid	-	8.2 ±0.2	0.7 ±0.1
Docosanoic acid	TR	48.7 ±0.9	6.9 ±0.1
Tricosanoic acid	-	17.5	4.3 ±0.1
Tetracosanoic acid	3.1 ±2.4	75.3 ±1.1	6.2 ±0.1
Pentacosanoic acid	-	-	10.5 ±4.2
Hexacosanoic acid	-	TR*	TR
Octacosanoic acid	-	TR	65.1 ±2.4
Tricontanoic acid	-	TR	33.7 ±0.3
Total saturated fatty acids	**212.9 ±24.2**	**1515 ±11.7**	**277.3 ±10.4**

TR* = *trace* amounts

Saturated fatty acids have the potential to be used in a wide range of applications including soaps, detergents, cleaning polishes and lubricating oils.[129, 130]

Three unsaturated acids were identified in the extracts; oleic acid (one double bond, $C_{18:1}$), linoleic acid (two double bonds, $C_{18:2}$) and linolenic acid (three double bonds, $C_{18:3}$) were also identified in lipophilic extracts. These were identified through molecular weights of each compound as obtained by GC-FI in addition to EI-mass fragmentation patterns. The unsaturated fatty acid concentration in the various sugarcane extracts is summarised in Table 2-4.

Table 2-4 Type and quantity of unsaturated fatty acids in the rind, leaves and bagasse in µg/g of dry plant.

Unsat. fatty acids	Rind (µg/g of dry plant)	Leaves (µg/g of dry plant)	Bagasse (µg/g of dry plant)
9-octadecenoic acid	3.4	8.2 ±0.2	1.3 ±0.1
9,12-Octadecadienoic acid	3.2 ±0.4	215.8 ±2.1	19.9 ±10.2
9,12,15-Octadecatrienoic acid	1.5 ±0.2	197.2 ±2.2	21.3 ±8.5
Total unsaturated fatty acids	**8.1 ±0.6**	**421.2 ±4.5**	**42.5 ±18.8**

The leaf wax contained considerable amounts of unsaturated C_{18} fatty acids with a total composition of 421.2 ±4.5 µg/g of dry plant while in contrast, there was very little unsaturated fatty acids in the rind wax (8.1 ±0.6 µg/g of dry plant). In the supercritical extraction of sugarcane waxes from rum factories, oleic acid and linoleic acid were detected in the sugarcane wastes, comprising 15% and 2% of the total fatty acids respectively, while no linolenic acid was detected.[114]

Polyunsaturated fatty acids are known to have an effect on serum cholesterol in humans.[131] The hypocholesterolemic effect of linoleic acid has been well established.[132-134] Horrobin and Huang have shown that increasing the intake of linoleic in one's diet leads to a reduction in plasma cholesterol, though large amounts need to be consumed.[133] It is thought that a metabolite of linoleic acid, which is metabolised in the body via a number of routes, brings about this cholesterol-lowering effect. Studies

indicate that in normolipidemic men, α-linolenic acid is just as effective in lowering blood cholesterol as linoleic acid.[134] Research has shown that different types of diets, which varied in the composition of unsaturated fatty acids, had similar cholesterol-lowering results. Other studies have also shown that an α-linolenic acid-rich diet has significant cardioprotective effects.[135]

Furthermore, linoleic acid, α-linolenic acid and other polyunsaturated fatty acids could be used as platform molecules to produce a variety of bio-transformation products that can be used for a variety of applications as summarised in Figure 2-7.[131]

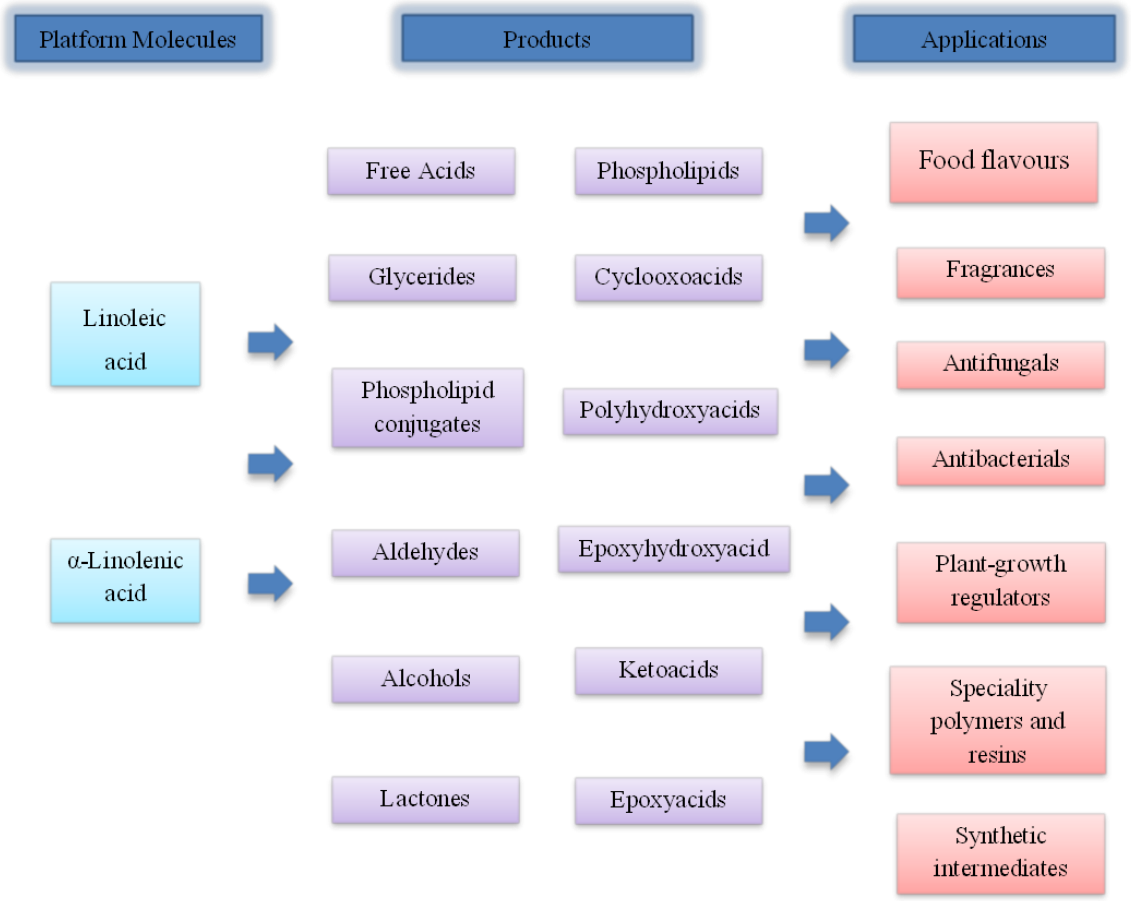


Figure 2-7 Applications of unsaturated fatty acids.[131]

In all extracts, small quantities of dicarboxylic acids were detected. The rind extract had dicarboxylic acids ranging from C_8 to C_{10} with C_9 dioic acid (azelaic acid) being the dominant chain length while in the bagasse and leaf wax, only azelaic acid was identified. The characteristic mass fragments of azelaic acid may be seen in Figure 2-8.

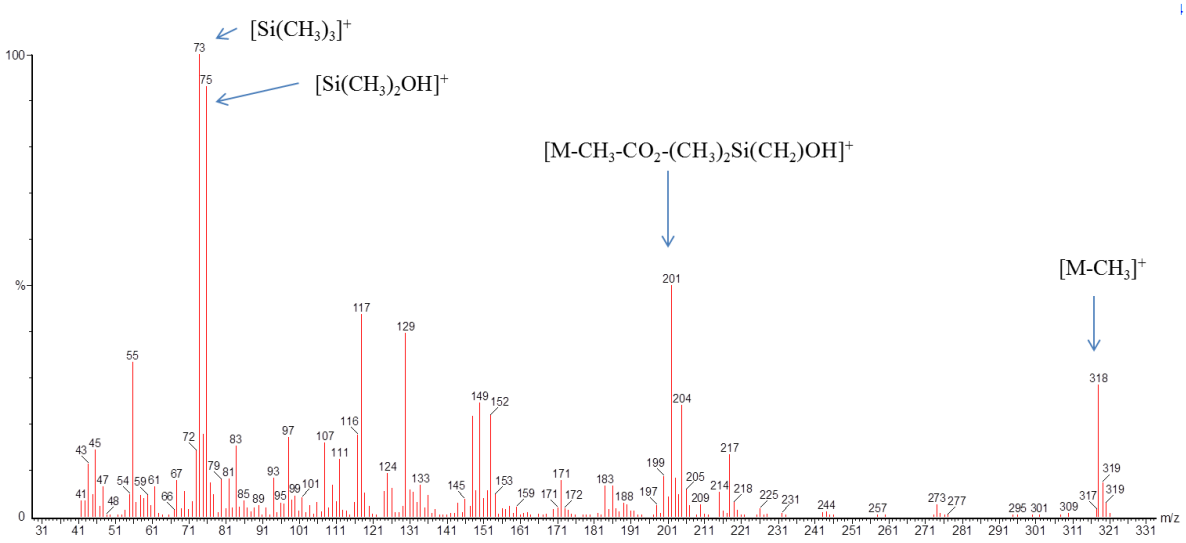


Figure 2-8 Mass spectrum of silylated azelaic acid from rind wax.

In plants, dicarboxylic acids play a role in systemic defense responses when plants are attacked by pathogens.[136] Although quite polar, the solubility of dicarboxylic acids such as azelaic acid has shown to increase in $scCO_2$ with increasing temperature once the crossover pressure has been reached (170 – 200 bar).[137] Table 2-5 summarises the types and quantities of dicarboxylic acids present in the extracts. No dicarboxylic acids have been previously recorded in the literature.[113, 114, 138]

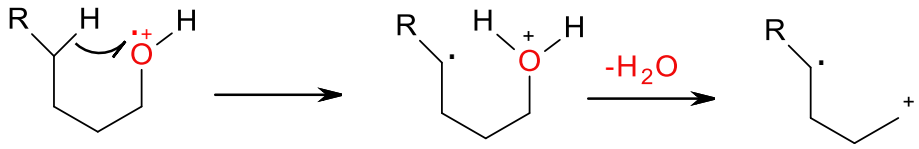
Table 2-5 Distribution and quantity of dicarboxylic acids present in rind, leaf and bagasse extracts, in µg/g of dry plant.

Dicarboxylic acid type	Rind (µg/g of dry plant)	Leaf (µg/g of dry plant)	Bagasse (µg/g of dry plant)
Octanedioic acid	2.2 ±0.4	-	-
Nonanedioic acid	8.2 ±1.5	11.9 ±0.8	3.7 ±0.1
Decanedioic acid	0.7 ±0.1	-	-
Total saturated difatty acids	**11.1 ±2**	**11.9 ±0.8**	**3.7 ±0.1**

Azelaic acid (nonanedioic acid) has been shown to display significant pharmaceutical properties. It is very effective in treating comedonal and inflammatory acne and has also shown to have a cytotoxic and antiproliferative effect on the human malignant melanocyte.[139] Furthermore, sebacic and azelaic acid esters are utilised as plasticisers in a variety of applications.[130, 140]

2.3.3 Long chain fatty alcohols

From the FI-mass spectrum of a typical fatty alcohol, there is a peak that occurs at M – 18 resulting from loss of water. This is a distinct and prominent peak found in the spectra of primary alcohols. A mechanism for this elimination by electron-impact has been deduced, involving the loss of a δ-hydrogen. The mechanism is highlighted in Scheme 2-2. The thermal decomposition of higher alcohols on hot inlet surfaces often exaggerates the M – 18 peak.



Scheme 2-2: Loss of water resulting in the formation of the M-18 ion.

Furthermore silylation of long-chain alcohols gives EI-mass spectra where the $[M-15]^+$ ion, corresponding to the loss of a methyl group from the trimethylsilyl ether group, is the most prominent peak in the spectrum as shown in Figure 2-9, which is a typical mass spectrum for a silylated C_{28} alcohol.

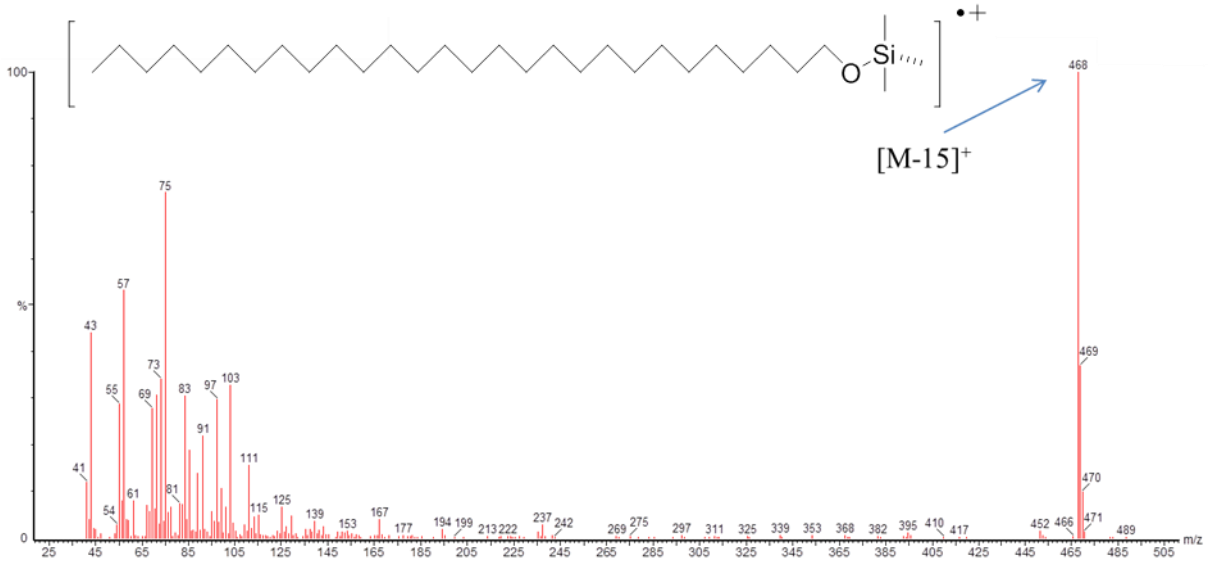


Figure 2-9 Mass spectrum of silylated 1-octacosanol from rind wax.

Table 2-6 Distribution and quantity of long chain fatty alcohols present in rind, leaf and bagasse extracts, in µg/g of dry plant.

Long-chain alcohol type	Rind (µg/g of dry plant)	Leaf (µg/g of dry plant)	Bagasse (µg/g of dry plant)
Tetracosanol	4.5 ±0.4	-	3.1 ±0.1
Hexacosanol	129.1 ±14.5	19.6 ±1.5	43.3 ±0.2
Octacosanol	1902.8 ±242.3	110 ±3.3	716.7 ±4.7
Triacontanol	142.7 ±19	90.9 ±1.1	91.5 ±1.4
Dotriacontanol	23.3 ±1.7	393.2 ±2.1	50.5 ±1.1
Tetratriacontanol	5.8 ±0.6	-	8.5 ±2.4
Total saturated fatty alcohols	**2208.2 ±278.5**	**613.7 ±8**	**913.6 ±9.9**

Both the rind and bagasse extracts had the widest distribution of long-chain fatty alcohols (*n*-policosanols) with chain lengths ranging from C_{24} to C_{34}. Large quantities of *n*-policosanols were detected in the rind wax extract, with total concentrations of 2208.2 ±278.5 µg/g of plant. 1-octacosanol was found to dominate the rind and bagasse extracts, with concentrations of 1902.8 ±242.3 and 716.7 ±4.7 µg/g of dry plant respectively which is in agreement with prior literature on the composition of fatty alcohols from sugarcane.[115] However, in the leaf wax, the dominant alcohol was found to be 1-dotriacontanol (393.2 ±2.1 µg/g of dry plant).

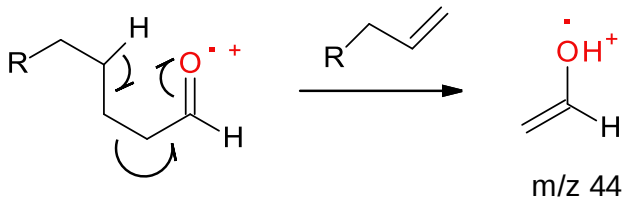
Policosanols have a wide variety of potential applications, most notably in the prevention and treatment of a variety of cardiovascular-related conditions such as poor arterial function, hypercholesterolemia, poor antioxidant status and intermittent claudication.[141] Policosanol therapy has been found to improve risk factors that are linked with arteriosclerosis which leads to significant improvement in cardiovascular health. Policosanols also exhibit anti-platelet effects, whereby platelet aggregations are significantly reduced when policosanols are administered. Platelets play a vital role in the formation of blood clots, which could cause a decrease in the rate of blood flow leading to an embolism or stroke.[141] The reduction of serum cholesterol was found to be more effective when administering policosanol supplements than when taking prescription medications.[142] It has also been suggested that policosanols can act as

potent antioxidants, inhibiting low-density lipoprotein (LDL)-cholesterol peroxidation.[141, 143]

Policosanols were found to be ideal components in cosmetics as anti-acne agents, as an emollient and for the control of sebum secretion.[144]

2.3.4 Long-chain fatty aldehydes

The long-chain fatty aldehydes were determined by a combination of GC and GC-FI data. The FI spectrum showed the molecular weight of the compound and a peak that occurs at M – 18 resulting from loss of water,characteristic of aldehydes and alcohols. In order to determine which of the two functional groups was present the sample was silylated. Silylation of the sample results in a shift in retention time of the alcohol peak in the silylated extract when compared to the underivatised extract while the aldehyde peak does not shift as it does not undergo silylation. In addition, aldehydes can undergo McLafferty rearrangement to give an ion at *m/z* 44.



Scheme 2-3: McLafferty rearrangement in aldehydes.

In the rind wax, long-chain fatty aldehydes having even chain lengths varying from C_{26} to C_{36} were identified while in the leaf wax, chain lengths of C_{24} to C_{32} were detected. The bagasse wax had aldehyde chain lengths of C_{26} to C_{34}.

Table 2-7 Distribution and quantity of long chain fatty aldehydes present in rind, leaf and bagasse extracts, in µg/g of dry plant.

Long-chain aldehyde type	Rind (µg/g of dry plant)	Leaf (µg/g of dry plant)	Bagasse (µg/g of dry plant)
Tetracosanal	-	23.6 ±1.3	-
Hexacosanal	137.5 ±19.8	35.2 ±4.5	36.6 ±0.4
Octacosanal	3001.5 ±694.8	81.3 ±11.1	807.1 ±5.8
Triacontanal	825.6 ±315.9	199.3 ±14.1	260.3 ±2
Dotriacontanal	286.7 ±102.2	TR	111.7 ±1.4
Tetratriacontanal	177.3 ±68.8	-	78.8 ±0.1
Hexatriacontanal	16.4 ±6.1	-	-
Total saturated fatty aldehydes	**4445 ±1207.6**	**339.4 ±31**	**1294.5 ±9.7**

Substantial quantities of long-chain fatty aldehydes were present in the rind extract (4445 ±1207.6 µg/g of dry plant). Previous work has shown that long-chain fatty aldehydes are abundant in sugarcane waxes.[113, 114, 145-147] The bagasse wax also exhibited significant quantities of long-chain fatty aldehydes (1294.5 ±9.7 µg/g of dry plant). Octacosanal was the most abundant fatty aldehyde in the rind and bagasse extracts while triacontanal predominated in the leaf extracts.

Long-chain fatty aldehydes, such as octacosanal, have been found to have health benefits, as effective agents for preventing and treating osteoporosis.[148] It has been shown that these long-chain fatty aldehydes can be safely and effectively administered in food and drink.[148]

2.3.5 Wax Esters

Previous literature on the hydrophobic composition of sugarcane waxes gave no information on the type and quantity of wax esters present in sugarcane wax.[49, 113, 114, 120] In this study, wax esters have been identified in the sugarcane waxes albeit in relatively small quantities. The molecular ion of wax esters is a key component in the determination of the wax ester chain length.

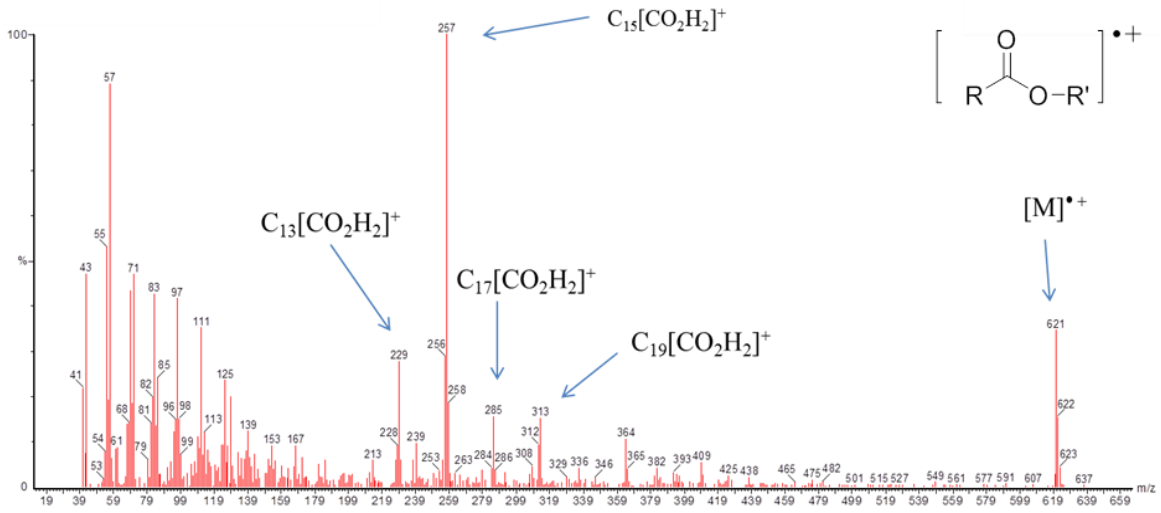
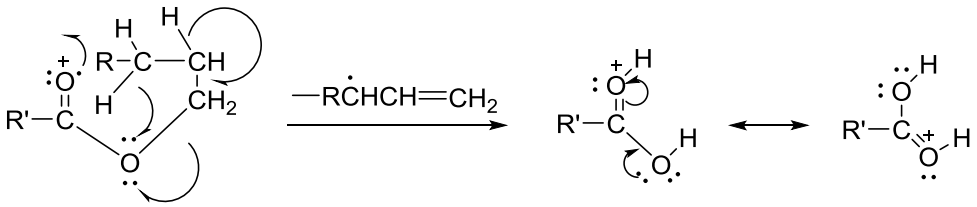


Figure 2-10 Mass spectrum of a C_{42} wax ester from the rind extract.

Figure 2-10 is a typical mass spectrum for a wax ester of chain length C_{42}. The acid moiety shows a diagnostic peak at *m/z* 257. This peak is a result of the ion $[RCO_2H]^+$, a protonated carboxylic acid residue. A number of studies have looked into the formation of this ion and it is thought to arise as a result of the transfer of two protons to the acid moiety and the elimination of the alkyl group of the alcohol moiety resulting in the protonated acid.[149-152] The proposed mechanism for the formation of this peak is found in scheme 2-4. The base peak, together with the molecular ion, is the most important peak in the spectrum since the mass of the alcohol moiety can subsequently be deduced.



Scheme 2-4: Formation of protonated carboxylic acid residue.

The other peaks at m/z 229, 285 and 313 also arise as a result of a rearrangement process where protons are transferred from the alcohol chain to the acid chain and correspond to protonated tetradecanoic, octadecanoic and eicosanoic acids respectively. This indicates that for each wax ester chain length there are different isomers having different acid : alcohol moieties.

Table 2-8 Distribution and quantity of wax esters present in rind, leaf and bagasse extracts, in µg/g of dry plant.

Wax Ester Type	Rind (µg/g of dry plant)	Leaf (µg/g of dry plant)	Bagasse (µg/g of dry plant)
Wax ester C_{38}	-	20.6 ±0.7	1.2
Wax ester C_{40}	1.4 ±0.2	12.6 ±2	2.8
Wax ester C_{42}	6.9 ±1.2	15.1 ±1.6	8.4 ±0.3
Wax ester C_{43}	1.7 ±0.3	8.2 ±0.4	3.1
Wax ester C_{44}	37.2 ±2.9	41.1 ±0.3	46.7 ±0.6
Wax ester C_{45}	1.6 ±0.1	14.7 ±1.8	6 ±0.2
Wax ester C_{46}	14 ±2.1	37.8 ±1.9	28 ±0.2
Wax ester C_{47}	1.4 ±0.1	2.8 ±0.2	3.4 ±0.2
Wax ester C_{48}	11 ±1.1	54.2 ±3.9	26.6 ±0.4
Wax ester C_{49}	0.8 ±0.2	4.3 ±0.4	3.5 ±0.1
Wax ester C_{50}	6.2 ±0.1	29 ±4.2	14.5 ±0.2
Wax ester C_{51}	1.1 ±0.1	-	3.7 ±0.1
Wax ester C_{52}	3.7 ±0.4	20.6 ±0.7	11.1 ±0.1
Wax ester C_{53}	1.3 ±0.1	-	3.3 ±0.1

Wax ester C_{54}	3.4 ±0.6	11 ±0.6	7.1 ±2.7
Wax ester C_{55}	2.5 ±0.2	-	2.2 ±0.8
Wax ester C_{56}	18.4 ±4.6	TR	17.6 ±1.7
Wax ester C_{58}	7.3 ±3.1	TR	7 ±0.3
Total Wax esters	**119.9 ±17.4**	**272 ±18.7**	**196.2 ±7.9**

2.3.6 Sterols

Phytosterols are present in the leaf and bagasse lipophilic extractives. Three phytosterols were determined in the bagasse wax; β-sitosterol, stigmasterol and campesterol while the former two where present in the leaf wax. The structures of the three phytosterols in the sugarcane extractives are shown below (Figure 2-11). Stigmasterol and campesterol are derived from β-sitosterol originally and therefore all sterols are similar in structure.[153]

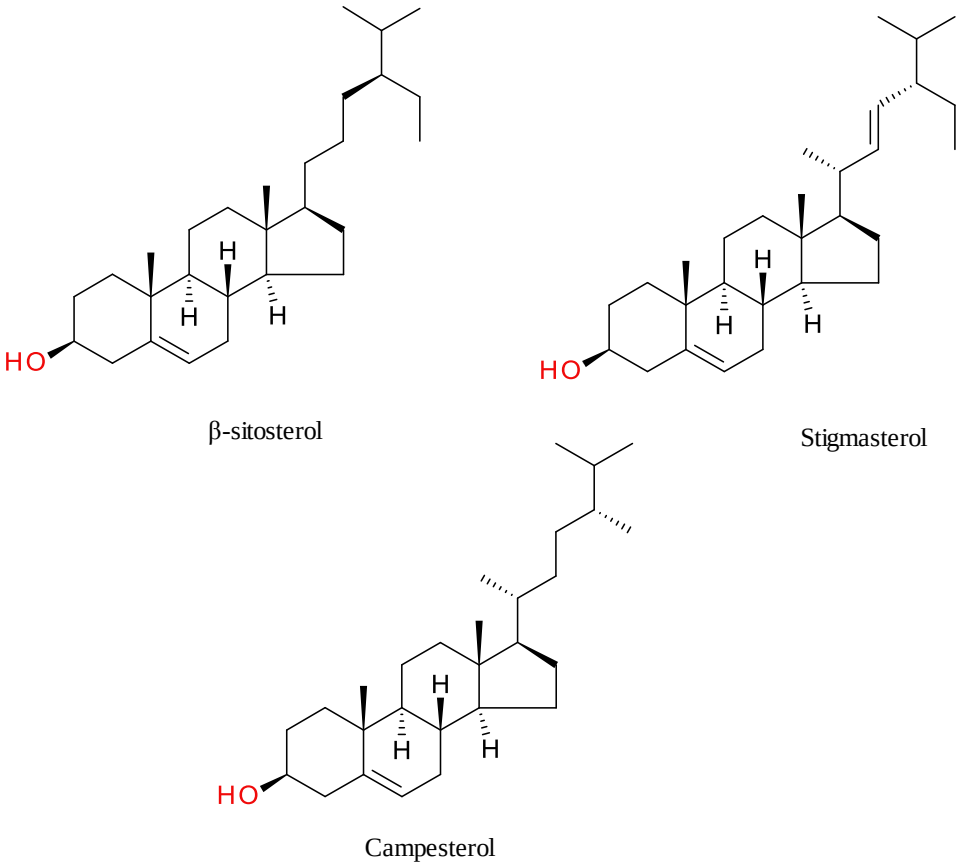


Figure 2-11 Structures of sterols present in the leaf and bagasse extracts.

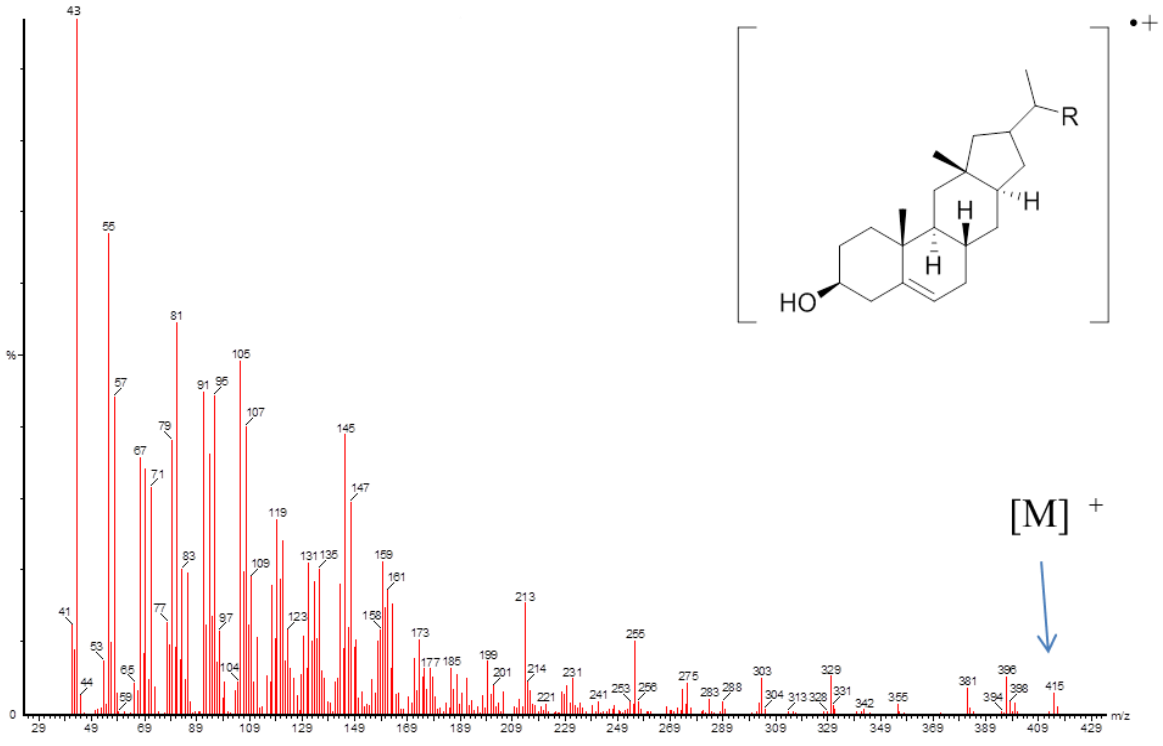
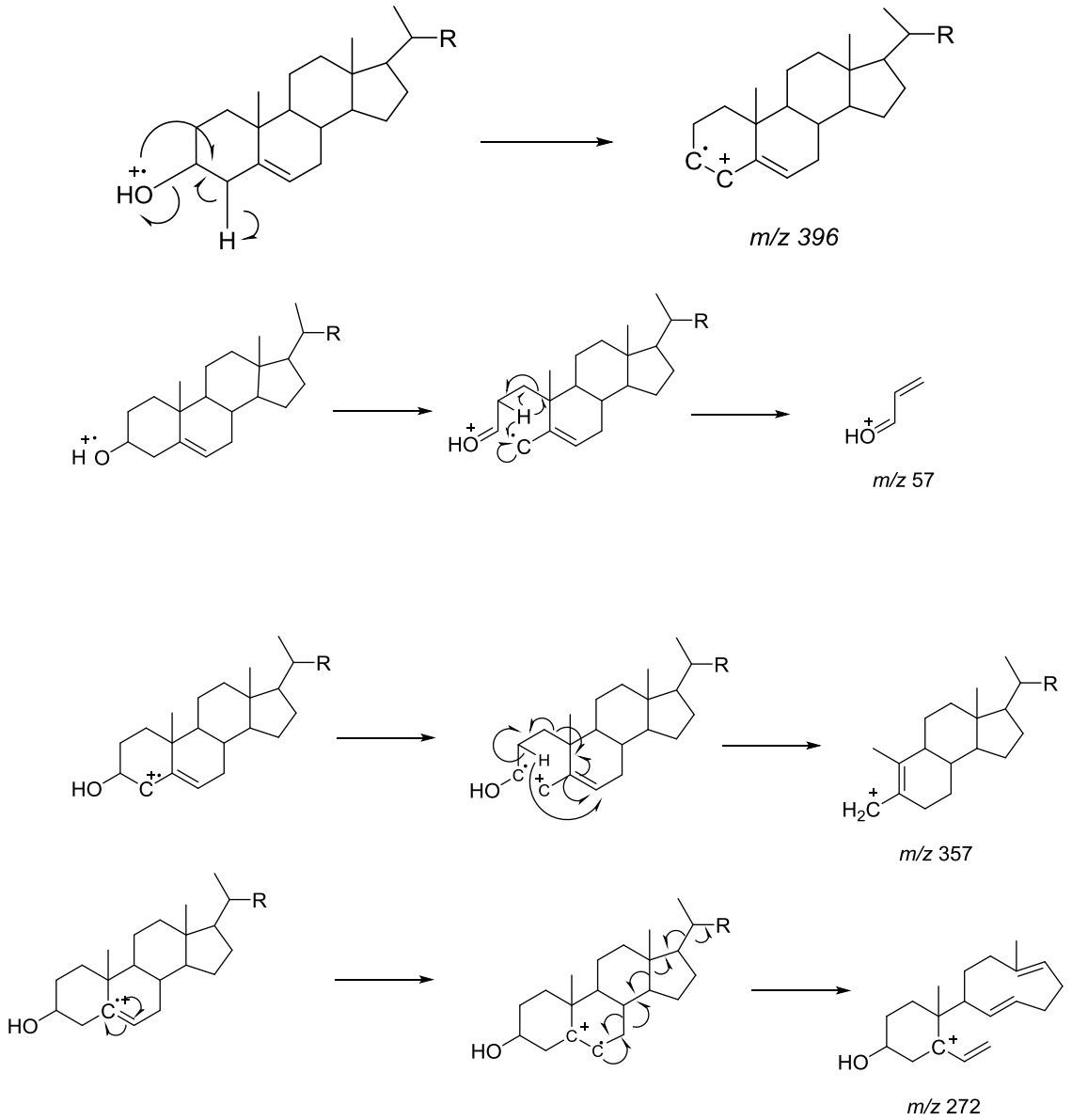


Figure 2-12 Mass spectrum of β-sitosterol from leaf wax.

The three phytosterols were determined by structure elucidation using GC-EI and by means of standards. Figure 2-12 illustrates the fragmentation pattern of β-sitosterol. The OH group situated on the first carbon ring can undergo a dehydration step giving rise to a stable conjugated cation with *m/*z = 396. A characteristic peak at *m/z* = 57 arises as a result of β-cleavage of the ring and subsequent formation of a diene. β-cleavage of the same ring followed by hydrogen migration results in a 3-rings ion giving a characteristic mass ion at *m/z* = 357. A stable tertiary cation with *m/z* = 272 can arise as a result of the cleavage of three rings.



Scheme 2-5: Formation of major ions *m/z* = 397, 57, 357 and 272 in sterols.

Table 2-9 Distribution and quantity of sterols present in rind, leaf and bagasse extracts, in µg/g of dry plant.

Types of sterol	Rind (µg/g of dry plant)	Leaf (µg/g of dry plant)	Bagasse (µg/g of dry plant)
Campesterol	-	-	80.2 ±7.3
Stigmasterol	-	464.4 ±0.8	79.9 ±23.3
β-Sitosterol	-	623.4 ±114.9	115 ±6.1
Total sterols	**-**	**1087.8 ±115.7**	**275.1 ±36.7**

The leaf wax had the largest quantity of sterols with 1087.8 ±115.7 µg/g of dry plant. Campesterol was only found in the bagasse wax while no sterols were found in the rind wax.

Phytosterols are of particular interest as they have a variety of potential biological and physiological applications. The three main phytosterols that are present in the human diet are β-sitosterol, campesterol and stigmasterol.[154] These sterols are widely known to act as efficient anticancer compounds.[154] It has been estimated that the risk of cancer can significantly decrease by as much as 20% with a phytosterol-enriched diet.[17, 155-161]

β-sitosterol may also have a possible preventative role in patients suffering from benign prostatic hypertrophy (BPH), which is an enlargement of the central area of the prostate.[161, 162] Dosages of 20 mg or 65 mg of β-sitosterol were given to patients with BPH three times daily. The studies indicated that symptomatic improvements were observed in prostate function.[161, 162]

Another important application of phytosterols is their involvement in cholesterol metabolism and atherosclerosis. It is known that phytosterols are effective in reducing plasma LDL-cholesterol levels with minimal side-effects and without significantly changing high-density (HDL)-cholesterol and triglycerides.[163]

2.3.7 Triterpenoids

It is very difficult to identify triterpenoid compounds found in wax extracts as the majority of plants contain a large number of different triterpenoids that are very similar structurally and have similar polarities. In addition, a number of them are isomers which makes their separation all the more difficult. The determination of triterpenoids found in the wax extracts of sugarcane was attempted by fractionation of the crude wax and

isolation of the triterpenoid fractions. This was carried out using a combination of thin-layer chromatography (TLC), flash chromatography and GC. The primary aim was to try and isolate the triterpenoid fraction, which will be discussed later in this chapter.

TLC was used in order to find a suitable solvent system to carry out the fractionation. A hexane: ethyl acetate solvent system with a ratio of 3:1 was found to give adequate separation of the various groups of compounds making up the sugarcane wax. This solvent system was selected and used in order to separate the various groups of compounds via flash chromatography. Figure 2-13 is a photograph illustrating the fractionation of the sugarcane wax. The different fractions were collected in test tubes as shown in Figure 2-14.

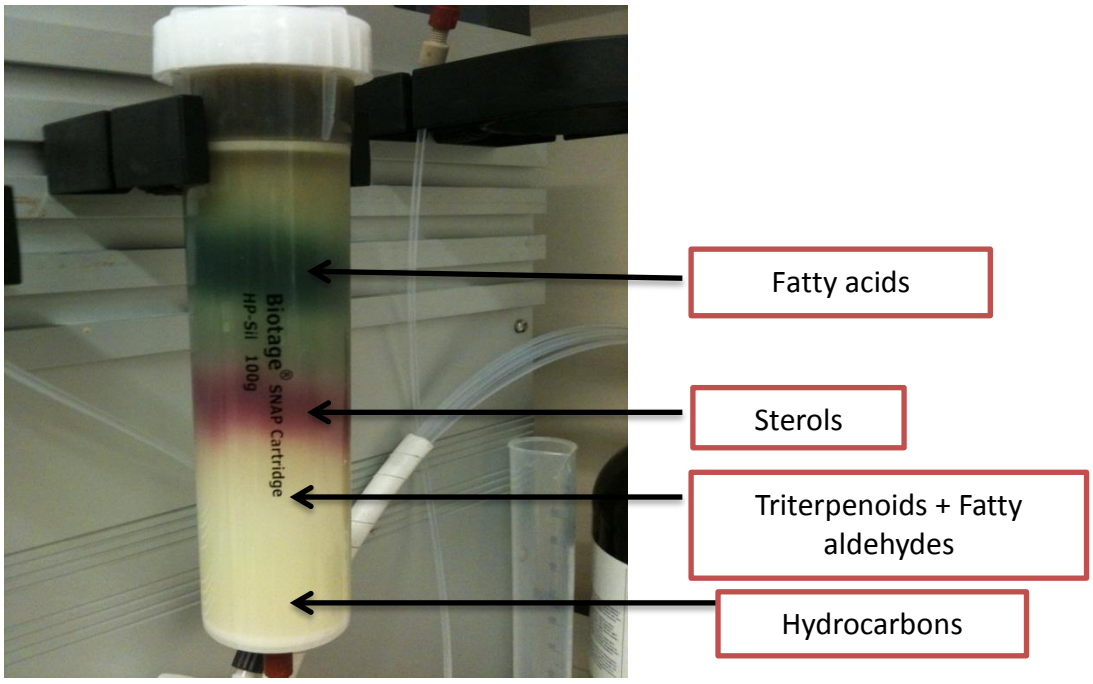


Figure 2-13 Photograph showing the fractionation of the different groups making up the sugarcane wax.

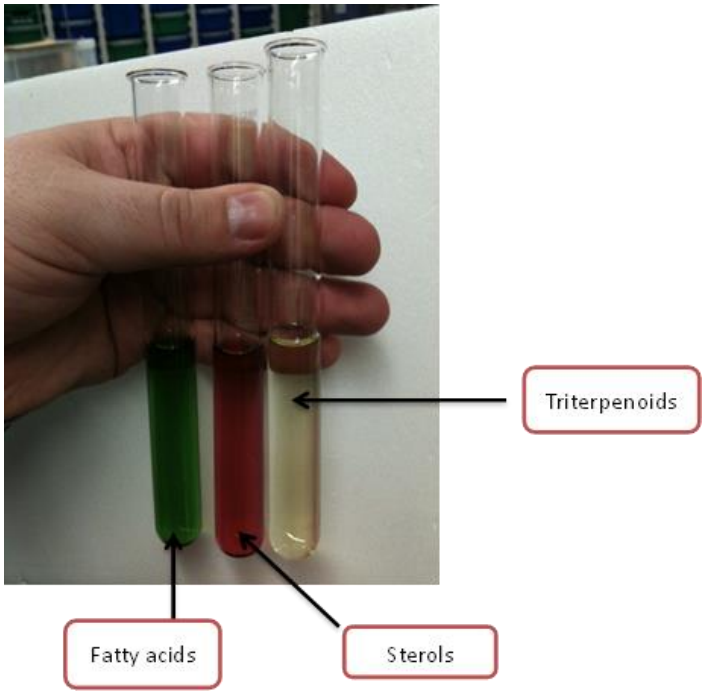


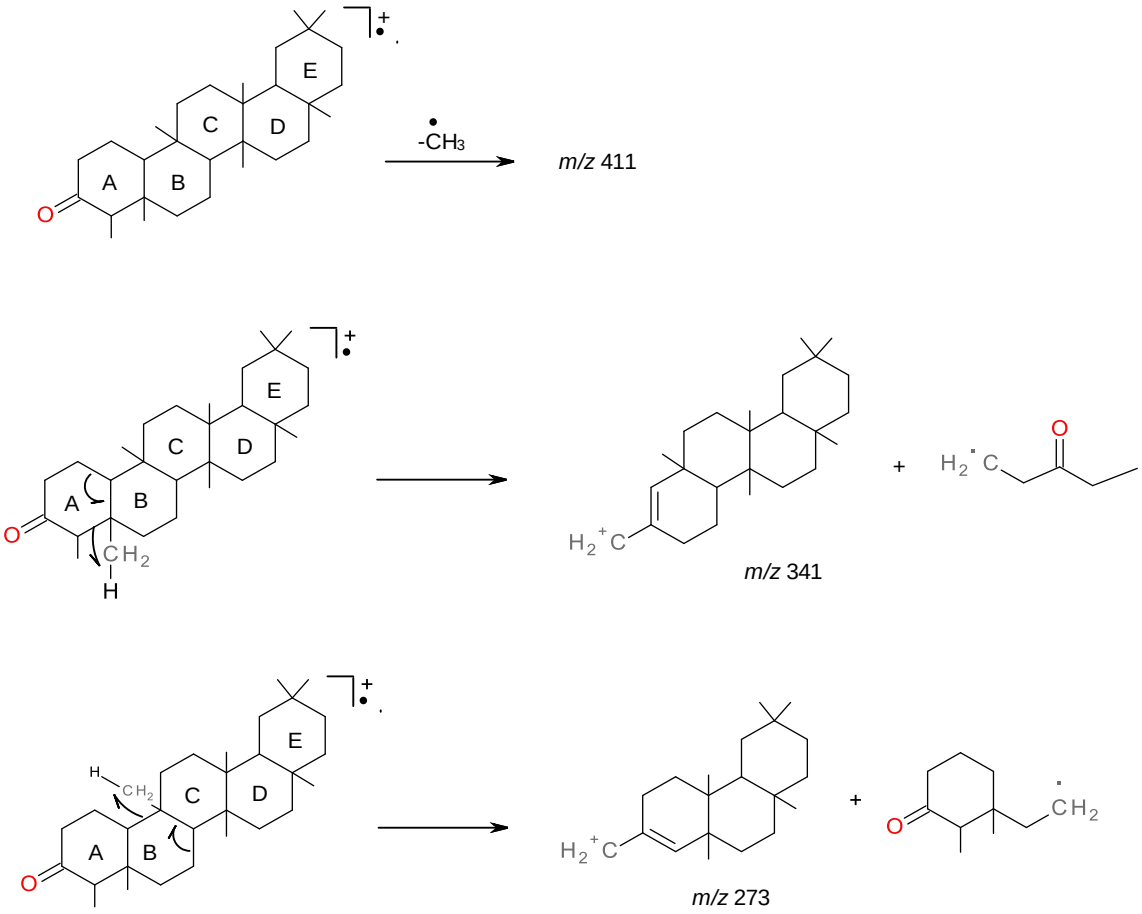
Figure 2-14 Tubes containing a) Fatty acids b) Sterols and c) Triterpenoids.

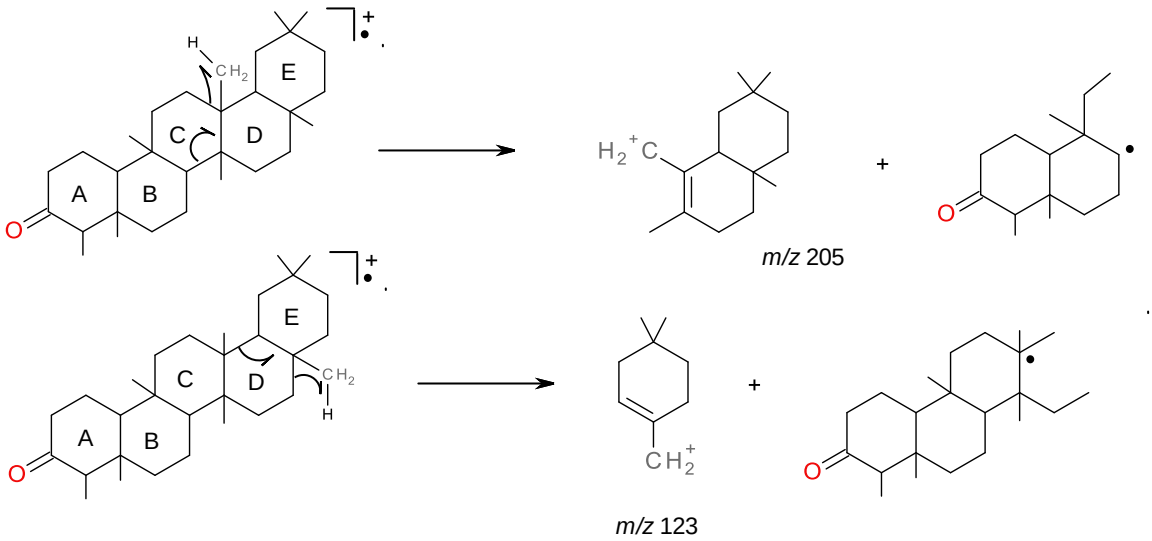
Structural analysis of the isolated triterpenoids was carried out by means of GC, EI-mass spectrometry and ^{13}C NMR. From interpretation of the EI-mass spectrum of the crude wax, a considerable number of triterpenoids were found in the leaves. Around five compounds in the leaf extract had fragmentation patterns which resemble those of triterpenoids. Two of these compounds were successfully isolated and were analysed by ^{13} C NMR.

2.3.7.1 Friedelin

One triterpenoid found in the leaves which was successfully isolated from the other triterpenoids, was identified as friedelin. A standard of friedelin was purchased and therefore a direct comparison was made between the pure standard and the compound found in the leaves. The GC retention times of the unknown compound (Unknown A) and the pure standard of Friedelin were found to be the same (Figure 2-15). Figure 2-16 compares the EI-mass spectrum of the pure standard and the unknown compound. It can be noted that the two spectra are almost identical. In the EI-mass spectrum of the unknown compound, a number of fragmentation patterns characteristic of friedelin can be identified.

The compound showed a molecular ion peak of 426, identical to that of friedelin. Diagnostic peaks found at 411, 341, 273, 205 and 123 were present in the spectrum. The peak at 411 occurs as a result of the loss of a methyl group from the molecular ion. The ions with a *m/z* of 341, 273 205 and 123 arise due to the fragmentation of rings A, B, C and D respectively as shown in Scheme 2-6. GC-MS data provides strong evidence to suggest that the compound found in the sugarcane leaves is friedelin.





Scheme 2-6: Formation of characteristic ions (*m/z* = 341, 273, 205 and 123) in Friedelin.

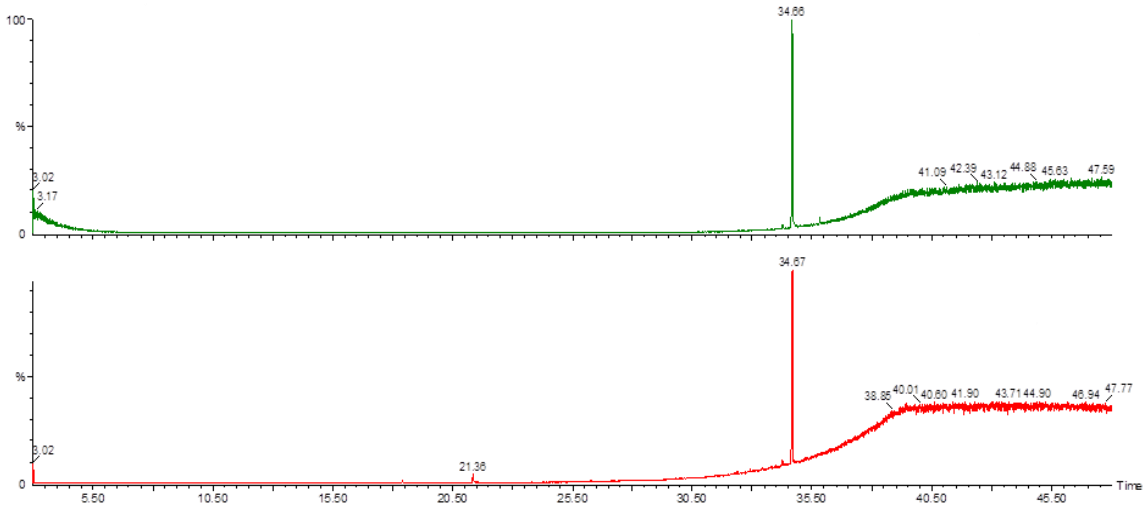


Figure 2-15 GC Chromatogram of a) Friedelin Standard b) Triterpenoid extracted from sugarcane leaves.

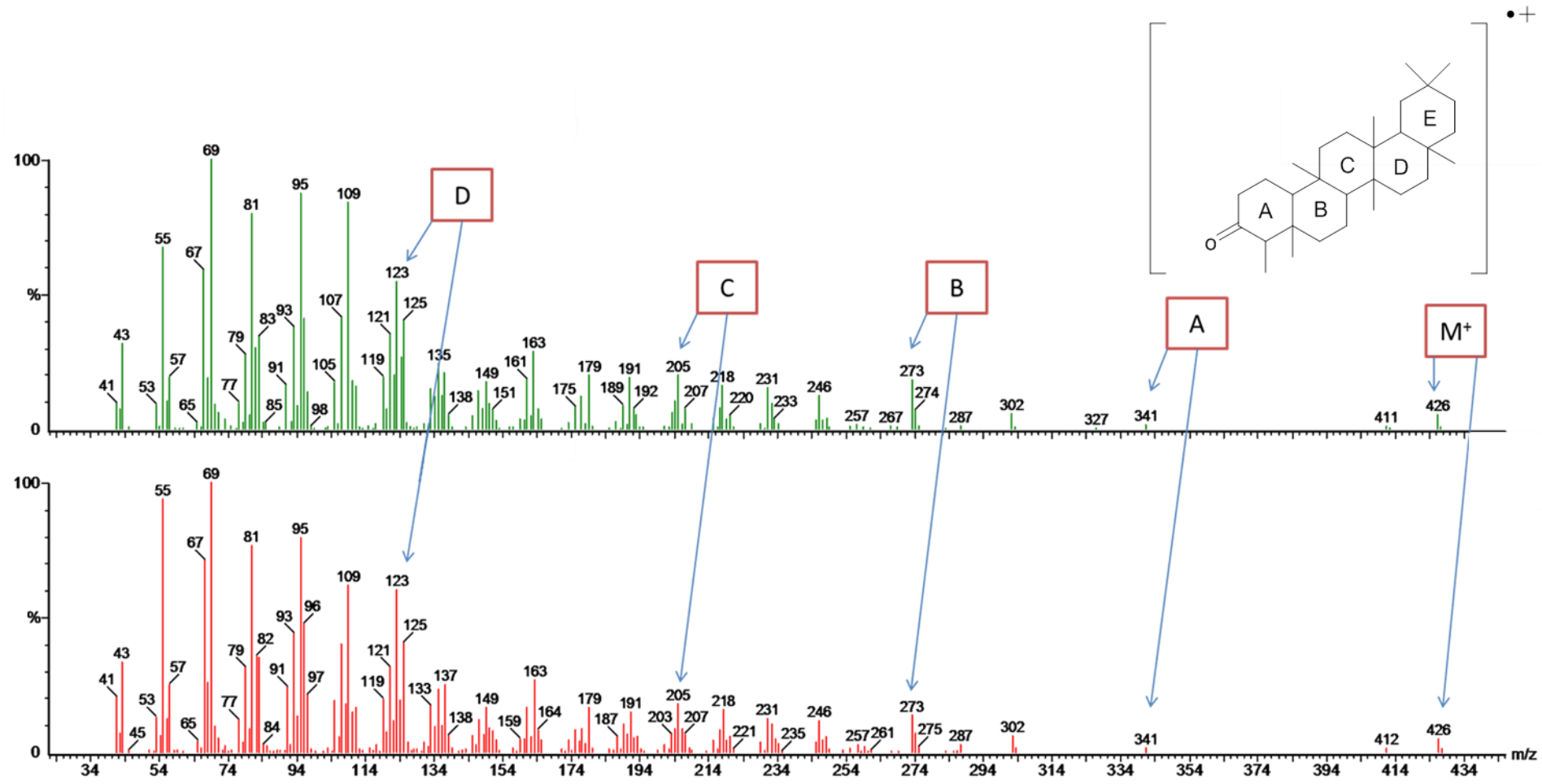


Figure 2-16 EI-Mass spectrum of a) Compound from sugarcane leaves b) friedelin.

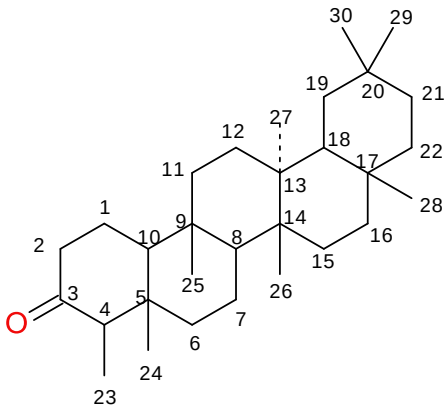


Figure 2-17 Structure of Friedelin.

Figure 2-18 shows the ^{13}C NMR spectrum for unknown compound A. The two spectra are similar. The ^{13}C NMR spectrum for unknown A is expanded in Figures 2-19 and 2-20. All the carbon atoms are assigned to their respective signals and are consistent with literature.[164] These are summarised in Table 2-10.

From Figures 2-18 and 2-20, it can be seen that there are a few impurities present along with friedelin. However, the majority of these impurities are also present in the ^{13}C NMR spectrum of the standard which therefore shows that the friedelin extracted from sugarcane wax is no less pure than the commercial sample.

Table 2-10 Carbon signals in the ^{13}C NMR of triterpenoid isolated from sugarcane wax.

Carbon no.	Chemical shift (δ, ppm)
1	22.35
2	41.61
3	213.36
4	58.26
5	42.22
6	41.34
7	18.31

8	53.19
9	37.52
10	59.55
11	35.69
12	30.58
13	39.79
14	38.38
15	32.51
16	36.09
17	30.07
18	42.86
19	35.41
20	28.26
21	32.83
22	39.34
23	6.89
24	14.70
25	18.01
26	20.34
27	18.74
28	32.16
29	31.87

30	35.10

Hence, from the GC, EI-MS and ^{13}C NMR data, it can be concluded that this triterpenoid found in the wax extracted from sugarcane leaves is friedelin.

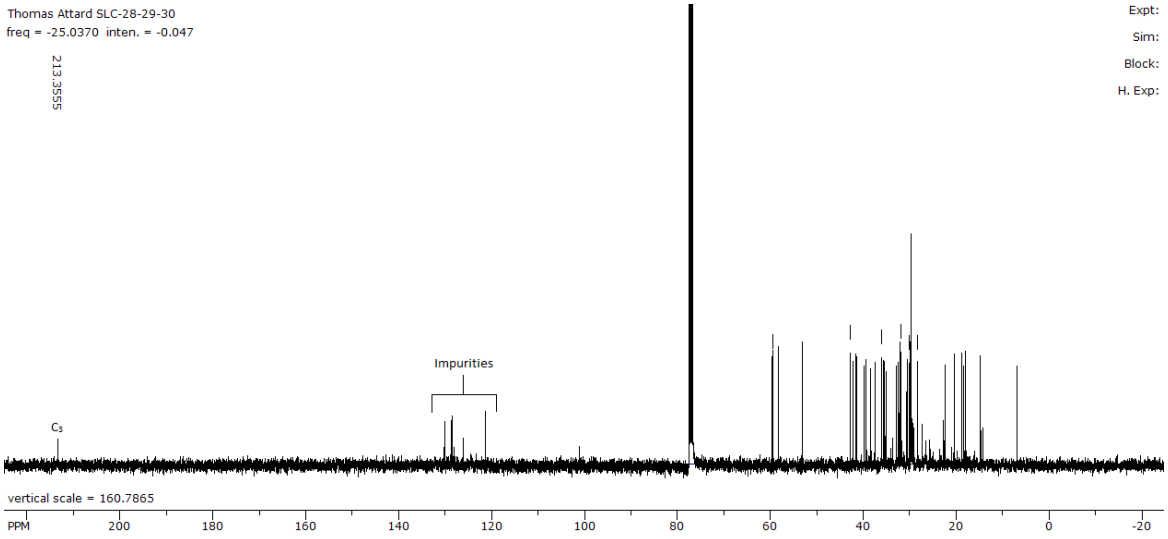


Figure 2-18 ^{13}C NMR spectrum of unknown compound A.

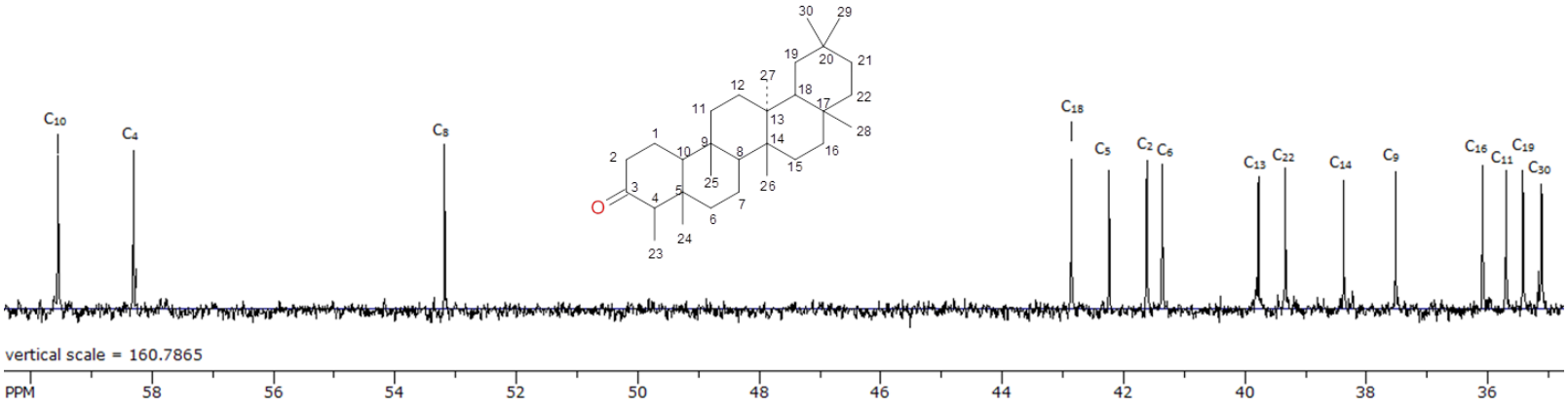


Figure 2-19 ^{13}C NMR spectrum of compound suspected to be friedelin expanded (34 – 64 ppm).

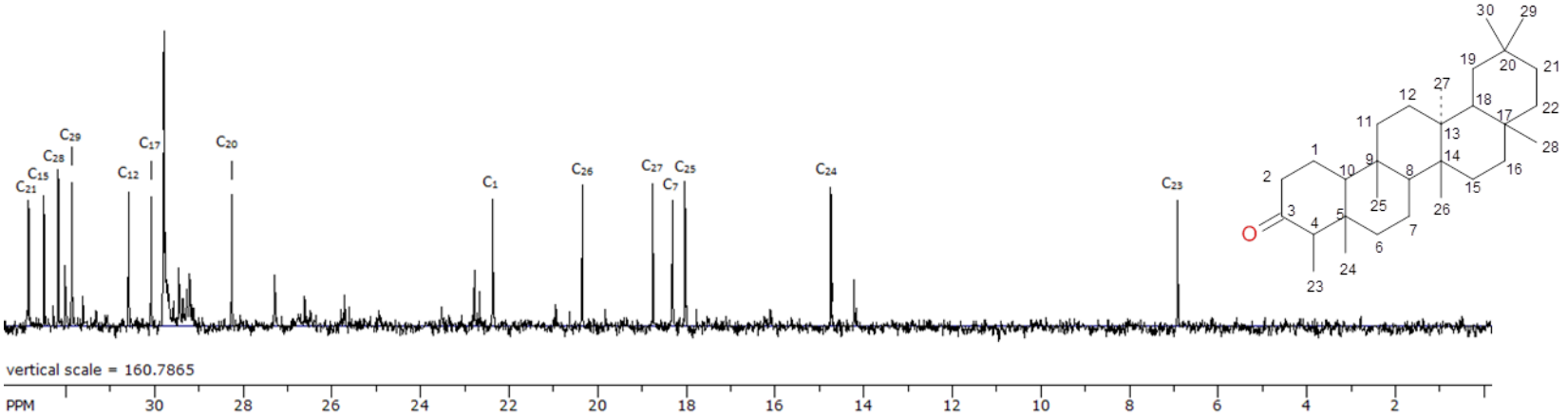


Figure 2-20 ^{13}C NMR spectrum of compound suspected to be friedelin expanded (0 – 32 ppm).

Simiarenol

Figure 2-21 illustrates the successful isolation (by flash chromatography) of another triterpenoid compound from the crude wax. The compound was analysed by a combination of EI-MS fragmentation data, NIST (v. 2.2) library data and ^{13}C NMR data, all of which give strong evidence to suggest that the compound is simiarenol ($C_{30}H_{50}O$). This is a pentacyclic, hopane-derived triterpene alcohol. Figure 2-21 illustrates the structure of simiarenol.

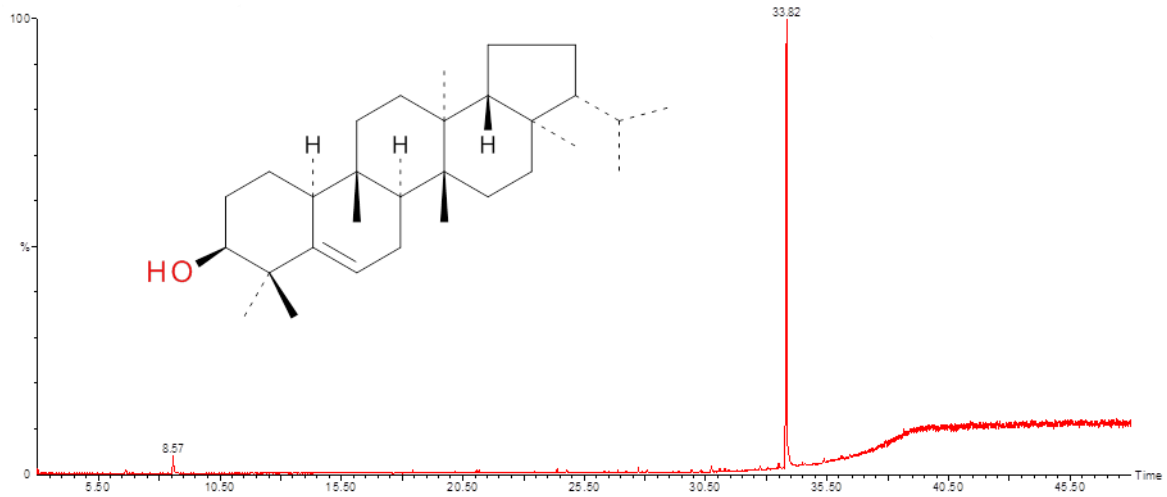


Figure 2-21 GC chromatogram fraction containing triterpenoid that is suspected to be simiarenol.

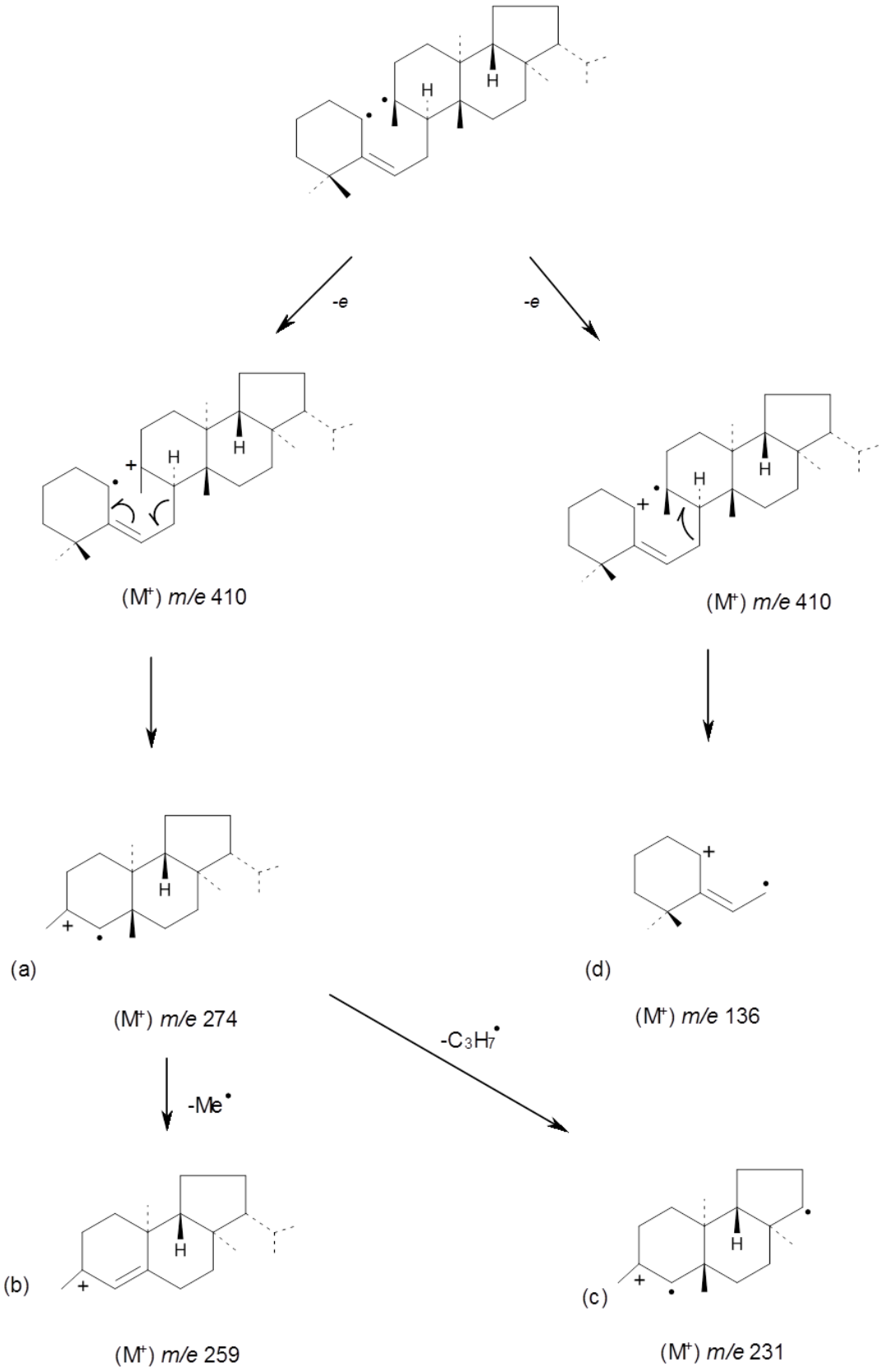
Figure 2-22 is the EI-mass spectrum of the compound that is suspected to be simiarenol. Figure 2-23 compares this EI-mass spectrum with that of the NIST (v. 2.2) library simiarenol standard. The mass fragmentation patterns of the compound and the standard are very similar.

The ion at *m/z* 426 is the molecular ion of simiarenol.

Scheme 2-7 illustrates the major fragmentation patterns of simiarenol. The base peak at *m/*z 274 occurs as a result of a retro-Diels-Alder cleavage of the second ring resulting in species *(a)*.[181] Another major fragment arises at *m/z* 259 (species (*b)*) as a result of the loss of the allylic C-26 methyl from species *(a).* There is an important weak peak at *m/z*

231 (species (*c)*) which arises as a result of the loss of the isopropyl side chain from species (*a)*. This is important as it differentiates the EI-spectrum of simiarenol from another triterpenoid β-glutinol. These two compounds have very similar mass spectra, but the ion at *m/z* 231 is found only in the spectrum of simiarenol as the loss of the isopropyl chain from *m/z* 274 occurs more easily in simiarenol than in β-glutinol.

Therefore, the EI-mass spectrum of the compound isolated from the crude wax gives strong evidence to suggest that it is simiarenol as it indicates the presence of the isopropyl group (loss of M-43 from *m/z* 274 to give *m/z* 231) as well as the Δ^5 double bond (*m/z* 274). The presence of the weak peak at 231 indicates that the compound in question is not β-glutinol.[165]



Scheme 2-7: Major fragmentation patterns in simiarenol.

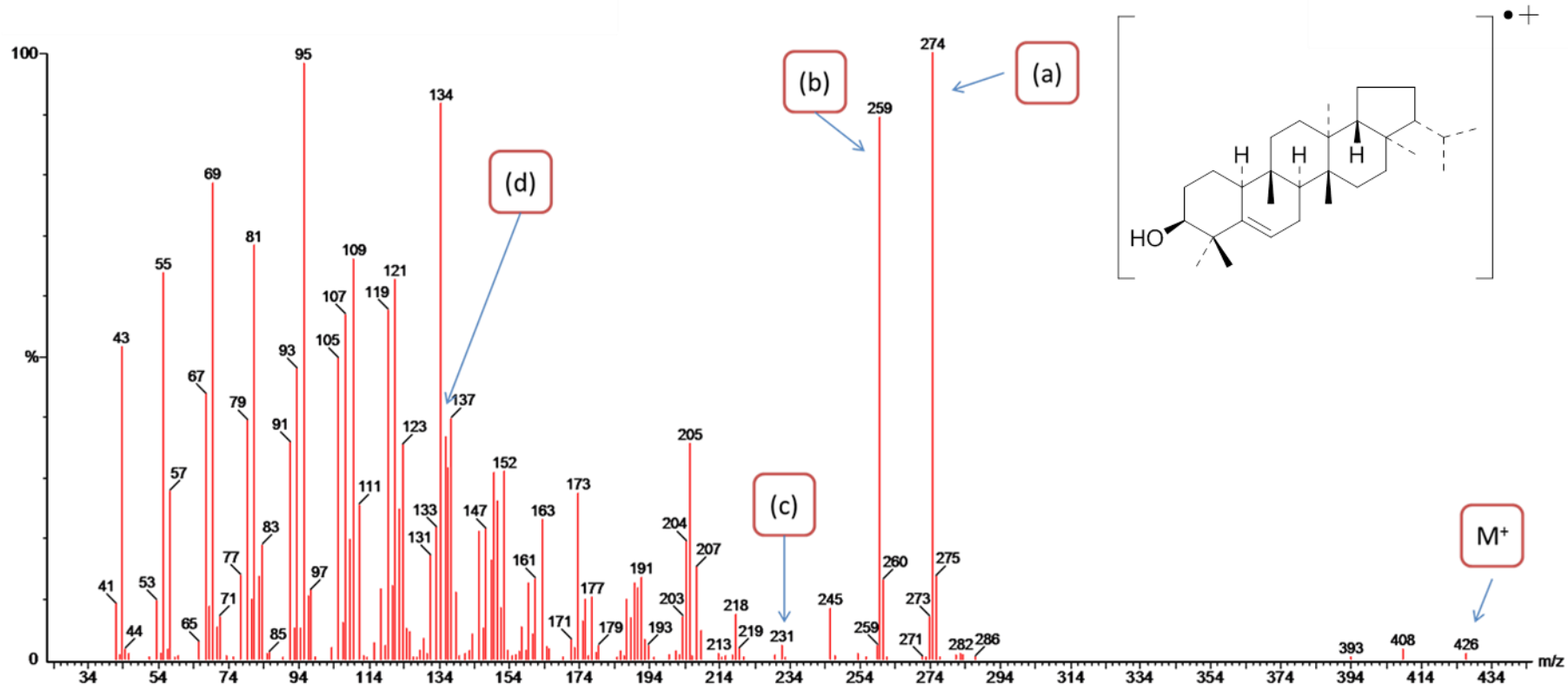


Figure 2-22 Mass spectrum of compound suspected to be simiarenol.

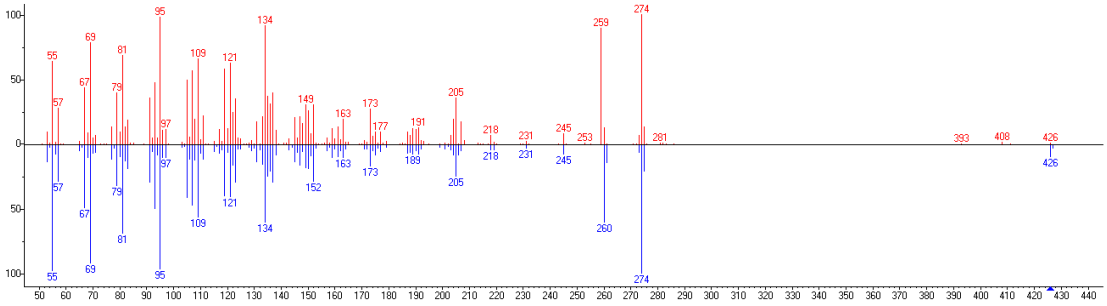


Figure 2-23 Comparison of EI-mass spectrum of unknown compound with simiarenol (NIST).

Figure 2-24 is the ^{13}C NMR spectrum obtained for the triterpenoid compound suspected to be simiarenol. The chemical shifts are comparable to those that are found in the literature for simiarenol. The two carbon signals at δ 142 and δ 122 indicate the presence of two olefinic carbon atoms (carbon atoms no.5 and no.6) which therefore highlights the presence of an unsaturated double bond in the structure.

Another carbon signal which is found in the NMR spectrum of simiarenol and in the NMR spectrum of the unknown compound is a carbon signal at δ 76.5 (found adjacent to the d-chloroform peaks as seen in Figure 2-25 which is an oxygenated carbon signal (carbon atom no.3). This signal indicates the presence of a hydroxyl group attached to the third carbon of the unknown compound.

^{13}C NMR spectrum is consistent with literature precedent but some discrepancies were found. Some of the carbon shifts (ppm) of the compound in this study did not match those that were found in literature.[166, 167] This could be due to the sample being too dilute. There are also discrepancies between different sets of published data for simiarenol.[166, 167] However, the easily identifiable peaks (described above) showed good correlation and this therefore gives further evidence showing that the structure is simiarenol.

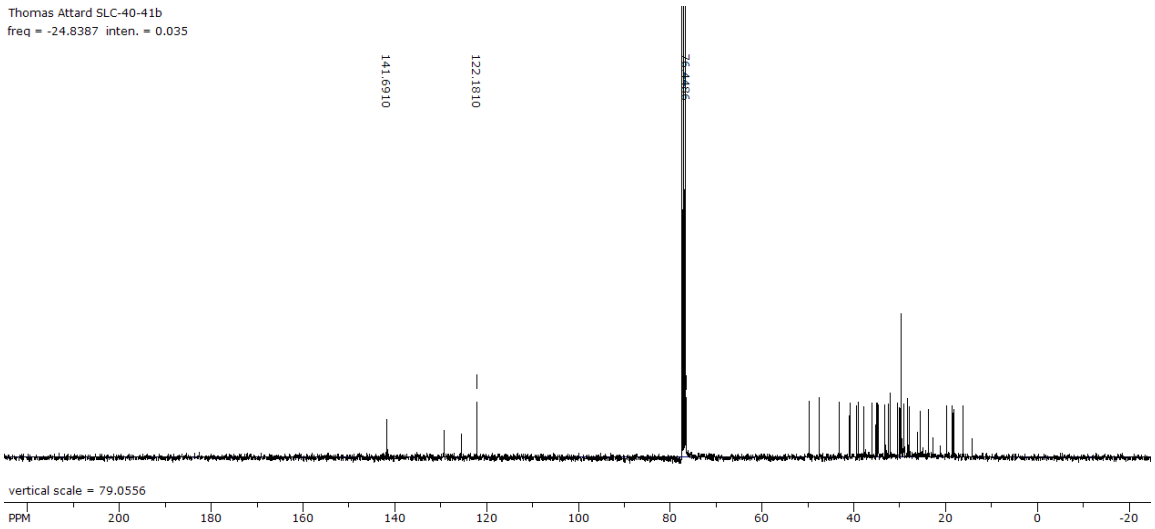


Figure 2-24 ^{13}C NMR of triterpenoid which is suspected to be simiarenol.

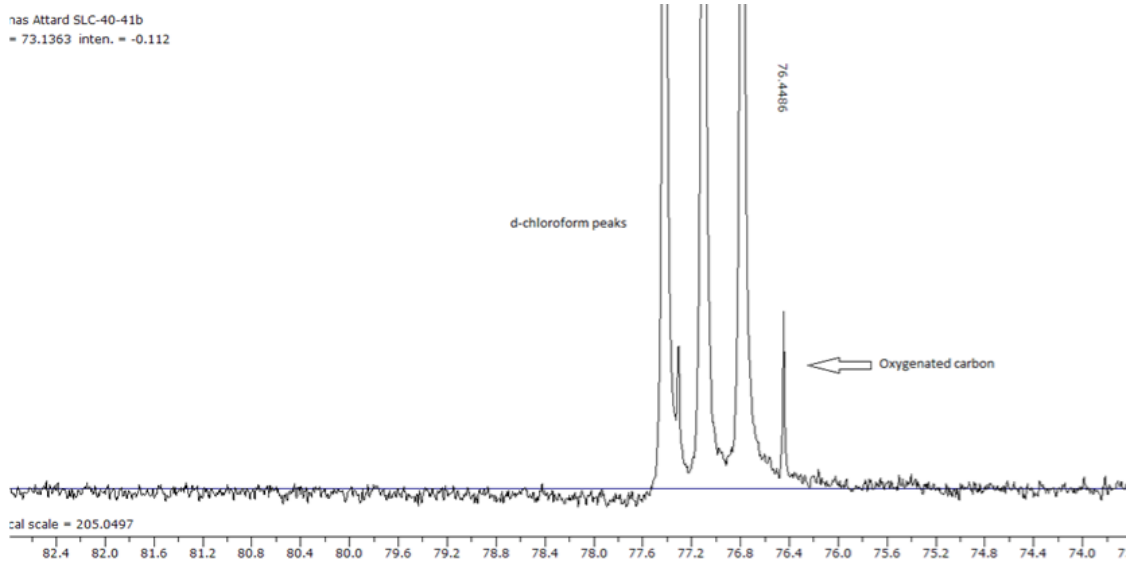


Figure 2-25 ^{13}C NMR indicating the oxygenated carbon.

2.3.7.2 Other Triterpenoids

The remaining triterpenoids were collected in one fraction together with the long-chain fatty aldehydes and it was not possible to carry out ^{13}C NMR. Therefore, it was only possible to analyse the compounds using GC-MS data together with the NIST (v. 2.2) library data. Further work would involve isolation of each triterpenoid in order to carry out additional analysis.

Figure 2-26 is a GC chromatogram showing the fraction containing the remaining triterpenoids (labelled as compound 1, 2 and 3). The EI-mass spectra of the three compounds are quite similar and share certain common characteristics (Figure 2-27). They all have a molecular mass of 440 amu (*m/z* 440), as well as two fragments at *m/z*

408 $(M-32)^{+\cdot}$ and *m/z* 393 $[(M-15)-32)]^{+}$. These fragments strongly point to the loss of a methoxy group during fragmentation as a neutral methanol molecule.[138] The fragmentation pattern of each of the three compounds strongly resembles that of triterpene ethers (as will be explained below) and this, together with the characteristics described previously, gives strong evidence to suggest that these three compounds are unsaturated methoxy triterpenoids having the formula $C_{31}H_{52}O$.

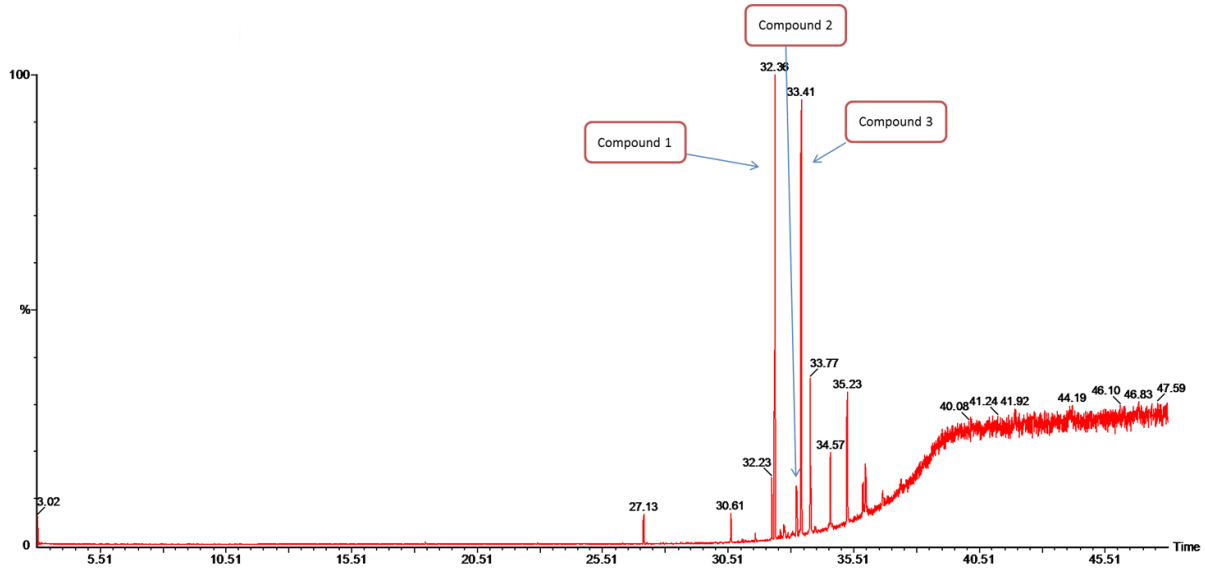


Figure 2-26 GC chromatogram of the fraction containing the three triterpenoids and the free long chain aldehydes.

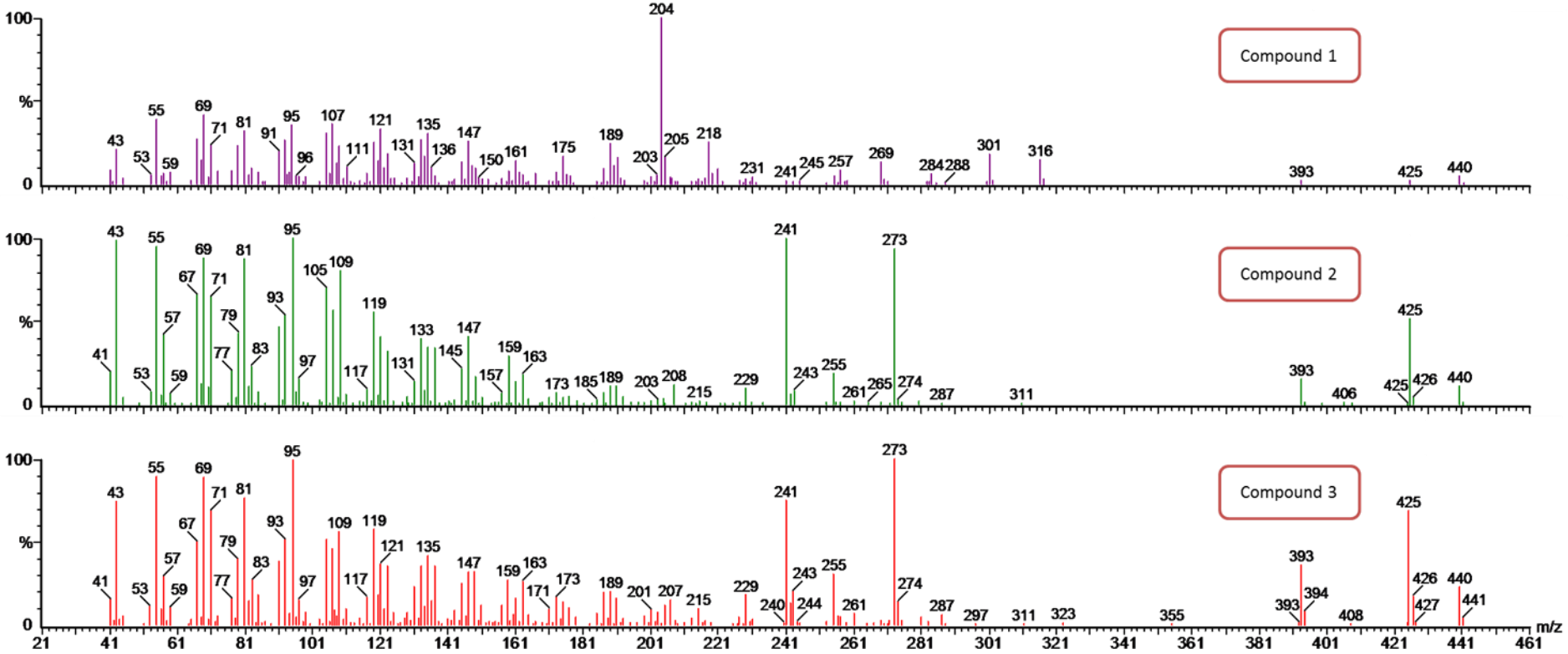


Figure 2-27 EI-Mass Spectra of Triterpenoids : a) compound 1 b) compound 2 c) compound 3.

Closer inspection showed that the EI-spectra of compounds 2 and 3 are very similar while that of compound 1 is different. The mass spectrum of compound 1 is dominated by fragments at *m/z* 218, 203 and 189 while the mass spectra of compounds 2 and 3 are dominated by mass fragments at 273 and 241.

2.3.7.2.1 Compound 1

The intense fragments at *m/z* 218, 204 and 189 found in the mass spectrum of compound 1 could be a result of the D/E moiety of normal and D-friedo- triterpenes after C-ring breaking and possible retro-Diels Alder rearrangement.[138] Apart from these fragments, there are other intense peaks at *m/z* 316, 301, 284 and 269. These fragments have been reported by Bryce *et al.* as being typical fragments of crusgallin (Figure 2-28) (taraxer-14-en-3β-ol).[138]

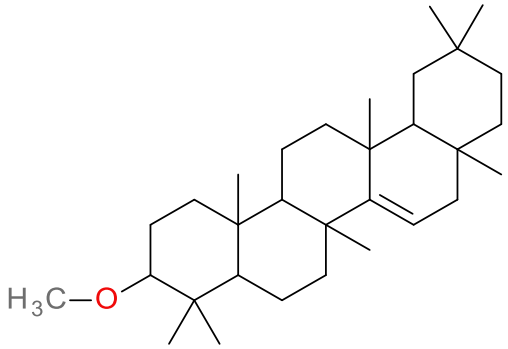


Figure 2-28 Structure of crusgallin.

NIST (v. 2.2) library indicated that the mass spectrum of compound 1 was that of crusgallin. Furthermore, Bryce *et al.* reported the presence of crusgallin in sugarcane wax.[138] This and the above data indicate that compound 1 is crusgallin.

2.3.7.2.2 Compounds 2 and 3

Compounds 2 and 3 have very similar mass spectra. The EI mass spectrum of compound 3 is found in Figure 2-29. The major fragment at *m/z* 273 $[M-167]^+$ is characteristic of D:C or E:C-friedo triterpenes of the arborane, bauerane, fernane or multiflorane type having a trisubstituted double bond in the 9(11) position.[168, 169] The fragment at *m/z* 241 arises via the loss of methanol from the fragment at *m/z* 273. A very small peak at *m/z* 365 could have arisen as a result of the loss of an isopropyl side-chain from the *m/z* 408 fragment indicating that compounds 2 and 3 are D:C or E:C friedo triterpene methyl ethers having an isopropyl group on the fifth ring and a tri-substituted double bond in the $\Delta^{9(11)}$ position.[168]

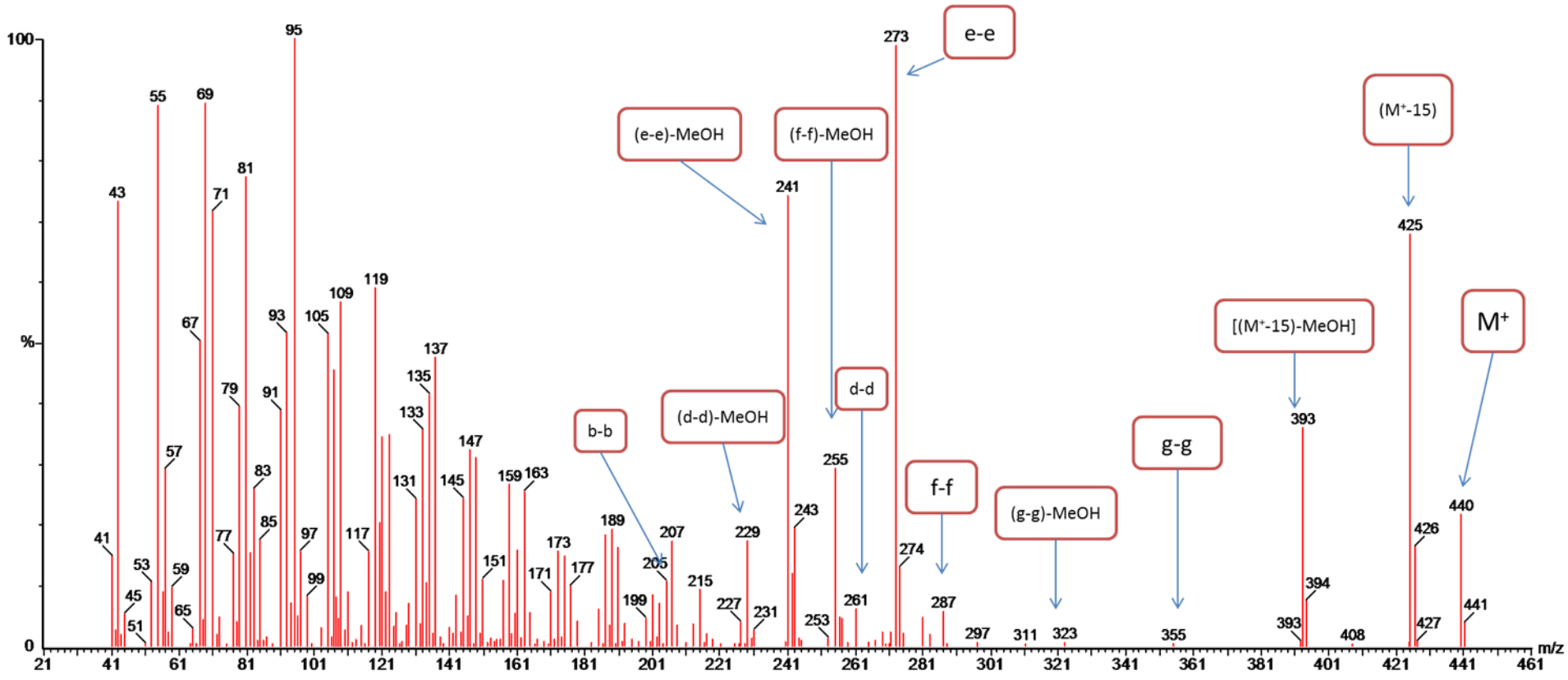
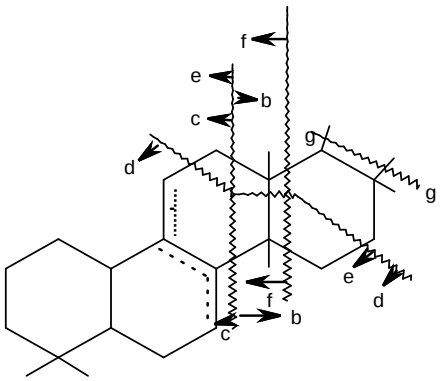


Figure 2-29 EI-Mass spectrum of Compound 3.

Scheme 2-8 illustrates the fragmentation of D:C or E:C- friedo triterpenoids and Table 2-11 summarises the resulting fragments.



Scheme 2-8 Major fragmentations in D:C or E:C friedo triterpenoids.

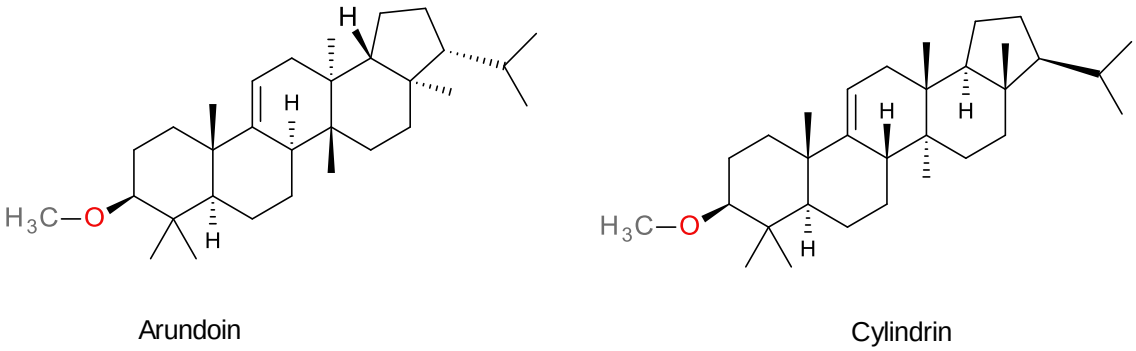
Table 2-11 Comparison of mass spectra of D:C and E:C-Friedo-Triterpenoids.

Compound	b-b	c-c	d-d	e-e	f-f	g-g	M^+-15	M^+
2	205	-	261	273	287	355	425	440
		-	229*	241*	255*	323*	393*	
3	205	-	261	273	287	355	425	440
		-	229*	241*	255*	323*	393*	

* Figures shown in the second line indicate those corresponding to the peak shown in the first line – MeOH.

NIST (v. 2.2) indicated that the structure of compounds 2 and 3 was found to be arundoin (arbor-9(11)-en-3β-ol ME) which is a D:C friedo-type triterpene methyl ether with a double bond in the 9(11) position. A number of published reports have shown that arundoin is present in wax extracted from sugarcane along with another D:C friedo-type triterpene methyl ether called cylindrin.[114, 138] The mass spectra of cylindrin and arundoin are almost identical. The only difference between the two compounds is the retention times on the GC chromatogram, where arundoin elutes off before cylindrin. Therefore, this suggests that compounds 2 and 3 are arundoin and cylindrin respectively due to the similar mass spectra and the fact that both triterpenoids have already been identified in other types of sugarcane wax. However, confirmation by direct comparison

with pure standards of the compounds for retention times and mass spectra or isolation of the two triterpenoids and analysis by ^{1}H NMR and ^{13}C NMR, would be required.



Arundoin Cylindrin

Figure 2-30 Structures of a) Arundoin and b) Cylindrin.

Table 2-12 summarises the type and quantity of triterpenoids found in the leaf wax.

Table 2-12 Distribution and quantity of triterpenoids present in the leaf extract, in µg/g of dry plant.

Triterpenoid type	Leaf (µg/g of dry plant)
Crusgallin	748.6±23.1
Cylindrin	209.1±31.1
Arundoin	712.4±3.2
Simiarenol	533.7±23.7
Friedelin	495±14.1
Total Triterpenoids	**2698.8±95.2**

Triterpenoids are high-value compounds, as they are known to have medicinal and pharmacological applications. It has been demonstrated that friedelin, as well as its derivatives, have a variety of properties including analgesia and anti-inflammatory abilities, anti-cancer activity, anti-bacterial activity and is a vascularising agent.[170-175] Furthermore, it has the potential to be utilised in pharmaceutical products or in functionalised food for treating cardiovascular and cerebrovascular tumours and other diseases.[175]

Table 2-13 summarises all of the compounds found in the sugarcane rind, leaf and bagasse extracts.

Table 2-13 Quantification of compounds found in lipophilic extractives from rind, leaves and bagasse of sugarcane (in µg/g of plant), obtained from GC and GC-MS.

Compounds	Rind (µg/g of dry plant)	Leaves (µg/g of dry plant)	Bagasse (µg/g of dry plant)
Hexanoic acid	17.2 ±1.6	1.4 ±0.1	3.3 ±0.04
Heptanoic acid	-	1.3 ±0.05	0.3 ±0.03
Octanoic acid	25.1 ±1	2.8 ±0.1	1.2 ±0.05
Nonanoic acid	-	2.7 ±0.2	1.6 ±0.03
Decanoic acid	0.8 ±0.1	7.7	0.5
Dodecanoic acid	4.5 ±0.3	112.4 ±2.4	3.1
Tetradecanoic acid	3.5 ±0.4	104.5 ±0.3	3 ±0.04
Pentadecanoic acid	2.2 ±0.2	13.1	1.9 ±0.03
Hexadecanoic acid	126.6 ±15.3	866.8 ±4.2	99.9 ±1.1
Heptadecanoic acid	-	29.5 ±0.1	1.8 ±0.04
Octadecanoic acid	15.9 ±1.7	94.1 ±0.7	13.8 ±1.4
Nonanoic acid	-	9.1 ±0.3	0.4
Eicosanoic acid	14 ±1.2	119.9 ±1.1	18.1 ±0.3
Heneicosanoic acid	-	8.2 ±0.2	0.7 ±0.1
Docosanoic acid	TR	48.7 ±0.9	6.9 ±0.1
Tricosanoic acid	-	17.5	4.3 ±0.1

Tetracosanoic acid	3.1 ±2.4	75.3 ±1.1	6.2 ±0.1
Pentacosanoic acid	-	-	10.5 ±4.2
Hexacosanoic acid	-	TR	TR
Octacosanoic acid	-	TR	65.1 ±2.4
Tricontanoic acid	-	TR	33.7 ±0.3
Total saturated fatty acids	**212.9 ±24.2**	**1515 ±11.7**	**277.3 ±10.4**
9-octadecenoic acid	3.4	8.2 ±0.2	1.3 ±0.1
9,12-Octadecadienoic acid	3.2 ±0.4	215.8 ±2.1	19.9 ±10.2
9,12,15-Octadecatrienoic acid	1.5 ±0.2	197.2 ±2.2	21.3 ±8.5
Total unsaturated fatty acids	**8.1 ±0.6**	**421.2 ±4.5**	**42.5 ±18.8**
Octanedioic acid	2.2 ±0.4	-	-
Nonanedioic acid	8.2 ±1.5	11.9 ±0.8	3.7 ±0.1
Decanedioic acid	0.7 ±0.1	-	-
Total saturated di-fatty acids	**11.1 ±2**	**11.9 ±0.8**	**3.7 ±0.1**
Tetracosanol	4.5 ±0.4	-	3.1 ±0.1
Hexacosanol	129.1 ±14.5	19.6 ±1.5	43.3 ±0.2
Octacosanol	1902.8 ±242.3	110 ±3.3	716.7 ±4.7
Triacontanol	142.7 ±19	90.9 ±1.1	91.5 ±1.4

Dotriacontanol	23.3 ±1.7	393.2 ±2.1	50.5 ±1.1
Tetratriacontanol	5.8 ±0.6	-	8.5 ±2.4
Total saturated fatty alcohols	**2208.2 ±278.5**	**613.7 ±8**	**913.6 ±9.9**
Tetracosanal	-	23.6 ±1.3	-
Hexacosanal	137.5 ±19.8	35.2 ±4.5	36.6 ±0.4
Octacosanal	3001.5 ±694.8	81.3 ±11.1	807.1 ±5.8
Triacontanal	825.6 ±315.9	199.3 ±14.1	260.3 ±2
Dotriacontanal	286.7 ±102.2	TR	111.7 ±1.4
Tetratriacontanal	177.3 ±68.8	-	78.8 ±0.1
Hexatriacontanal	16.4 ±6.1	-	-
Total saturated fatty aldehydes	**4445 ±1207.6**	**339.4 ±31**	**1294.5 ±9.7**
Tricosane	3.6 ±0.3	8.2 ±0.1	1
Pentacosane	7.4 ±0.2	5 ±2.7	4.1
Heptacosane	113.1 ±3.3	20.2 ±0.1	41.1 ±0.3
Octacosane	2 ±0.7	-	1.8 ±0.03
Nonacosane	41.4 ±8.2	45.5 ±0.5	22.5 ±0.3
Hentriacontane	315.8 ±23.3	167.2 ±2.4	33.6 ±1.7
Triatriacontane	-	303.6 ±13	32.4 ±0.5
Pentatriacontane	-	44.6 ±1.9	-
Total hydrocarbons	**483.3 ±36**	**594.3 ±20.7**	**136.5 ±2.8**

Campesterol	-	-	80.2 ±7.3
Stigmasterol	-	464.4 ±0.8	79.9 ±23.3
β-Sitosterol	-	623.4 ±114.9	115 ±6.1
Total sterols	-	**1087.8 ±115.7**	**275.1 ±36.7**
Crusgallin	-	748.6 ±23.1	-
Cylindrin	-	209.1 ±31.1	-
Arundoin	-	712.4 ±3.2	-
Simiarenol	-	533.7 ±23.7	-
Friedelin	-	495 ±14.1	-
Total Triterpenoids	-	**2698.8 ±95.2**	-
Wax ester C_{38}	-	20.6 ±0.7	1.2
Wax ester C_{40}	1.4 ±0.2	12.6 ±2	2.8
Wax ester C_{42}	6.9 ±1.2	15.1 ±1.6	8.4 ±0.3
Wax ester C_{43}	1.7 ±0.3	8.2 ±0.4	3.1
Wax ester C_{44}	37.2 ±2.9	41.1 ±0.3	46.7 ±0.6
Wax ester C_{45}	1.6 ±0.1	14.7 ±1.8	6 ±0.2
Wax ester C_{46}	14 ±2.1	37.8 ±1.9	28 ±0.2

Wax ester C_{47}	1.4 ±0.1	2.8 ±0.2	3.4 ±0.2
Wax ester C_{48}	11 ±1.1	54.2 ±3.9	26.6 ±0.4
Wax ester C_{49}	0.8 ±0.2	4.3 ±0.4	3.5 ±0.1
Wax ester C_{50}	6.2 ±0.1	29 ±4.2	14.5 ±0.2
Wax ester C_{51}	1.1 ±0.1	-	3.7 ±0.1
Wax ester C_{52}	3.7 ±0.4	20.6 ±0.7	11.1 ±0.1
Wax ester C_{53}	1.3 ±0.1	-	3.3 ±0.1
Wax ester C_{54}	3.4 ±0.6	11 ±0.6	7.1 ±2.7
Wax ester C_{55}	2.5 ±0.2	-	2.2 ±0.8
Wax ester C_{56}	18.4 ±4.6	TR	17.6 ±1.7
Wax ester C_{58}	7.3 ±3.1	TR	7 ±0.3
Total Wax esters	**119.9 ±17.4**	**272 ±18.7**	**196.2 ±7.9**
2-Pentadecanone-6,10,14-trimethyl	5.6	234 ±6.9	14.9 ±0.1
Phytol	-	126.1 ±0.7	-
Branched alcohol 1	-	183.5 ±2.6	-
Total 'Other' compounds	**5.6**	**543.6 ±10.2**	**14.9 ±0.1**

2.4 Comparison of Rind, Leaf and Bagasse wax

The visual appearance and texture of the waxes (Appendix A, Figure A1) differed for each type of sugarcane waste. GC and GC-MS analyses indicate that there is a significant difference in both the composition and quantity of hydrophobic compounds found within the waxes from the rind, leaves and bagasse.

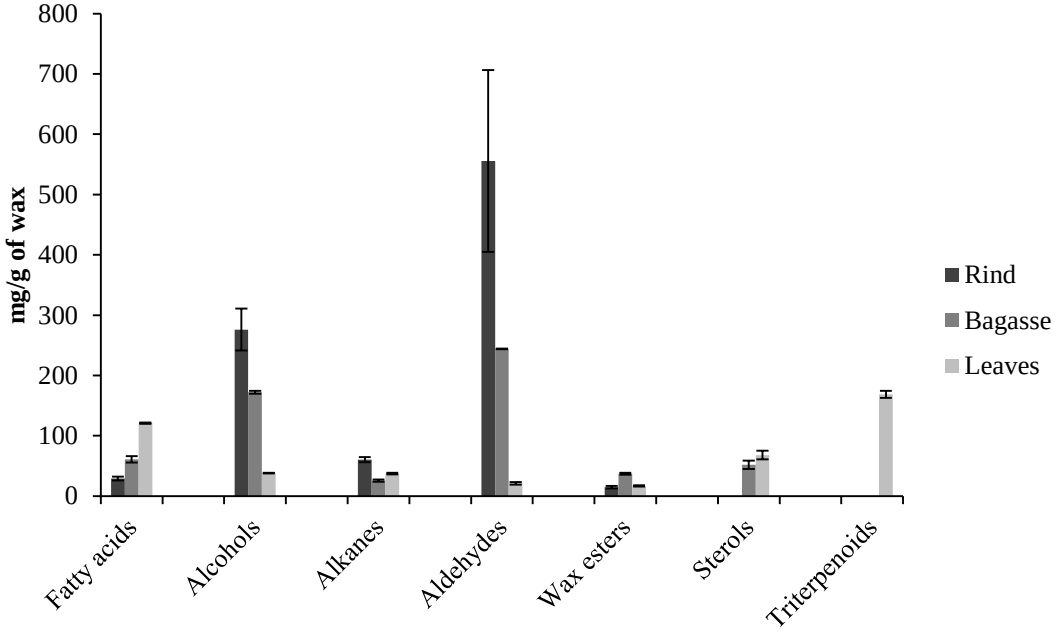


Figure 2-31Distribution of compounds in the rind, bagasse and leaf wax.

The wax from sugarcane rind contains substantial quantities of long-chain fatty aldehydes and long-chain policosanols, which comprised 56% and 27% of the wax composition, respectively. Octacosanal (375.2±86.9 mg/g of wax) and 1-octacosanol (237.9±30.3 mg/g of wax) were the predominant chain length for each group (Figure 2-32 and 2-33).

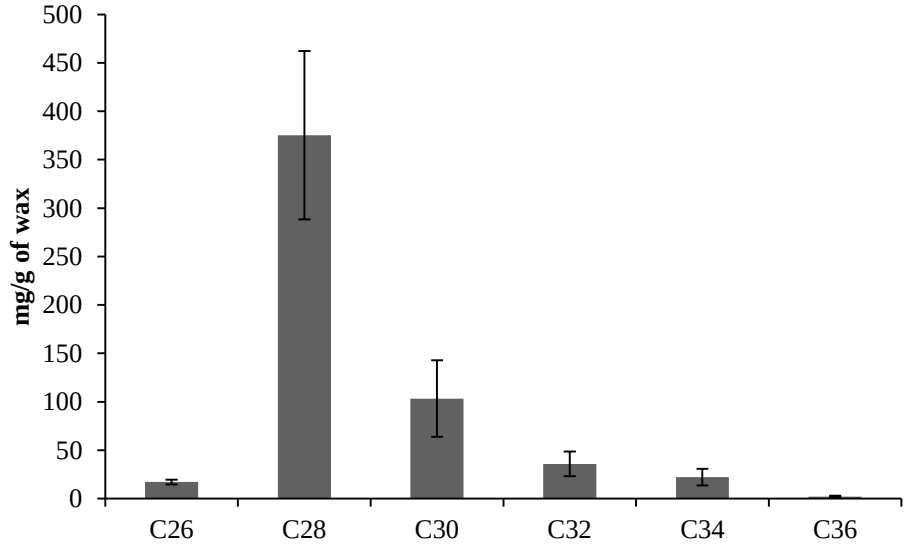


Figure 2-32 Distribution of long-chain fatty aldehydes in rind wax.

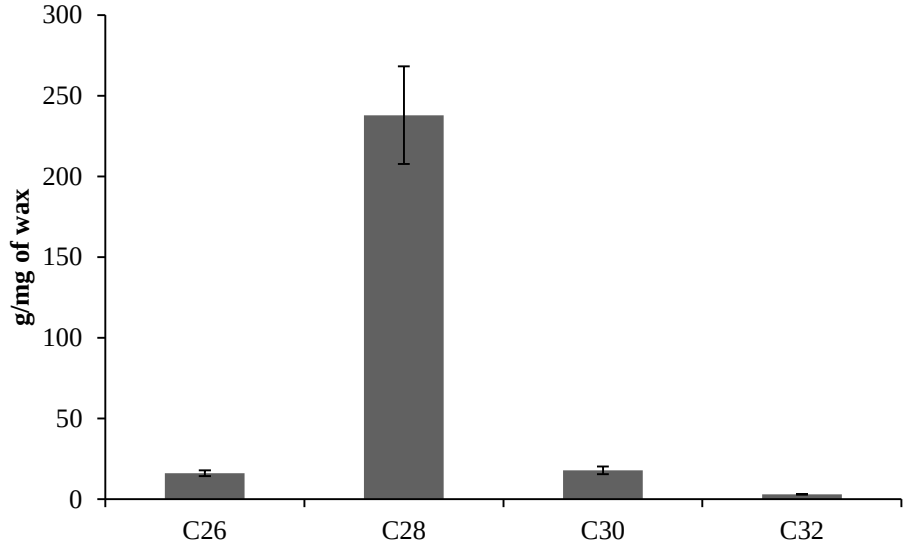


Figure 2-33 Distribution of long-chain fatty alcohols in rind wax.

The simple isolation and purification of long chain aldehydes/policosanols from the extract could be of commercial significance. Apart from these compounds, relatively small quantities of long-chain alkanes, long-chain fatty acids and wax esters were also present.

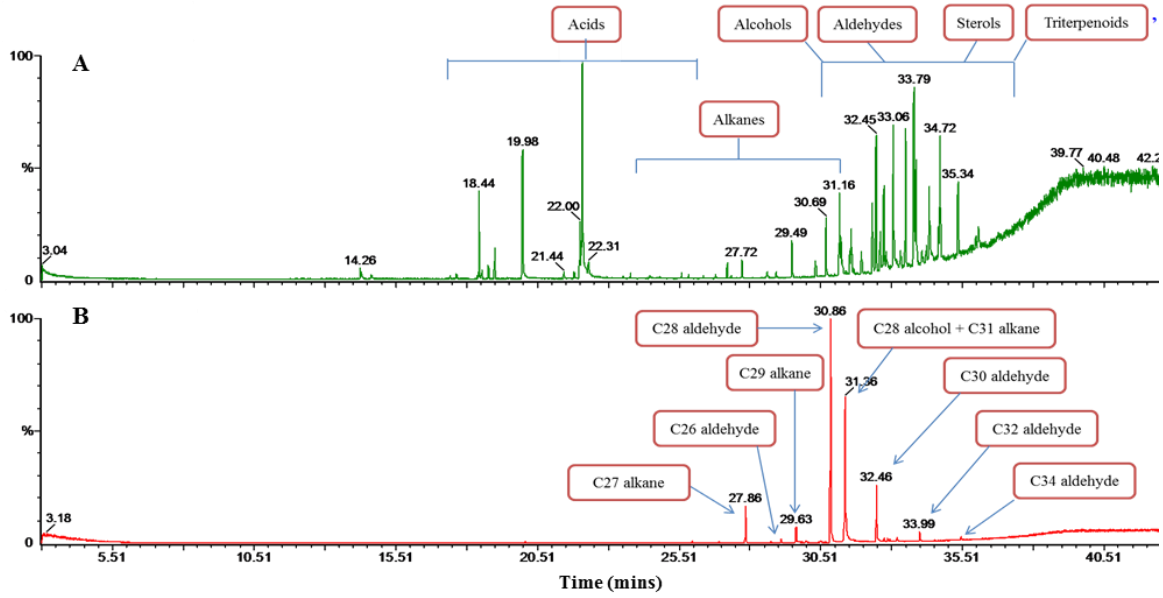


Figure 2-34 Comparison of GC-MS chromatograms of unsilylated A) Leaf wax B) Rind wax.

The results obtained from the crude extract of sugarcane rind are comparable with literature.[113, 114, 145-147] However, a much wider variety of compounds were identified in the wax obtained from the leaves, as compared to the rind. Figure 2-34 shows GC-MS chromatograms of unsilylated waxes from the sugarcane leaves and rind, where a stark contrast may be observed when comparing the extractives. A greater variety of compounds are present in the leaf wax including *n*-alkanes, saturated and unsaturated fatty acids, fatty-alcohols, fatty aldehydes, wax esters, sterols, triterpenoids and tocopherols amongst other compounds. Triterpenoids were only found to be present in the leaf waxes.

Previous studies have demonstrated that sugarcane wax is typically predominantly composed of long-chain aldehydes. However, only small quantities of long-chain fatty aldehydes were detected in the leaf wax.[113, 114, 145-147] These results are consistent for those obtained for rind wax. The main group of compounds constituting the leaf wax were found to be triterpenoids (169 ±6 mg/g of wax), followed by long-chain fatty acids and sterols.

Other compounds detected in the leaf were phytol, tocopherol (vitamin E) and phylloquinone (vitamin K1). Phytol is of considerable interest as it is an extensively used fragrance material. It is used in a number of applications, including fine fragrances, cosmetics, toilet soap, shampoos and other toiletries, detergents and household cleaners.[176] Vitamin K_1 has interesting medicinal properties, where it has been shown to reduce postmenopausal bone loss in patients.[177]

Limited work has been carried out on the full characterisation of the sugarcane leaf wax and no previous studies have reported the extraction of valuable products from leaf trash using $scCO_2$. Specifically, methyl ether triterpenoid structure elucidation in sugarcane leaves has previously been carried out, but this current study reports the first full characterisation of sugarcane leaf wax.[138, 178]

The exploitation of sugarcane leaf trash for wax extraction has previously not been considered. The wax from leaf trash is very different to the sugarcane wax reported from other sugarcane waste in literature, in that it has very small amounts of long-chain aldehydes and policosanols, but has appreciable amounts of triterpenoids (169±6 mg/g of wax). The large quantities of triterpenoids combined with an increase in the amount of leaf trash due to green harvesting means that there is a steady source of high-value triterpenoids.

The profile of the bagasse wax had regions similar to the rind extract and other regions similar to the leaf wax. Long-chain fatty aldehydes (24.4% of total composition) and fatty alcohols (17.2% of total composition) were the dominant groups of compounds with 1-octacosanol (135±1.3 mg/g of wax) and octacosanal (152±1.1 mg/g of wax) predominating. However, like the leaf wax there were also appreciable quantities of phytosterols with campesterol, stigmasterol and β-sitosterol detected. Sugarcane bagasse had the highest amount of wax esters present (37±1.5 mg/g of wax).

The melting point profile of the waxes was also investigated. DSC was used to study the thermodynamic changes that occur when waxes transform from one physical state to another.[179] The samples that were analysed were subjected to a first heating cycle, in order to remove any prior thermal character. In all DSC thermograms, the second heating cycle is shown. DSC thermograms of commercial waxes candelila wax, beeswax and carnauba wax were obtained for comparison (Figure 2-35).

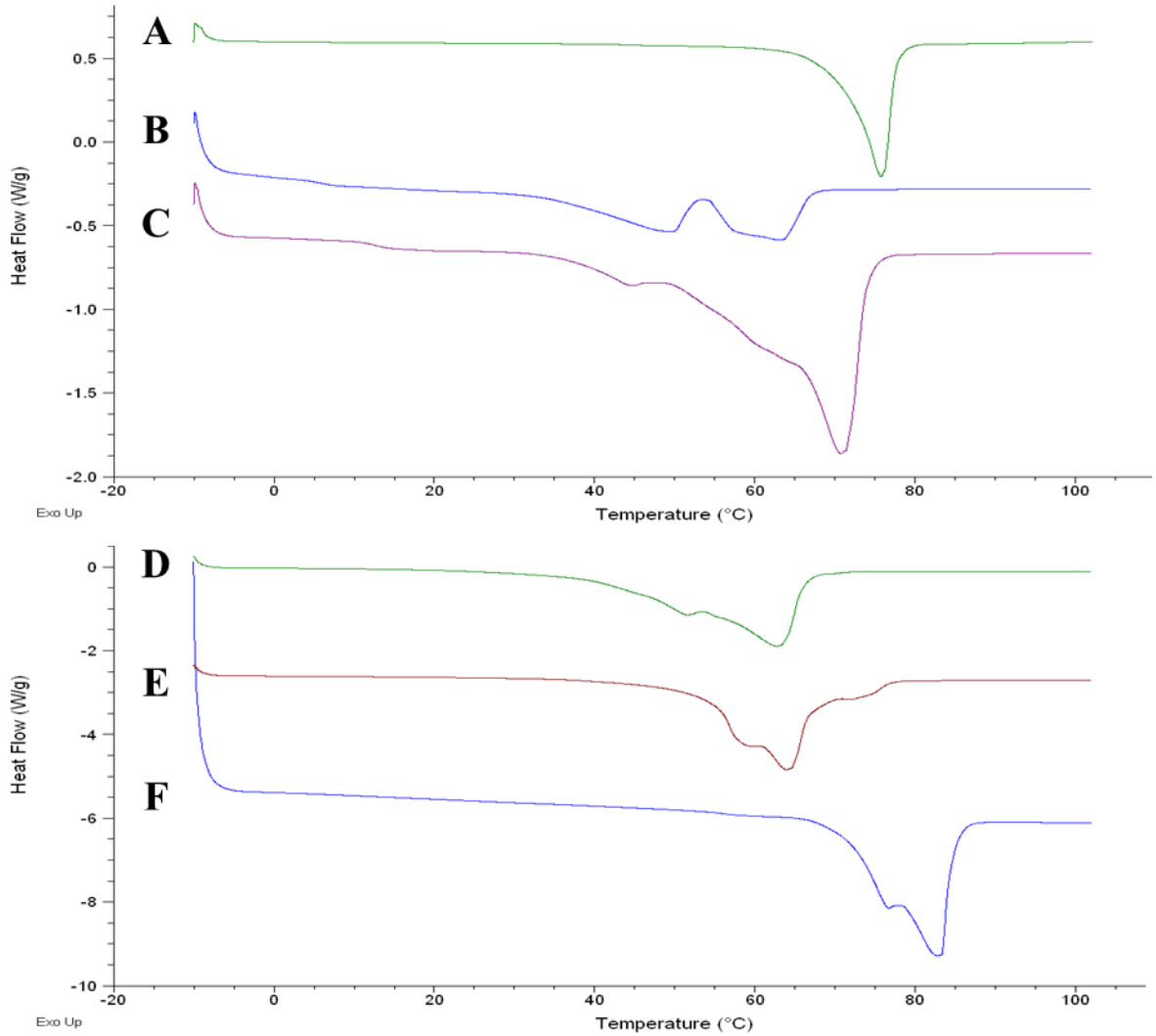


Figure 2-35 DSC thermograms (second heating) of A) Sugarcane rind wax B) Sugarcane leaf wax C) Sugarcane bagasse wax D) Commercial beeswax E) Commercial candelila wax F) Commercial carnauba wax.

It is interesting to note that there is an appreciable difference between the DSC thermogram of the sugarcane rind wax (A), leaf wax (B) and bagasse wax (C). Since natural waxes comprise a complex mixture of long-chain hydrophobic compounds, it is rare to find clearly defined thermal transitions for them.[179] This appears to be the case for the sugarcane leaf wax, which has a broad DSC trace with two endothermic minima centred at around 48 ^{o}C and 63 ^{o}C respectively, as a result of the large variation in the family of compounds constituting the wax. These endothermic events are most likely a result of phase transitions occurring for families of compounds (melting). The DSC curve for sugarcane leaf wax is similar to that found in the commercial beeswax (63 ^{o}C) and candelila wax (64 ^{o}C).

In contrast, the sugarcane rind wax has a well-defined narrow peak, with a minimum at 76 ^{o}C. This could potentially be attributed to the high abundance of specific types of

compounds (≈83% of the rind wax is composed of long-chain alcohols and long-chain aldehydes) which would therefore be expected to give a sharper more clearly-defined thermal transition when compared to the leaf wax. The commercial waxes have sharper thermal transitions than crude waxes due to their greater level of purification. The melting profile of the crude rind wax is comparable to the commercial waxes, which suggests that minimal purification is required. The sugarcane rind wax has the highest melting profile as a result of the large abundance of long-chain fatty alcohols and fatty aldehydes present. Furthermore, the DSC trace shows that there is only one transition when compared to the other types of sugarcane wax as well as the commercial waxes (the other waxes all have minor transitions as a result of broader range of compounds present). The bagasse wax has an endothermic minimum at 71 ^{o}C.

The difference in wax composition, together with the variation in the DSC traces, suggests that waxes obtained from various parts of the plant could be utilised in different applications. The sugarcane leaves have a broader range of compounds and have a high abundance of triterpenoids suggesting that these could be utilised in nutraceutical or pharmacological applications. The DSC profile of the rind wax exhibits a slightly lower melting profile compared to that of the carnauba wax (83 ^{o}C) and could be used in similar applications, such as automobile and instrument polishes. Furthermore, the high abundance of policosanols in the rind and bagasse wax would make them an ideal source for the production of cholesterol-reducing nutraceuticals.

2.5 Comparison of supercritical carbon dioxide extraction with hexane (soxhlet) extraction

It is important to assess the supercritical extraction of sugarcane waxes by comparison with conventional extraction solvents. Conventional extraction techniques, such as soxhlet extraction, are still considered to be a benchmark to compare other extraction techniques.[180] Several extraction techniques using different technologies have been introduced to extract natural products, yet conventional solvent extraction still remains the most common technique.[39] Hexane, which is the most commonly used extraction solvent, is petroleum-based, a hazardous air pollutant (as listed by the US EPA in the Clean Air Act 1990) and a neurotoxin having severe adverse effects on the nervous system.[54] It is extremely flammable and its vapour can form explosive mixtures when mixed with air.[181]

Soxhlet extractions were carried out on the leaf and rind biomass using hexane and the results were directly compared to those obtained for the supercritical extractions.

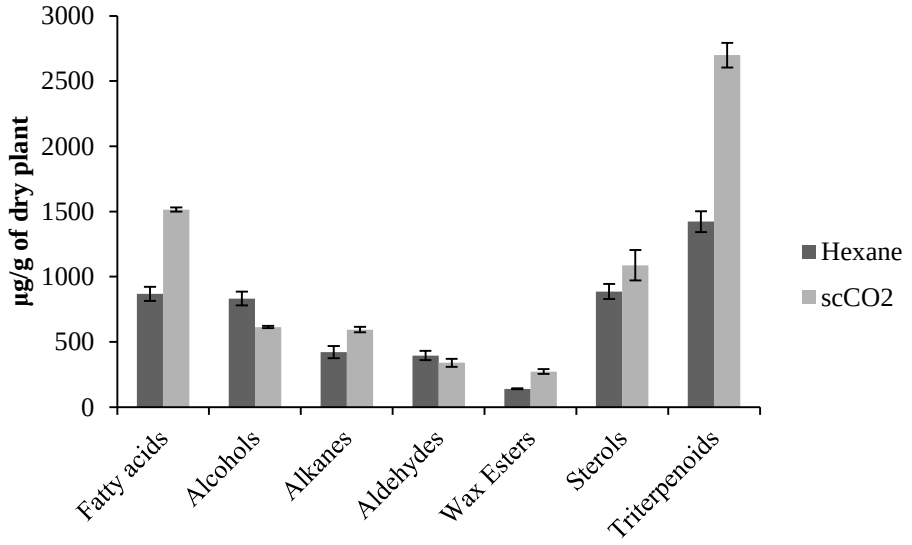


Figure 2-36 Composition of waxes extracted from leaf trash using hexane and scCO2 in µg/g of dry plant.

From the results that were obtained, shown in Figure 2-36, it was found that the same type of compounds were extracted for both types of extraction techniques, however there was a variation in the distribution and quantities of compounds extracted. Figure 2-36 shows that for most families of compounds, $scCO_2$ extracts larger quantities of molecules than hexane. This phenomenon could be due to the entrainer effect of solute molecules in the supercritical fluid phase, whereby solute molecules acts as co-solvents enhancing the solubility of other less soluble compounds.[73] Therefore, as compounds are incorporated into the supercritical phase, the solvation properties of $scCO_2$ change significantly resulting in an enhanced extraction of compounds. [73]

There was a significant variation in the quantities of fatty acids extracted when using $scCO_2$ and hexane. Significantly larger quantities of these molecules were extracted with $scCO_2$ (1515±16.1 µg/g of dry plant) when compared to hexane (868.7±53.5 µg/g of dry plant). One of the reasons for this is attributed to the much higher concentrations of unsaturated fatty acids (linolenic acid, linoleic acid and oleic acid) present in the $scCO_2$ wax extract (421.2 µg/g of dry plant compared to 99.3 µg/g of dry plant for the hexane extract), as shown in Figure 2-37.

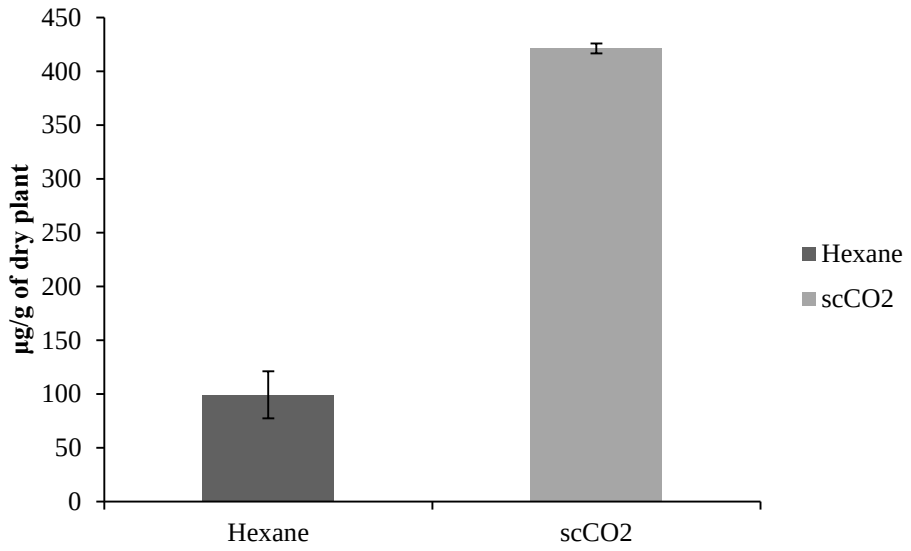


Figure 2-37 Amount of unsaturated fatty acids extracted using hexane and $scCO_2$.

One problem with using hexane soxhlet extraction is the exposure of the extracts to air which can lead to the oxidation of unsaturated compounds.[182] This is significantly decreased in $scCO_2$, reducing the chances of free radical reactions occurring and has been observed in this study. This is supported by the fact that in the hexane extracts there are significantly larger amounts of the dicarboxylic acid azelaic acid when compared to the $scCO_2$ extracts. Azelaic acid is a product of unsaturated fatty acid oxidation and Figure 2-38 indicates the higher amount of dicarboxylic acid present in the hexane extracts when compared to the $scCO_2$ extracts.

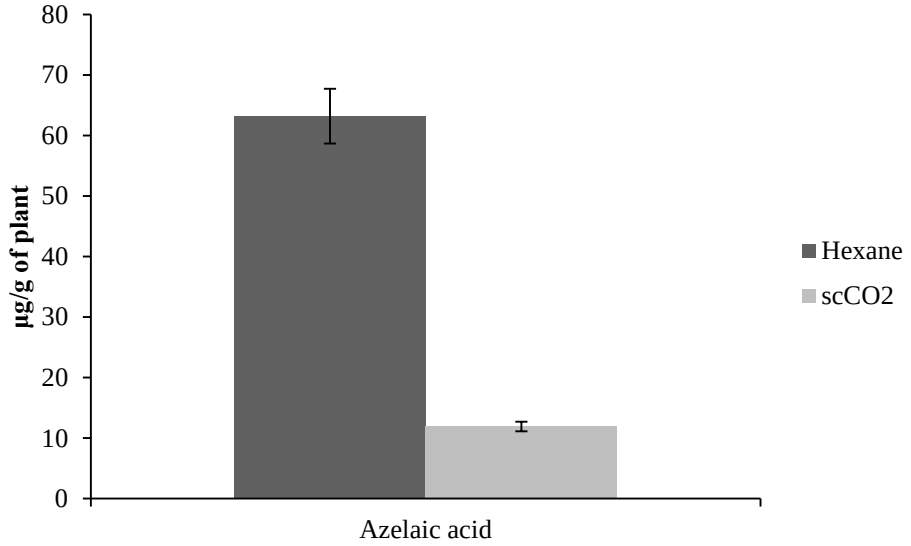
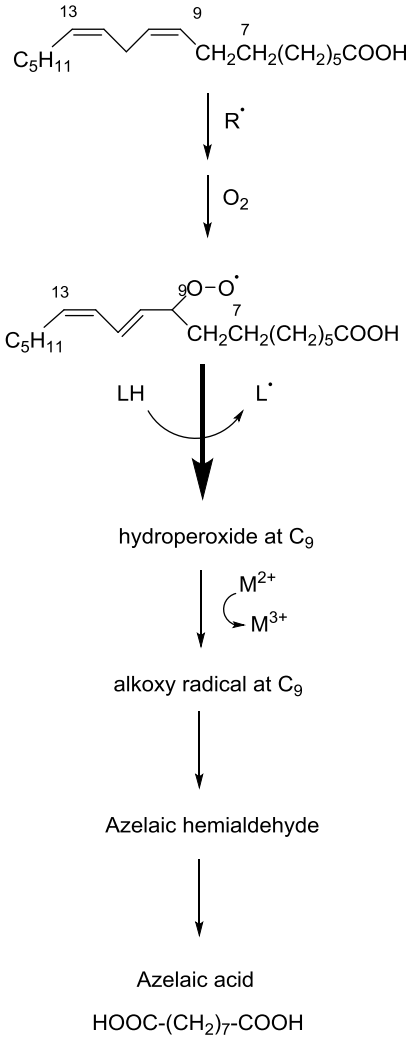


Figure 2-38 Amount of azelaic acid in hexane extract and $scCO_2$ extract.

Scheme 2-9 indicates the mechanism by which azelaic acid is formed from linoleic acid which involves an oxidative attack resulting in the formation of a peroxy radical at carbon no.9 followed by direct degradation to give azelaic acid.[183]



Scheme 2-9: Formation of linoleic acid from azelaic acid (LH = linoleic acid).

Similar results to this study were obtained by Bernardo-Gil *et al.* when extracting unsaturated fatty acids from hazelnut oil, where a higher concentration of unsaturated fatty acids was obtained when using $scCO_2$ when compared to hexane.[184] In addition, the same observation was made by Hunt, who carried out wax extraction from heather.[185] Significantly larger quantities of unsaturated fatty acids were found in $scCO_2$-extracted heather waxes than the hexane-extracted ones.[185]

The most abundant saturated fatty acids, $C_{12} - C_{22,}$ were found in much larger quantities in the $scCO_2$ extract as summarised in Table 2-14. Morrison *et al.* found that there were significantly larger amounts of palmitic acid, stearic acid and eicosanoic acid in the

CO_2-extracted cuticular wax from flax waste than the hexane extract.[186] Similar observations were made in other studies.[187]

Table 2-14 Comparision of saturated fatty acid concentrations in the $scCO_2$ wax and hexane wax.

Saturated Fatty acid	$scCO_2$ (µg/g of dry plant)	Hexane (µg/g of dry plant)
Dodecanoic acid	112.4 ±2.4	61.2 ±2.6
Tetradecanoic acid	104.5 ±0.3	58.1 ±2.5
Palimitic acid	866.8 ±4.2	411.1 ±12.5
Stearic acid	94.1 ±0.7	55 ±0.7
Eicosanoic acid	119.9 ±1.1	33 ±3
Docosanoic acid	48.7 ±0.9	29.5 ±2.8

In contrast to the fatty acid concentration, the total *n*-policosanol content was smaller in the $scCO_2$ extracts when compared to hexane extracts with total concentrations of 613.7 ±8 µg/g of dry plant and 831.9 ±59.3 µg/g of dry plant respectively. Higher quantities of 1-hexacosanol were found in the supercritical extracts. This was also observed by Hunt in the extraction of waxes from heather.[185] In both extracts, the dominant alcohol was found to be 1-dotriacontanol constituting 64% of the total alcohol composition in the hexane extract and 66.4% of the total alcohol composition in the $scCO_2$ extract. Similar results were obtained for the long-chain fatty aldehydes, with hexane exhibiting greater extraction yields.

Larger quantities of *n*-alkanes were present in the $scCO_2$ extracts (594.3 ±20.7 µg/g of dry plant) compared to the hexane extracts (421.8 ±46.2 µg/g of dry plant). The dominant chain length in both the hexane and $scCO_2$ extracts was found to be triatriacontane, comprising 41.3% and 51.1% of the total alkane composition respectively. Extraction of wax esters was greater with $scCO_2$ extraction (272 ±18.7 µg/g of dry plant) than hexane extraction (140 ±4 µg/g of dry plant).

The total phytosterol concentration was higher in the $scCO_2$ wax. Castola *et al.* have shown that higher concentrations of phytosterol can be extracted with $scCO_2$ at 200 –

250 bar and 50 ^{o}C than with soxhlet extractions using DCM.[188] Furthermore, a study comparing $scCO_2$ and recirculated hexane extraction demonstrated that more phytosterols are extracted using $scCO_2$ (275 bar and 75 ^{o}C) than hexane.[189]

The triterpenoid profile of the $scCO_2$ extract was found to be drastically different to that of the hexane soxhlet. Significantly larger amounts of triterpenoids were extracted when using $scCO_2$ than hexane, with total concentrations of 2698.8 ±95.3 µg/g of dry plant in the $scCO_2$ extract and 1422.2 ±80.5 µg/g of dry plant in the hexane extract.

Similar results were obtained from the rind wax, as shown in Figure 2-39, with larger amounts of *n*-policosanols and long chain fatty aldehydes in the hexane extracts, while slightly higher concentrations of long-chain fatty acids, *n*-alkanes, and wax esters in the $scCO_2$ extracts.

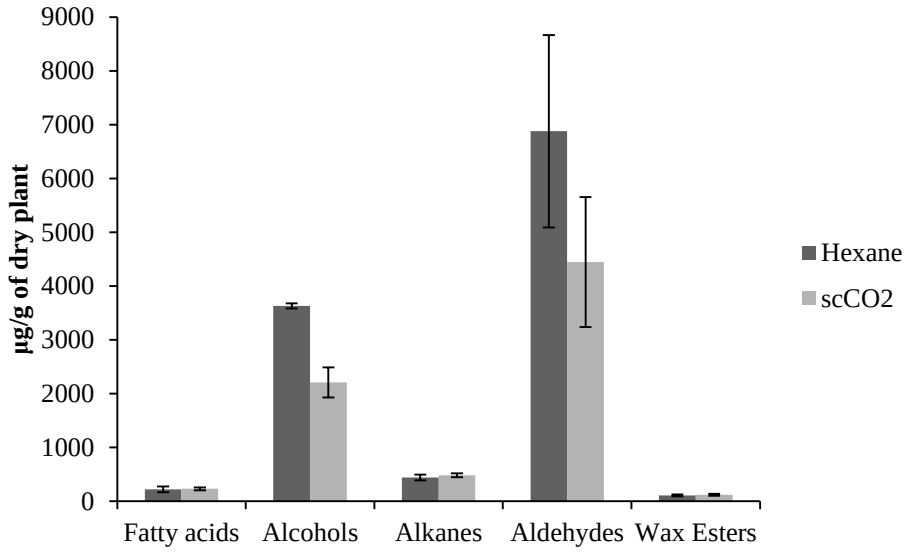


Figure 2-39 Composition of waxes extracted from sugarcane rind using hexane and scCO2 in µg/g of dry plant.

2.6 Advantage of $scCO_2$ for the extraction of waxes

In this study, CO_2 was utilised for the extraction of these waxes. A large number of these compounds have nutraceutical and medicinal properties and are often incorporated in number of food and drink products.

Furthermore, the supercritical extraction of waxes from the sugarcane waste can be utilised as a pre-treatment step within a biorefinery. Sugarcane bagasse and rind are already utilised as a large scale fermentation feedstock for ethanol production.

Supercritical extraction of these waxes has no detrimental effects on the biomass and it can be used post extraction for production of second generation biofuels.

2.7 Conclusion

The supercritical extraction of waxes from different types of sugarcane waste (rind, leaves and bagasse) has been successfully carried out in order to obtain high-quality compounds which would potentially add value to this waste biomass. This work demonstrates that different botanical components of sugarcane waste give rise to waxes with substantially different compositions. The rind wax provides substantially large quantities of *n*-policosanols and long-chain fatty aldehydes (83% of the total wax composition) while the leaf trash wax can be an excellent source of triterpenoids (169 mg/g of wax) along with unsaturated and saturated fatty acids and phytosterols. The exploitation of leaf trash as a potential source of wax has not been previously considered. Bagasse wax provides the highest amount of wax esters. These results, along with the DSC traces, have shown that these different waxes can therefore be used in different applications. Furthermore, the use of $scCO_2$ could allow the direct use of molecules within food or drink applications.

Chapter 3

Supercritical CO_2 extraction of waxes as an effective pre-treatment step in a maize stover biorefinery.

3 Chapter 3

3.1 Introduction

Ever-increasing world demands are being placed on fossil resources as a feedstock for the manufacture of chemicals and fuels.[190] A recent resurgence in the use of renewable feedstocks in holistic integrated biorefineries can aid in reducing the reliance on petroleum-based products.[191, 192] C_4 plants, such as maize, display high photosynthetic activity, high rates of carbon dioxide-fixation, high radiation and high water use efficiencies resulting in rapid growth and excellent productivity.[10, 13-16] The abundance of maize stover makes it a promising strategic feedstock for the manufacture of bio-based products as well as for bioenergy.[38, 40] After the maize grain is harvested, the maize stover is partially incorporated back into the soil, leaving a significant amount that can be used as potential feedstock.[36]

A first stage in an integrated maize stover biorefinery could be the extraction of high value waxes from the plant surface prior to the application of destructive technologies such as hydrolysis. This chapter may be seen as divided into two parts. The first part compares and contrasts waxes extracted by $scCO_2$ from three different parts of the maize stover; the stem, the leaves and the husks. The second part focuses on the use of $scCO_2$ extraction as a pre-treatment step in a holistic maize stover biorefinery whereby maize stover (as a whole) was subjected to $scCO_2$ extraction prior to hydrolysis and fermentation. The wax extracted from the maize stover was fractionated, characterised and applications for the different wax fractions are indicated. Furthermore, the effects of $scCO_2$ extraction on the downstream processing of the maize stover were investigated by comparing the properties of $scCO_2$-extracted maize stover to untreated-maize stover.

3.2 Characterisation and quantification of waxes obtained from different parts of the maize stover (stem, leaves and husks).

3.2.1 Extraction yields of supercritical extraction from the different parts of the maize stover.

The conditions for $scCO_2$ extractions used within this study are 350 bar and 50 ^{o}C. Typical commercial plants operate at below 400 bar thus these conditions were implemented. The yields of lipophilic extractives from the stem, leaves and husk are summarised in Figure 3-1. The highest yield of wax was obtained from the leaves which

accounted for 1.02% of the dry biomass, followed by the husk (0.64%) and the stem (0.31%).

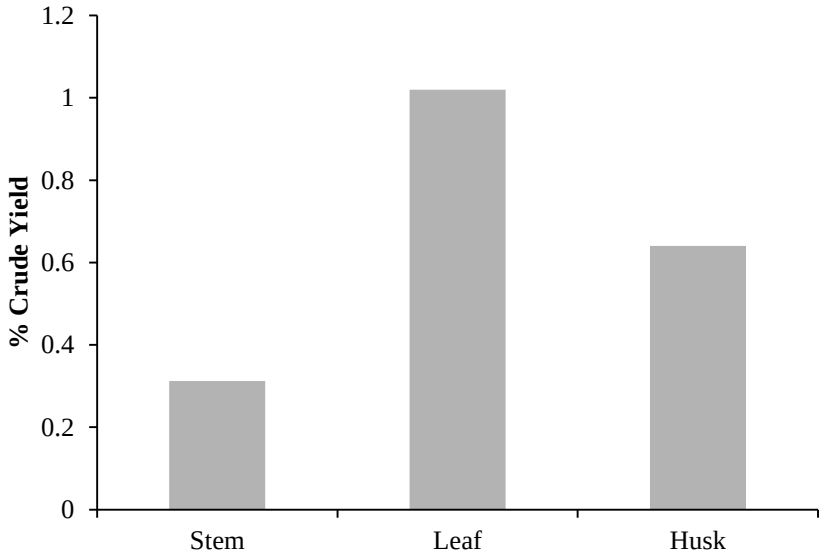


Figure 3-1 % $scCO_2$ extraction yields of wax from stems, leaves and husks of corn stover.

3.2.2 Characterisation and quantification of compounds found in waxes from the maize stem, leaves and husk.

GC and GC-MS analyses were used to characterise the underivatised and silylated extracts using a high temperature capillary column and methods which allowed for the elution and determination of high-molecular weight compounds such as sterols, steroid ketones and wax esters (section 7.9.2 and 7.9.3 in Chapter 7). The GC chromatogram of the silylated wax extract from the maize husk is illustrated in Figure 3-2.

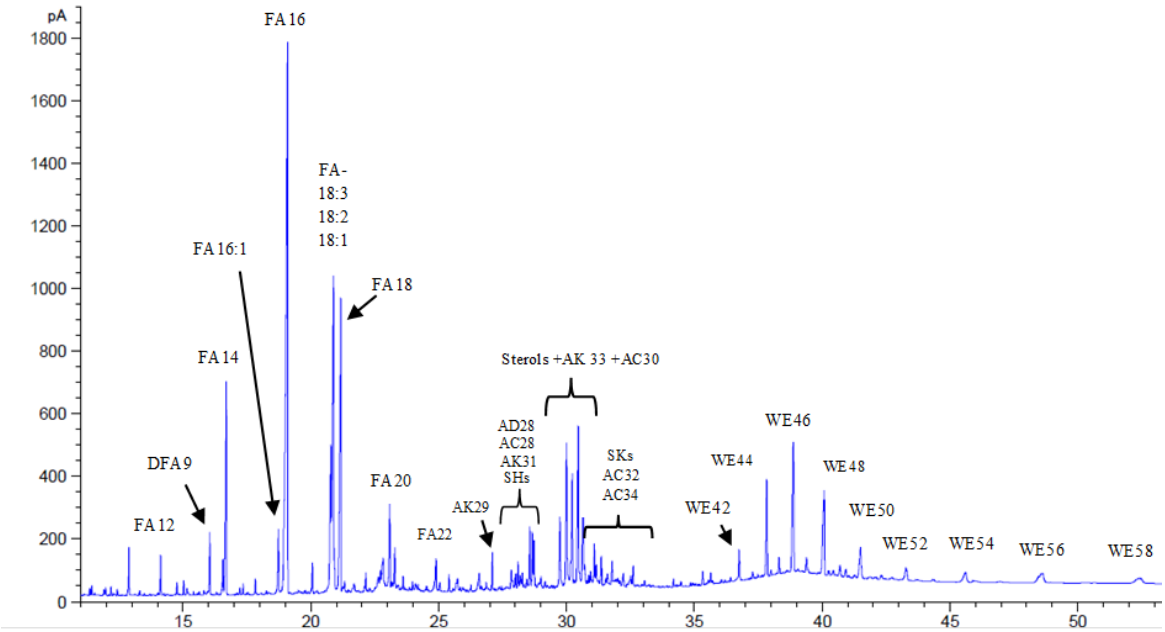


Figure 3-2 GC chromatogram illustrating some of the hydrophobic compounds found in the lipophilic extractive from the maize husk. FA (Fatty acid), DFA (Difatty acid), AK (Alkane), AC (Alcohol), AD (Aldehyde), SH (Steroid hydrocarbon), SKs (Steroid ketones), WE (Wax esters).

Table 3-1 Composition of compounds found in lipophilic extractives from stems, husks and leaves of maize (in µg/g of dry plant).

Compounds	Stem (µg/g of plant)	Husk (µg/g of plant)	Leaf (µg/g of plant)
Pentanoic acid	2.3 ±0.3	0.7 ±0.1	3.9 ±0.2
Hexanoic acid	9.7 ±0.3	5.3 ±0.8	18.1 ±0.3
Heptanoic acid	0.9 ±0.1	1.3 ±0.2	2.4 ±0.1
Octanoic acid	1.5 ±0.1	4.8 ±0.5	6.3 ±0.1
Nonanoic acid	2.8 ±0.6	7.3 ±0.3	12 ±1.8
Decanoic acid	0.5 ±0.06	2 ±0.2	5.8 ±0.1
Dodecanoic acid	4.2 ±0.4	1.2 ±0.1	65.6 ±0.9
Tetradecanoic acid	7.8 ±0.7	82.8 ±5.8	102 ±0.1

Pentadecanoic acid	2.3 ±0.3	5.9 ±0.8	7.3 ±0.5
Hexadecanoic acid	154.9 ±15.2	428 ±46.9	453.4 ±3.7
Heptadecanoic acid	6 ±0.8	11.3 ±0.9	13.5 ±0.2
Octadecanoic acid	43.9 ±4.6	143.5 ±2.8	173.7 ±1.8
Nonanoic acid	1.8 ±0.1	2.7 ±0.2	74.6 ±0.7
Eicosanoic acid	18.4 ±0.8	39.4 ±0.1	68.4 ±0.8
Heneicosanoic acid	2.9 ±0.04	6.2 ±0.5	8.3 ±1.1
Docosanoic acid	6.5 ±1.3	18.6 ±1.2	58.8 ±1
Tricosanoic acid	5.9 ±1.4	10.8 ±0.1	26.8 ±1.8
Tetracosanoic acid	8.1 ±2	13.2 ±1.8	55.5 ±1.3
Pentacosanoic acid	1.2 ±0.1	2 ±0.05	16 ±0.3
Hexacosanoic acid	0.6 ±0.02	1.8 ±0.1	9.9 ±0.3
Octacosanoic acid	TR*	TR	TR
Tricontanoic acid	TR	TR	TR
Dotriacontanoic acid	TR	TR	TR
Total saturated fatty acids	**282.9 ±29.3**	**788.7 ±63.5**	**1182.3 ±17.1**
9-hexadecenoic acid	22.7 ±1.1	27.2 ±2.1	-
9-octadecenoic acid	7 ±0.01	12.9 ±0.9	4.3 ±0.1
9,12-Octadecadienoic acid	50.3 ±3.7	171.5 ±5	20.5 ±0.8
9,12,15-Octadecatrienoic acid	36 ±2.9	96.3 ±10.8	7.2 ±0.1

Total unsaturated fatty acids	**116 ±7.7**	**298.5 ±18.8**	**32 ±1**
Ethanedioic acid	0.3 ±0.2	TR	0.53 ±0.05
Butanedioic acid	0.4 ±0.1	0.2 ±0.03	1.8 ±0.1
Heptanedioic acid	0.5 ±0.04	0.6 ±0.1	1.6 ±0.1
Octanedioic acid	2.5 ±0.2	3.7 ±0.2	4.9 ±0.03
Nonanedioic acid	12.4 ±1.6	17.3 ±1.3	22.5 ±0.3
Decanedioic acid	1.4 ±0.1	2.4 ±0.03	TR
Total saturated difatty acids	**17.5 ±2.2**	**24.4 ±1.7**	**31.3 ±0.6**
Tetracosanol	-	-	8.1 ±0.2
Hexacosanol	TR	7.5 ±0.8	14.8 ±1.2
Octacosanol	12.5 ±0.3	7.9 ±1	34.4 ±2.3
Triacontanol	34.1 ±1.7	91.6 ±1	104.1 ±28.2
Dotriacontanol	-	15.1 ±2.8	61 ±5.2
Tetratriacontanol	-	4.2 ±0.3	6.2 ±0.3
Total saturated fatty alcohols	**46.6 ±2**	**126.2 ±5.9**	**228.8 ±37.4**
Octacosanal	4.1 ±1.7	12.3 ±0.3	37.2 ±0.3
Total saturated fatty aldehydes	**4.1 ±1.7**	**12.3 ±0.3**	**37.2 ±0.3**
Pentacosane	0.56 ±0.03	5.4 ±2.6	9.5 ±1.1

Heptacosane	0.91 ±0.01	5 ±0.7	13.4 ±1.3
Nonacosane	2.2 ±0.4	11.9 ±2.5	31.8 ±0.4
Hentriacontane	6.1 ±1.3	20.8 ±1.4	62.1 ±6.8
Triatriacontane	6.4	12.6 ±0.4	34.3 ±9.6
Total hydrocarbons	**16.2 ±1.7**	**55.7 ±7.9**	**151.2 ±19.2**
Campesterol	57.2 ±4.1	146.8 ±10.7	152.5 ±0.1
Stigmasterol	64.1 ±4.6	132.4 ±24.2	231.4 ±0.6
β-Sitosterol	156.2 ±10.2	184.8 ±11.8	231.4 ±0.7
Stigmastanol	-	23.2 ±2.2	-
Total sterols	**277.5 ±18.9**	**487.3 ±48.9**	**615.2 ±1.4**
Stigma-3,5-diene	15.3 ±0.3	31.4 ±1.2	118.8 ±0.5
Total Steroid hydrocarbons	**15.3 ±0.3**	**31.4 ±1.2**	**118.8 ±0.5**
Stigmasta-3,5-dien-7-one	7.7 ±0.2	22.9 ±1.4	45.6 ±1.2
Stigma-4-*en*-3-one	12.3 ±0.02	28.1 ±6.3	53 ±4.6
Stigmastan-3,6-dione	6.5 ±0.3	-	33.8 ±0.5
Total Steroid ketones	**26.6 ±0.5**	**51.1 ±8.7**	**132.4 ±6.3**
Wax ester C_{38}	1.9 ±0.1	3 ±0.007	3.6 ±0.1
Wax ester C_{40}	6.5 ±0.4	5.7 ±1.2	3.5 ±0.1

Wax ester C_{42}	5.2 ±0.1	16.5 ±2.9	11.1 ±1.7
Wax ester C_{43}	0.8 ±0.02	3.2 ±0.3	2.4 ±0.1
Wax ester C_{44}	8.9 ±0.3	52.7 ±8.2	44.4 ±0.5
Wax ester C_{45}	1.8 ±0.1	6.9 ±1.4	7 ±0.5
Wax ester C_{46}	9.1 ±0.1	75 ±11.8	60.3 ±6.8
Wax ester C_{47}	1.8 ±0.2	8.7 ±1.4	8.8 ±1
Wax ester C_{48}	5.6 ±0.1	58.5 ±9	61.5 ±5.9
Wax ester C_{49}	0.7 ±0.01	7.1 ±2	8 ±0.9
Wax ester C_{50}	5 ±0.2	24.1 ±3.6	38.1 ±3.5
Wax ester C_{51}	0.5 ±0.2	2.7 ±0.6	4.5 ±0.5
Wax ester C_{52}	3 ±0.1	10.3 ±1.9	24.6 ±0.9
Wax ester C_{53}	2 ±0.5	1.4 ±2.5	2.3 ±0.2
Wax ester C_{54}	2 ±0.1	11.8 ±0.7	16.4 ±0.1
Wax ester C_{56}	2 ±0.5	18.7 ±0.7	6.7 ±0.6
Wax ester C_{58}	-	11.9 ±1.1	TR
Total Wax esters	**57 ±3**	**318.2 ±48.6**	**303.4 ±23.4**
2-Pentadecanone-6,10,14-trimethyl	15.9 ±0.1	27.7	265.3 ±1.3
Total 'Other' compounds	**15.9 ±0.1**	**27.7**	**265.3 ±1.3**

*TR = *trace* amounts

Table 3-1 indicates the identities and quantities of the different lipophilic constituents making up the waxes extracted from the stems, husks and leaves of maize using $scCO_2$.

GC-MS analysis indicates that the type of hydrophobic compounds found within the waxes from the stems, husks and leaves are quite similar. However quantification data shows that there is a difference in the relative quantities of certain compounds and their families among the different parts of the plant. The predominant groups of compounds found in the lipophilic extractives include long-chain saturated and unsaturated fatty acids, sterols and long-chain wax esters followed by smaller quantities of long-chain fatty alcohols, fatty aldehydes, hydrocarbons and steroid ketones.

The most abundant group of compounds found in the $scCO_2$ extractives for the stems, husks and leaves were free saturated and unsaturated fatty acids. The saturated fatty acids had chain lengths varying from C_5 to C_{34} with the expected even-over-odd predominance.[91] The most abundant saturated fatty acid found in all parts (stem, husk and leaf) was palmitic acid (C_{16}) followed by stearic acid (C_{18}) and myristic acid (C_{14}). Odd-chain fatty acids were detected in the extractives albeit in smaller quantities. The husk and leaf waxes had the highest abundance of saturated fatty acids (123.2 ±9.9 mg/g of wax and 115 ±1.7 mg/g of wax respectively). A study carried out by Avato *et al.* looked into the fatty acid distribution in the seedlings and leaves of the mature maize plant.[109] Chain lengths from C_{14} to C_{34} were detected. While in the seedlings C_{14}-C_{16} fatty acids dominated (similar to this study), the dominant fatty acids found in the leaves of the mature plant were found to be C_{30} and C_{28}. Contrary to this study, only small amounts of C_{14}-C_{16} and C_{18} were found.[109] Bianchi *et al*. found fatty acids of chain length C_{18} to C_{34} in the surface lipids of the husk, with C_{22} being the dominant acid.[193] Therefore a much wider variety of acids were identified in the husk wax in this current study using $scCO_2$. The solvent utilised in the previous studies was chloroform, which is a toxic, non-renewable solvent having a number of negative effects on health and the environment. The routine use of chlorinated solvents such as chloroform and DCM should be drastically reduced.[194]

Di-saturated carboxylic acids were also present in the $scCO_2$ lipophilic extractives with the major di-fatty acid being azelaic acid followed by suberic acid. Minor quantities of other short-chain di-fatty acids having chain lengths of C_2, C_4, C_7 and C_{10} may be found in Table 3-1. Disaturated acids were not previously reported in literature.[109, 193]

Interestingly, high abundances of unsaturated fatty acids were detected in the $scCO_2$ waxes from the husks and stems (46.6 ±2.9 mg/g of wax and 37.2 ±2.5 mg/g of wax respectively), while very small amounts of unsaturated acids were found in the lipophilic extractives of the leaves (3.1 ±0.1 mg/g of wax) (Figure 3-3). Palmitoleic

acid ($C_{16:1}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) were found in the husk and stem extractives with linoleic and linolenic acid being the predominant compounds. No palmitoleic acid was found in the leaves. C_{18} unsaturated fatty acids were detected in the leaves and husks in the studies carried out by Avato *et al.* and Bianchi *et al.* respectively, however the type and distribution were not stated.[109, 193] Khan *et al.* reported the presence of palmitoleic acid, oleic acid, linoleic acid and linolenic acid in maize silage.[195]

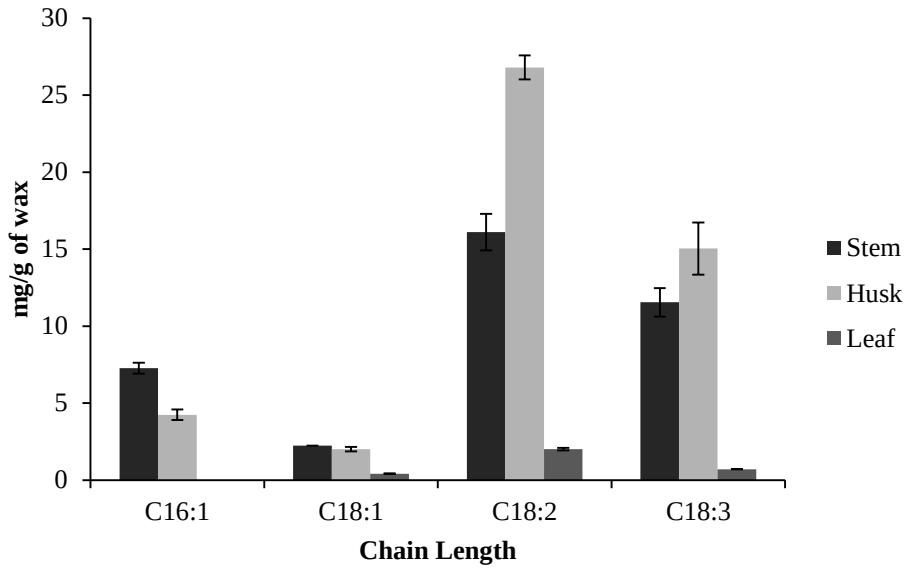


Figure 3-3 Unsaturated fatty acid distribution of the stem, husk and leaf extractives.

n-Policosanols were most abundant in the leaf $scCO_2$ extractives, with even chain lengths varying from C_{24} to $C_{34,}$ followed by the husk extractives (C_{26} - C_{34}) while the stem extractives had the lowest quantities and only three different fatty alcohols were present (C_{26} - C_{30}). In all cases, the major fatty alcohol was found to be triacontanol (C_{30}).[109] This is consistent with earlier studies on the alcohol composition of the leaves, where triacontanol was found to be the dominant alcohol. In the study carried out by Bianchi *et al.*, no alcohols were detected in the surface lipids from the husk.[193] This contrasts to what was found in this current study, where a considerable variety of alcohols were detected in the husk wax. The husk and leaf extractives had the largest amount of *n*-policosanols (19.7 ±0.9 mg/g of wax and 22.4 ±3.7 mg/g of wax respectively).

Odd-chained *n*-alkanes ranging from C_{25} to C_{33} were identified in all $scCO_2$ extractives. The most abundant alkane in the leaf and husk extractives was found to be hentriacontane (C_{31}) while relatively equal amounts of triatriacontane (C_{33}) and

hentriacontane were present in the stem extractives. Previous studies on the alkane composition in the mature leaves of the plant have shown alkane compositions ranging from C_{21} to C_{33}.[109] The dominant alkanes were found to be C_{31} and C_{29} (60% of total composition). Considerable amounts of C_{33} were also found in the leaves of the mature plant studied by Avato *et al.*, similar to this study. The other chain lengths mentioned were only found in small quantities.[109] A study on the lipid composition from the maize husk found three dominant homologues C_{27}, C_{29} and C_{31}, which differs to this current study where C_{33} was also found in significant quantities.[193]

Octacosanal (C_{28}) was the sole fatty aldehyde present in the $scCO_2$ lipophilic extractives. Previous studies have shown considerable amounts of long-chain fatty aldehydes in the leaves of the mature plant (ranging from C_{16} to C_{34}) with C_{30} and C_{28} being the two most dominant aldehydes.[109] Four aldehyde homologues were present in the husk surface lipids in the study by Bianchi *et al.*, with equal distribution of each.[193]

Quantification data indicates that sterols were the second most abundant class of compounds within the $scCO_2$ extractives, with β-sitosterol predominating. Other free sterols such as stigmasterol, campesterol and stigmastanol were also detected. In terms of wax composition, the stem and husk extractives contained the largest quantities of sterols, with 88.9 ±6.1 mg/g of wax and 76.1 ±7.6 mg/g of wax respectively. The studies carried out by Avato *et al.* and Bianchi *et al.* did not identify any sterols in the surface lipids of maize.[109, 193] Zhao *et al.* detected sitosterol, stigmasterol and campesterol in petroleum ether-extracted waxes from corn stalk but no quantification data was provided.[196]

Another interesting class of molecules determined was the steroid ketones. Three steroid ketones were identified in the lipophilic extractives; stigma-4-*en*-3-one, stigma-3,5-di*en*-7-one and 5α-stigmastan-3,6-dione, with stigma-4-*en*-3-one predominating.

Figure 3-5 is the EI-mass spectrum of stigmast-4-*en*-3-one from maize leaf wax. The base peak at *m/z* 124 is a typical fragmentation ion characteristic of Δ^4-3-ketosteroids (Figure 3-4).[197] The formation of this ion is shown in scheme 3-1. The steric compression of the fused A/B ring system is relieved by fission of the allylically activated C_9-C_{10} bond of the ionised steroid, giving rise to ion *a*. This is followed by hydrogen migration from carbon no.8 to the radical site found at carbon no. 10. Formation of the ionised diene *b* makes this process energetically favoured.

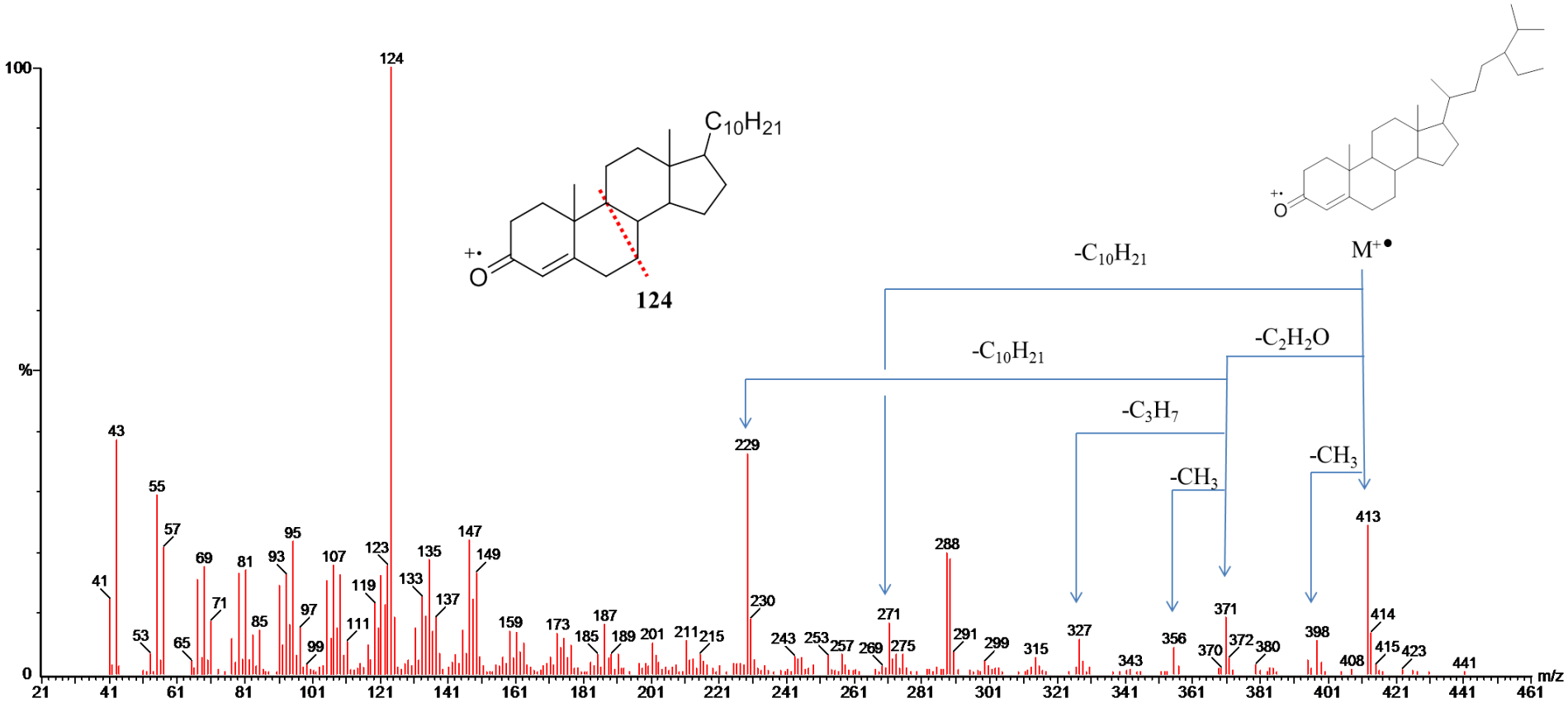


Figure 3-4 Mass spectrum of stigma-4-en-3-one from maize leaf wax

Scission of the C_6-C_7 bond is instigated by the transfer of an allylic hydrogen from carbon no.11 to the oxygen atom resulting in the ion fragment c having a *m/z* of 124 and a neutral fragment d.

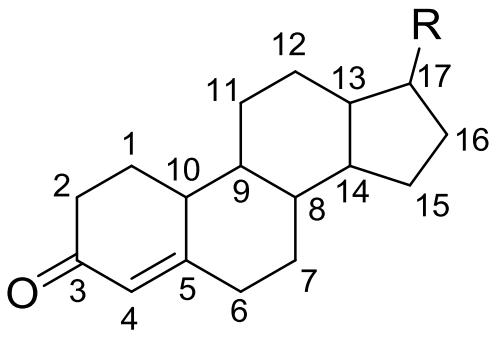
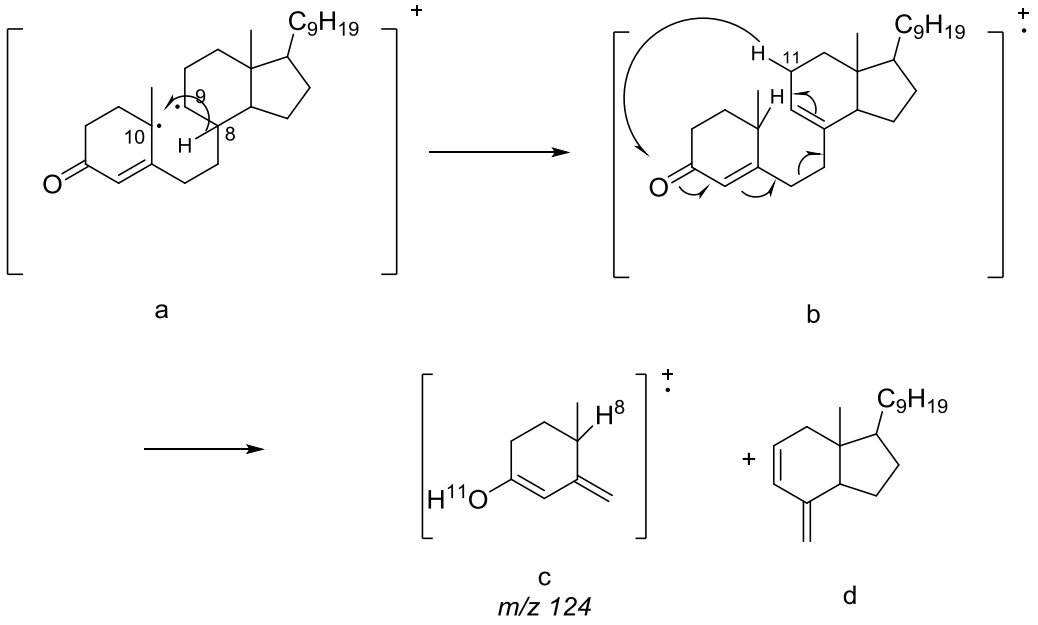
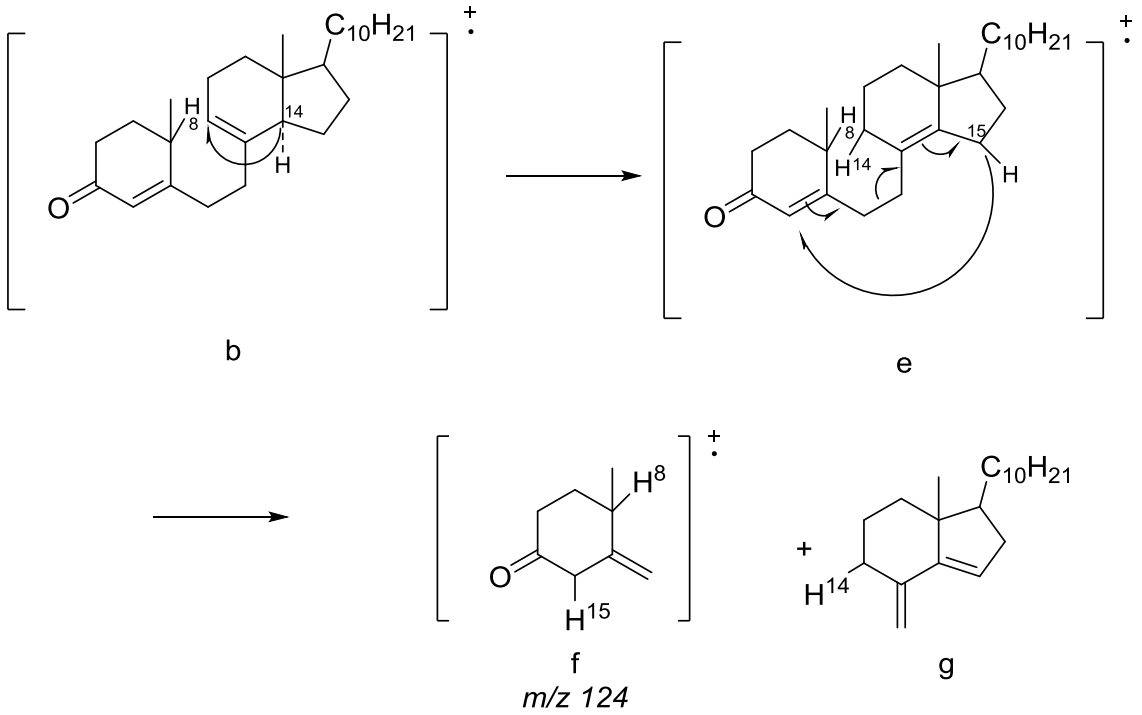


Figure 3-5 Structure of Stigmast-4-*en*-3-one with labelled C atoms.



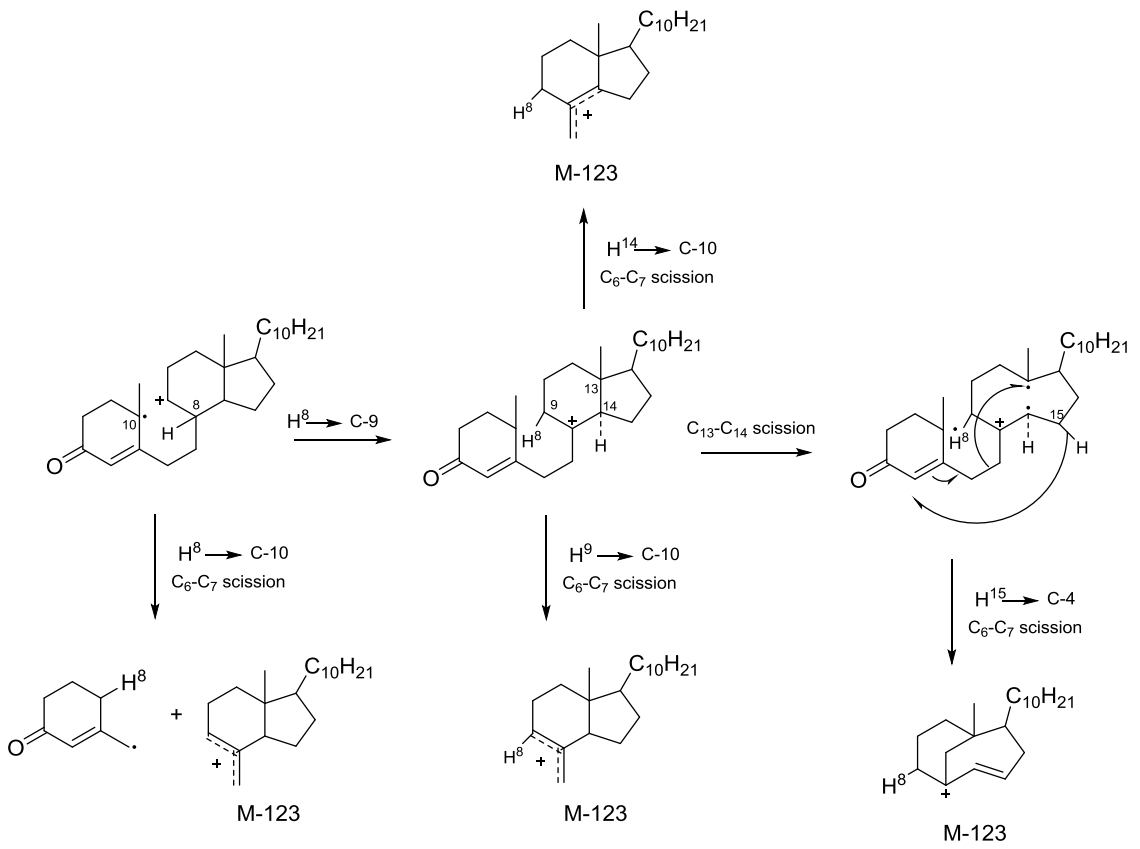
Scheme 3-1 Formation of base ion *m/z* = 124 in steroid ketones.

Studies have shown that stages a→c are fundamental contributors to the formation of the *m/z* 124 ion.[197] However in the second stage of the process (migration of the hydrogen at carbon no.11 to the oxygen atom) results have shown that migration of the hydrogen atom from carbon no.11 constitutes less than half of the second itinerant hydrogen. The other main source of hydrogen (42%) results from a hydrogen on carbon no.15. This process is shown below (scheme 3-2) and involves a 1,3-sigmatropic hydrogen shift in species b from carbon no. 14 to carbon no. 9 resulting in a thermodynamically stable diene e. This is followed by hydrogen migration from carbon no.15 to carbon no.4 resulting in C_6-C_7 bond fission yielding the *m/z* ion 124 ion f and a neutral diene g.



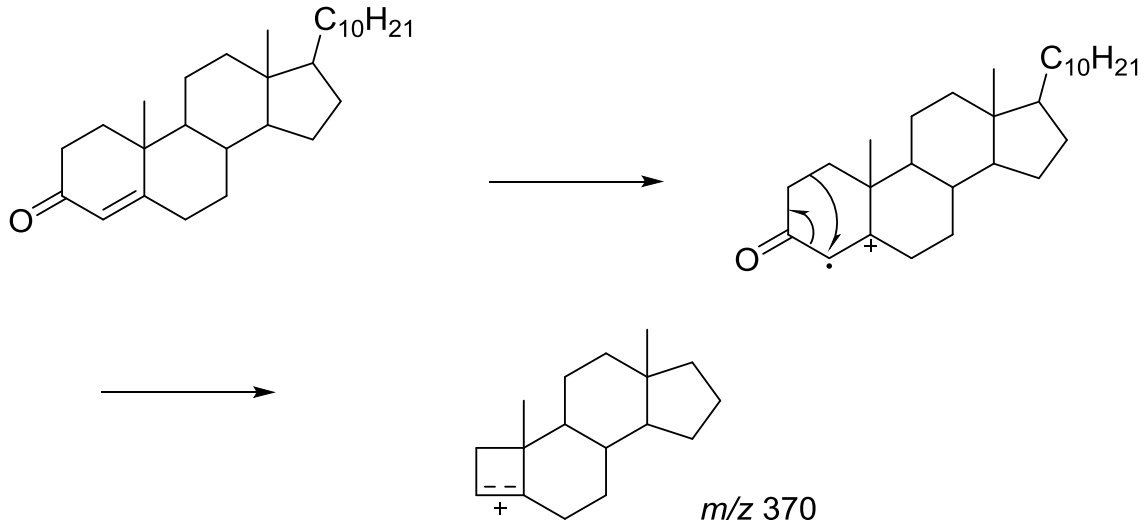
Scheme 3-2: Formation of base ion *m/z* = 124 by migration of hydrogen from carbon no.15 in second stage of the process.

As was the case in the formation of the base peak (*m/z* 124), the peak at *m/z* 288 is a result of the C_6-C_7 and C_9-C_{10} fission; however in this case the charge is located on the hydrocarbon fragment (i.e. rings C and D). A hydrogen atom is transferred away from the charged molecular ion, however migration is not specific – with migrations from C-8 (33%), 14 (25%), 15 (24%) and 9 (17%).



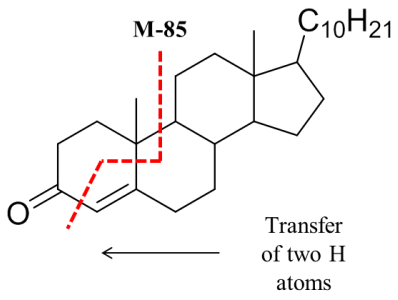
Scheme 3-3 Formation of ion at *m/z* = 288 (M-123).

Apart from the base peak and the M-123 peak (*m/z* 288), there are other ion fragments characteristic of Δ^4-3-ketosteroids. The peak at *m/z* 370 is a result of the loss of a ketene from ring A upon electron impact of stigmast-4-*en*-3-one as shown in Scheme 3-4. The ion at *m/z* 355 is a result of the loss of the ketene and a methyl radical.



Scheme 3-4: Formation of ion *m/z* = 360 as a result of the loss of a ketene.

Another diagnostic peak is that of *m/z* 327 (M-85 ion). It has been found that the process involves the loss of carbons no. 2, 3 and 19 while carbon no. 4, 6, 7 and 17 are retained in the charged fragment.[198] The ion is formed through a complicated procedure which involves the fission of carbon bonds 9-10, 5-10 and 3-4 with migration of two hydrogen atoms from the charged hydrocarbon fragment. Studies have shown that the molecular ion is not the parent ion involved in the process but the M-42 ion (resulting from the loss of the ketene; in the case of stigma-4-*en*-3-one this is the ion with *m/z* 370). No detailed mechanism for the process has been brought forward as this would require a large amount of speculation.[197]



Scheme 3-5: Formation of ion *m/z* = 327.

The other steroid ketone identified was 5α-stigmastan-3,6-dione. Characteristic fragments were found at *m/z* 428 (molecular ion), *m/z* 287 (M-$C_{10}H_{21}$ (side-chain)) and *m/z* 245 (M-$C_{10}H_{21}$ – 42) as shown in Figure 3-6.

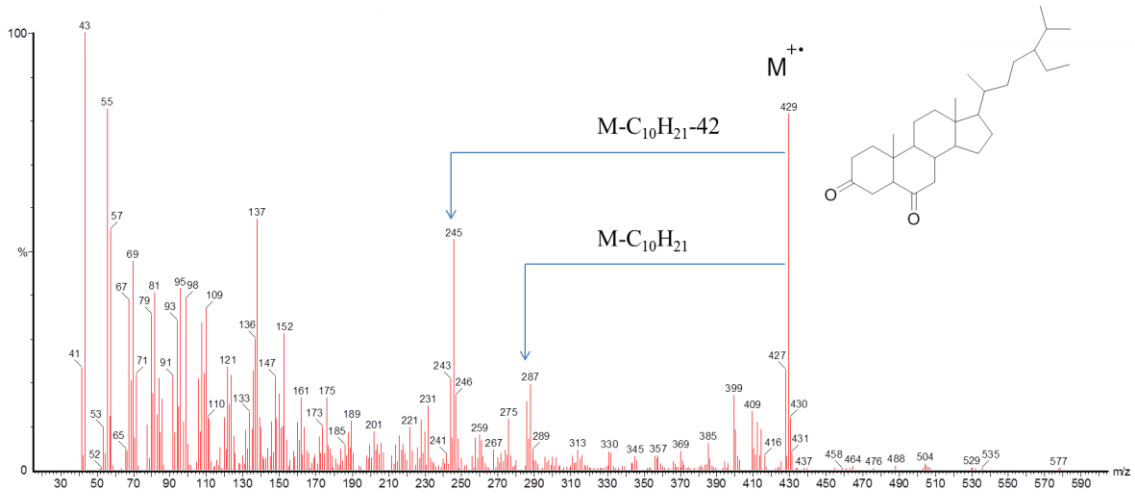


Figure 3-6 Mass spectrum of 5α-stigmastan-3,6-dione from maize leaf wax.

Stigma-4-*en*-3-one has shown to display hypoglycaemic effects.[199, 200] Extracts containing this molecule produced significant reductions in levels of blood glucose, whereby stigma-4-*en*-3-one was found to be the principle hypoglycaemic agent.[199]

Significant hypoglycaemic activity was observed when the pure compound was injected intravenously.[200]

The $scCO_2$ leaf extractives contained the highest amount of steroid ketones (13 ±0.6 mg/g of wax). Zhao *et al.* also detected stigma-4-*en*-3-one, stigma-3,5-diene-7-one and 5α-stigmastan-3,6-dione as well as three other steroid ketones (cholest-4-*en*-3-one, ergosta-4,22-dien-3-one and 4,22-stigmastadien-3-one) but does not mention the quantities found in the extracts.[196]

A number of wax esters were present in all $scCO_2$ lipophilic extractives but were found most abundant in the wax extracts from the husk (49.7 ±7.6 mg/g of wax) followed by the leaves (29.7 ±2.3 mg/g of wax).

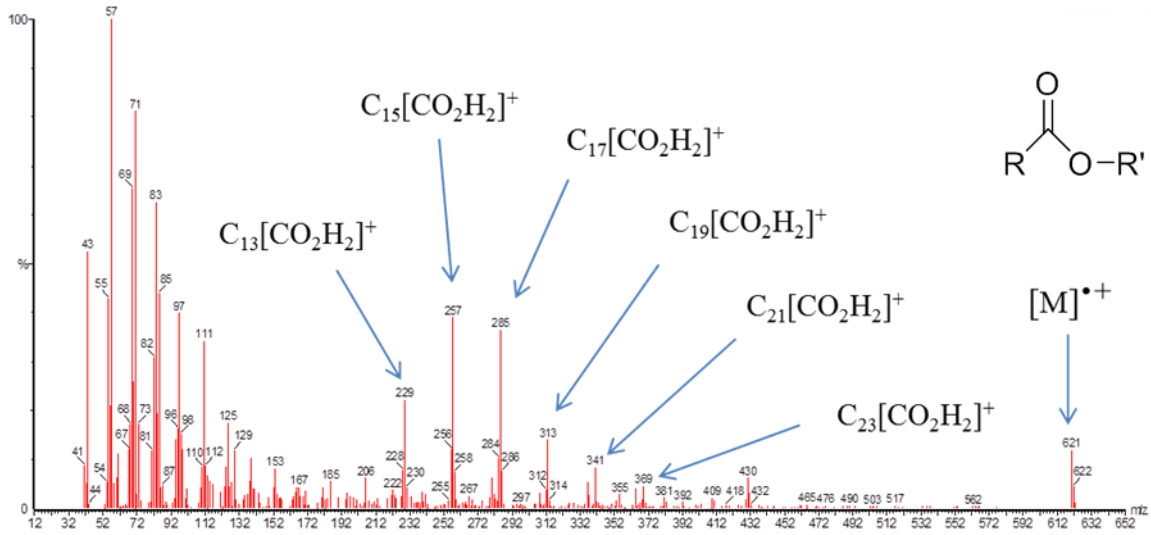


Figure 3-7 Mass spectrum for wax ester C_{42} from maize husk

Figure 3-7 is a typical spectrum of a wax ester chain length C_{42} from the husk lipophilic extractives. The peaks at *m/*z 229, 257, 285, 313, 341 and 369 arise as a result of a rearrangement process where protons are transferred from the alcohol chain to the acid chain and correspond to protonated hexadecanoic, octadecanoic, eicosanoic, docosanoic and tetracosanoic acids respectively. This indicates that for each wax ester chain length there are different isomers having different acid : alcohol moieties. Different isomers for wax esters up to C_{52} have been identified and may be found in Table 3-2.

Table 3-2 Wax ester isomers for each chain length up to C_{52}.

Chain length	Stem	Husk	Leaf
C_{38}	C_{14}:C_{24}	C_{14}:C_{24}	C_{14}:C_{24}
	C_{16}:C_{22}	C_{16}:C_{22}	C_{16}:C_{22}
	C_{18}:C_{20}	C_{18}:C_{20}	C_{18}:C_{20}
	C_{20}:C_{18}	C_{20}:C_{18}	C_{20}:C_{18}
	C_{22}:C_{16}	C_{22}:C_{16}	C_{22}:C_{16}
C_{40}	C_{14}:C_{26}	C_{14}:C_{26}	C_{14}:C_{26}
	C_{16}:C_{24}	C_{16}:C_{24}	C_{16}:C_{24}
	C_{18}:C_{22}	C_{18}:C_{22}	C_{18}:C_{22}
	-	C_{20}:C_{20}	C_{20}:C_{20}
	-	C_{22}:C_{18}	C_{22}:C_{18}
C_{42}	C_{14}:C_{28}	C_{14}:C_{28}	C_{14}:C_{28}
	C_{16}:C_{26}	C_{16}:C_{26}	C_{16}:C_{26}
	C_{18}:C_{24}	C_{18}:C_{24}	C_{18}:C_{24}
	C_{20}:C_{22}	C_{20}:C_{22}	C_{20}:C_{22}
	C_{22}:C_{20}	C_{22}:C_{20}	C_{22}:C_{20}
	-	C_{24}:C_{18}	C_{24}:C_{18}
C_{44}	C_{14}:C_{30}	C_{14}:C_{30}	C_{14}:C_{30}
	C_{16}:C_{28}	C_{16}:C_{28}	C_{16}:C_{28}
	C_{18}:C_{26}	C_{18}:C_{26}	C_{18}:C_{26}
	C_{20}:C_{24}	C_{20}:C_{24}	C_{20}:C_{24}
	C_{22}:C_{22}	C_{22}:C_{22}	C_{22}:C_{22}
	C_{24}:C_{20}	C_{24}:C_{20}	C_{24}:C_{20}
	-	C_{26}:C_{18}	C_{26}:C_{18}

C_{46}	C_{14}:C_{32}	C_{14}:C_{32}	C_{14}:C_{32}
	C_{16}:C_{30}	C_{16}:C_{30}	C_{16}:C_{30}
	C_{18}:C_{28}	C_{18}:C_{28}	C_{18}:C_{28}
	C_{20}:C_{26}	C_{20}:C_{26}	C_{20}:C_{26}
	C_{22}:C_{24}	C_{22}:C_{24}	C_{22}:C_{24}
	C_{24}:C_{22}	C_{24}:C_{22}	C_{24}:C_{22}
	C_{26}:C_{20}	C_{26}:C_{20}	C_{26}:C_{20}
	-	-	C_{28}:C_{18}
C_{48}	-	C_{14}:C_{34}	C_{14}:C_{34}
	C_{16}:C_{32}	C_{16}:C_{32}	C_{16}:C_{32}
	C_{18}:C_{30}	C_{18}:C_{30}	C_{18}:C_{30}
	C_{20}:C_{28}	C_{20}:C_{28}	C_{20}:C_{28}
	C_{22}:C_{26}	C_{22}:C_{26}	C_{22}:C_{26}
	C_{24}:C_{24}	C_{24}:C_{24}	C_{24}:C_{24}
	C_{26}:C_{22}	C_{26}:C_{22}	C_{26}:C_{22}
	-	-	C_{28}:C_{20}
C_{50}	C_{16}:C_{34}	C_{16}:C_{34}	C_{16}:C_{34}
	C_{18}:C_{32}	C_{18}:C_{32}	C_{18}:C_{32}
	C_{20}:C_{30}	C_{20}:C_{30}	C_{20}:C_{30}
	C_{22}:C_{28}	C_{22}:C_{28}	C_{22}:C_{28}
	C_{24}:C_{26}	C_{24}:C_{26}	C_{24}:C_{26}
	C_{26}:C_{20}	C_{26}:C_{20}	C_{26}:C_{20}
	C_{28}:C_{18}	C_{28}:C_{18}	C_{28}:C_{18}
C_{52}	C_{20}:C_{32}	C_{20}:C_{32}	C_{20}:C_{32}
	C_{22}:C_{30}	C_{22}:C_{30}	C_{22}:C_{30}

	C_{24}:C_{28}	C_{24}:C_{28}	C_{24}:C_{28}
	C_{26}:C_{26}	C_{26}:C_{26}	C_{26}:C_{26}
	C_{28}:C_{24}	C_{28}:C_{24}	C_{28}:C_{24}

In the stem extract, the major wax ester was C_{46} while in the leaf extract, the wax ester C_{48} predominated. Approximately equal amounts of C_{46} and C_{48} wax esters were found in the husk extracts. Previous studies have detected wax ester chain lengths of C_{38} to C_{60} which corroborates the finding for this study.[108, 109, 193] Bianchi *et al.* found that the dominant wax ester chain length in the leaves of the mature plant was found to be C_{44} followed by C_{46}.[108] A separate study by Bianchi *et al.* indicated that C_{46} was the dominant wax ester in the husk extractives.[193]

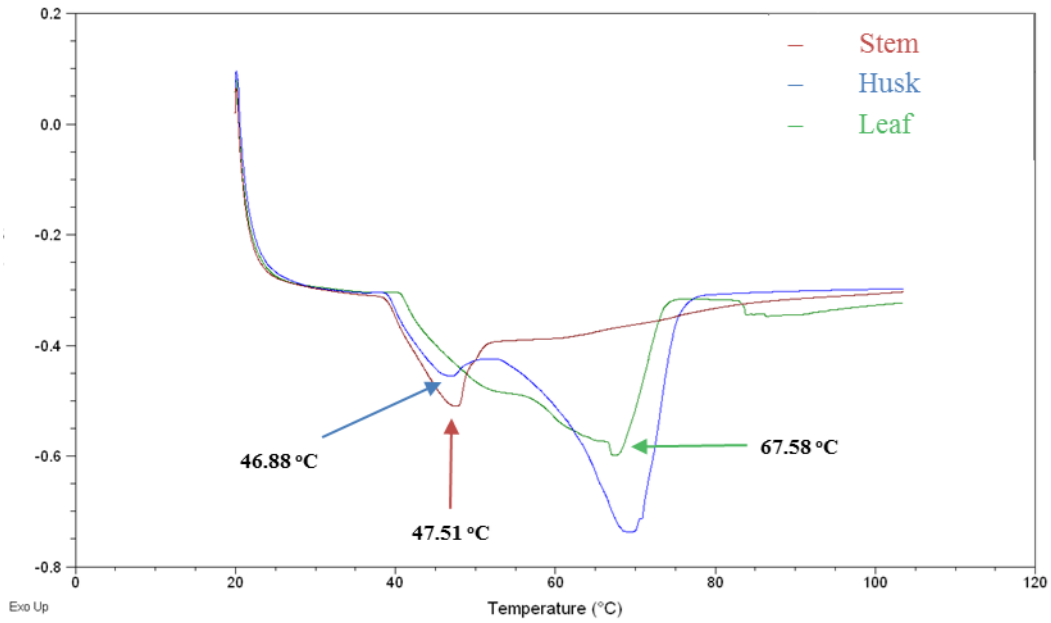


Figure 3-8 Melting temperature profiles for $scCO_2$ extractives.

DSC data for the $scCO_2$ waxes extracted from the stems, husks and leaves may be found in Figure 3-8. This data is in agreement with the quantification data and a correlation can be made between the melting point profiles with the families of compounds found in each lipophilic extractive. Figure 3-9 illustrates the distribution of compounds in each lipophilic extractive.

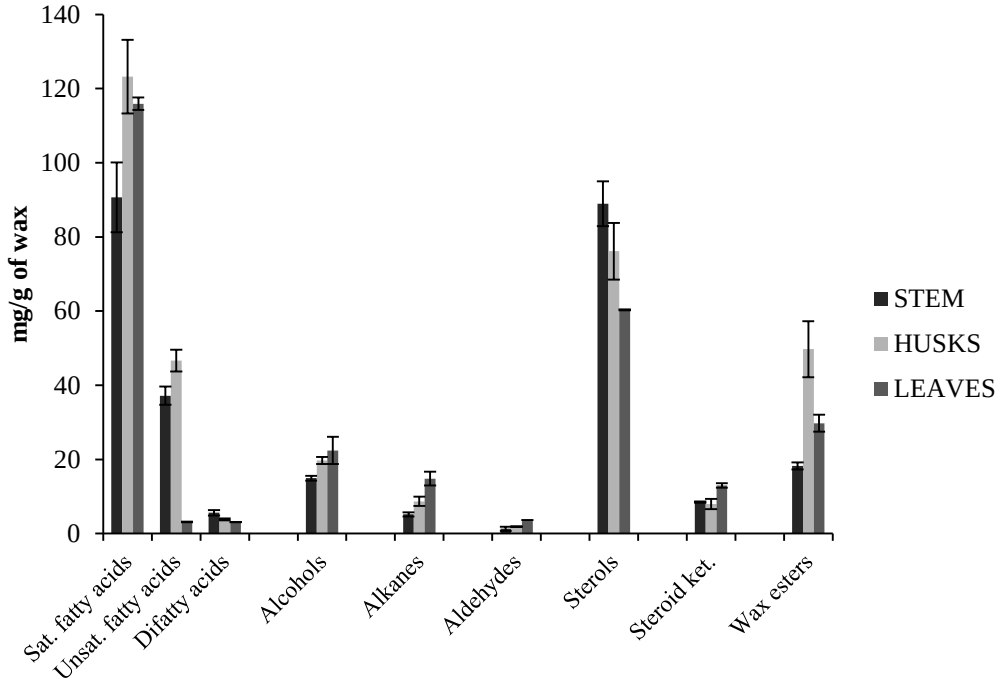


Figure 3-9 Distribution of hydrophobic compounds in the $scCO_2$ extractives from the stems, husks and leaves.

The lipophilic extractive from the husk exhibited the highest melting point profile (having an endothermic minimum of approximately 69.8^{o}C). The maize husk analysed as part of this study has significantly larger amounts of high-molecular weight wax esters (49.7 ±7.6 mg/g of wax) when compared to the stem (18.3 ±1 mg/g of wax) and leaf extractives (29.7 ±2.3 mg/g of wax) and as a result the melting temperature of the wax is higher. Furthermore, the DSC data shows two melting point curves (first curve with an endothermic minimum of 46.88 ^{o}C, the second 69.84 ^{o}C). The lower melting point range is probably resulting from the significant presence of unsaturated fatty acids that are found in the husk extractives (46.6 ±2.9 mg/g of wax) which is comparable to that of the stem.

Maize leaf extracts have a slightly lower melting point profile than the husk. The melting point profile is higher than the stem extract due to larger quantities of wax esters, steroid ketones, long-chain alcohols and hydrocarbons combined with a low concentration of unsaturated fatty acids. In addition, the low abundance of unsaturated fatty acid explains the absence of the lower melting point curve observed in the husk).

The stem extract has the lowest melting profile which is very similar to the first melting point profile curve found in the husk extract. Figure 3-9 shows that there is a high abundance of unsaturated fatty acids (37.2 ±2.5 mg/g of wax), comparable to the husk extract, which would explain why they have similar melting profiles. This together with

the low abundance of wax esters explains the low melting point profile of the stem extracts.

3.2.3 Optimisation of supercritical extraction of waxes from the maize leaves.

An attempt was made to optimise the % yield of wax extracted from the maize leaves by applying a factorial experimental design to the variation of temperature and pressure in the $scCO_2$ extraction.

Factorial experimental designs are an efficient way to enhance the value of research and cut down the time which is allotted for process development.[201] They aid to describe relationships which may exist between variables. A factor refers to any aspect of the experimental conditions that has a direct effect on the result obtained from an experiment.[201, 202] Normally, just one variable is of interest, referred to as the response (or dependent) variable which is dependent on a set of variables called explanatory (or independent) variables. In this case, the response variable is the extraction yield. In supercritical fluid extraction, the main explanatory variables include pressure, temperature, density flow rate, duration of the extraction and biomass preparation. In this current work, the explanatory factors which were investigated were temperature and pressure (hence a two-level factorial design) and these were varied according to the requirement of the experiment. The other independent variables were kept constant throughout the investigation. This experiment requires 2^f runs, where each factor is at two levels, those of the minimum and maximum extraction limits. A variety of temperature and pressure ranges was incorporated into this study as seen in Table 3-3 and Figure 3-10. Four hour extraction times were allotted for each set of experiments.

Table 3-3 Experimental design for optimisation process.

Experiment	Temperature	Pressure
1	High	High
2	High	Low
3	Low	High
4	Low	Low
5	Medium	Medium

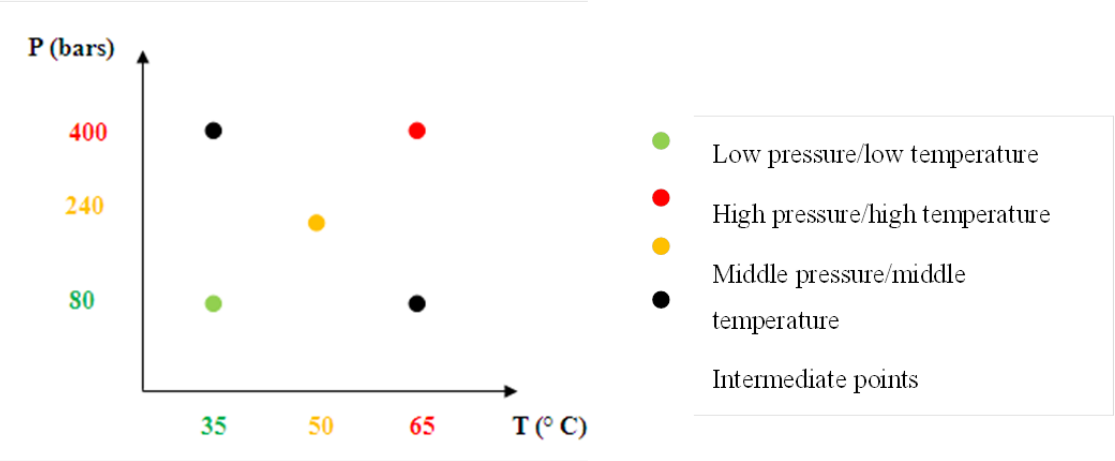


Figure 3-10 Experimental Design for optimisation process.

It is known that there is a relationship between the extraction capability of $scCO_2$ and its density. It was predicted that an increase in CO_2 density would lead to an increase in extraction yield.

Table 3-4 % Extraction yields obtained at different pressures and temperatures for maize leaves.

Experiment	Temperature (^oC)	Pressure (bar)	Extraction Yield (%)
1	**35**	**80**	**0.33**
2	**65**	**80**	**0.024**
3	**50**	**240**	**0.91**
4	**50**	**240**	**0.96**
5	**50**	**240**	**0.94**
6	**35**	**400**	**0.71**
7	**65**	**400**	**1.71**
8	**65**	**400**	**1.76**
9	**50**	**350**	**1.02**

The optimisation was carried out on the leaves due to the high wax yield. However, in industry, all parts of the biomass would be utilised in the extraction (stem, leaves etc.)

and therefore the overall % yield would be lower than that achieved solely for the leaves. The temperature range that was chosen for this work was in the range of 35 – 65^{o}C, while the pressure range was set from 80 – 400 bar.

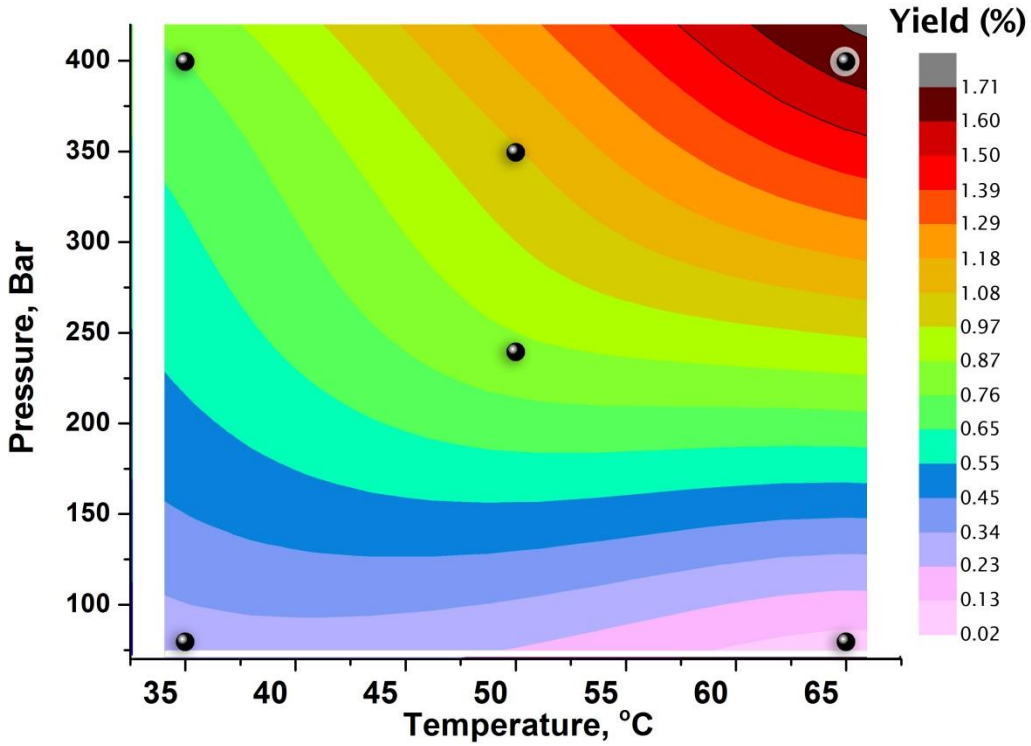


Figure 3-11 % crude yield of maize leaf waxes from scCO$_2$ extraction.

Changes in the solvation properties of CO_2 with different temperatures and pressures results in varying solubility of compounds in CO_2, leading to different extraction yields and compositions (Figure 3-11). The wax content extracted from the maize leaves under the different conditions implemented varied from 0.02 – 1.76%.

Temperature and pressure dictate the dielectric constant and density of CO_2. Increasing the pressure above 80 bar significantly increased the % yield of wax extracted. This indicated that the density of CO_2 is an important factor in the extraction process (0.33% at 80 bar/65 ^{o}C compared to 1.02% at 350 bar/50 ^{o}C). Moreover, the highest yields (1.76%) were achieved using a pressure of 400 bar and temperature of 65 ^{o}C. The density of CO_2 under these conditions is 0.873 kg/m^3 and the yields obtained were significantly higher than those obtained at 350 bar and 50 ^{o}C (1.02%), which has the highest density in this study of 0.899 g/cm^3. This shows that even though density has an important role, there are other factors that dictate the solubility of hydrophobic compounds in CO_2.

Temperature also plays an important role as higher yields are obtained at higher temperatures (65 ^{o}C). The cuticular waxes are semi-crystalline and therefore relatively high thermal energy is required to increase their solubility. As shown in the section 3.3, the melting range of the maize leaf wax was found to be around 40 – 65 ^{o}C with an endothermic minimum at 54 ^{o}C, showing that higher temperatures melt the wax aiding in the solubilisation (Figure 3-8).

The data obtained here seems to be in agreement with solubility studies carried out on minor lipid components in $scCO_2$.[203] In these studies, the effects of pressure and temperature on the solubility behaviour of various lipid components in $scCO_2$ were studied. It was found that the solubility of solutes under investigation increase with an increase in pressure; however there was a variation in pressure dependence of solubility with temperature. At constant pressure, the effects of temperature on solubility could be explained by two mechanisms; whereby pressure dictated the dominant mechanism. The first mechanism is temperature increase causes an increase in vapour pressure resulting in an increase in solubility. The second mechanism is a decrease in solvent density with temperature resulting in a decrease in solubility.[203]

It was suggested that there is a crossover pressure value, whereby below this value there is a decrease in solubility with temperature (and hence density is more important). Once this crossover value has been surpassed, then solubility increases with temperature.[203] This is in good agreement with this current study where at low pressures and lower temperature, higher yields were obtained (80 bar, 35 ^{o}C - 0.33%) compared to a higher temperature (80 bar, 65 ^{o}C - 0.024%). Increase in density caused the yield to increase. However at high pressure conditions – a significant increase in yield was obtained at higher temperatures (400 bar 65 ^{o}C – 1.74%) compared to lower temperatures (400 bar 35 ^{o}C – 0.71%).

3.3 Supercritical extraction as a pre-treament step in a maize stover biorefinery

In this section $scCO_2$ extraction was investigated as a pre-treatment technology in a holistic maize stover biorefinery. $ScCO_2$ extraction and fractionation was carried out prior to hydrolysis and fermentation of maize stover.

3.3.1 Supercritical extraction of maize stover and applications testing of maize stover wax.

Maize stover was obtained from plants grown under field conditions near York (UK). The maize was harvested after R6 stage (silage) and the cobs removed. In the work that was carried out in the first section, the supercritical extractions were carried out a lab-scale (≈100 g of biomass loaded into extractor) supercritical apparatus. The supercritical extraction and fractionation of the maize stover carried out in this section was done at semi-pilot scale (≈1.8 kg of biomass loaded into extractors) in order to have an appreciable amount of $scCO_2$-treated maize stover for hydrolysis and fermentation as well as to increase the amount of wax available for applications testing. A total of ≈20 kg of maize stover was pre-treated with $scCO_2$. The extractions were carried out using the optimised conditions obtained in the 2x2 factorial experimental design, i.e. a pressure of 400 bar and temperature of 65 ^{o}C.

The supercritical extractions gave rise to high-value waxes, containing a wide range of added-value compounds such as *n*-policosanols, long-chain fatty acids, fatty aldehydes, *n*-hydrocarbons, sterols, steroid ketones and wax esters (Table 3-5). These compounds could be used in a variety of applications, including nutraceuticals, ingredients for cleaning products, flavours, degreasers, cosmetics and lubricants.[124, 129, 131, 141, 144, 154, 204, 205]

One such application for waxes is their use as defoaming agents in washing machine formulations. Foam control in horizontal axis washing machines is an important issue. Due to mechanical agitation, elevated temperature, and high surfactant concentration, an excess of foam can be generated. Excessive foam has an adverse effect on washing performance due to impaired movement of the laundry itself, and inefficient rinsing and drainage of the machine. Furthermore, the electronic parts of the washing machine may be damaged. Several types of antifoam substances are currently used for foam control. Some of these have been reported as having a negative impact on the environment: phosphates (eutrophication), nitrogen-containing compounds (possible carcinogenic by-products nitrosamines), organic silicon compounds (persistent) and fluoro-compounds.[206] Waxes represent a potential environmentally friendly alternative, but it is crucial that the wax has a melting point range between 30-50°C and low saponification values. [207 207 207 207 207 207 207] This is because, nowadays, washing machine runs are generally carried out using temperature programmes ranging from 30 – 60 ^{o}C so it is important that the wax is expelled along with the detergent. Crude plant and mineral waxes generally have melting points that range between 41 and 87^{o}C.[187] Figure 3-12 is

the DSC thermogram of the crude maize stover wax showing a melting profile where the maximum melting occurs at around 55 ^{o}C, and is therefore too high for the crude wax to be used in defoaming applications.

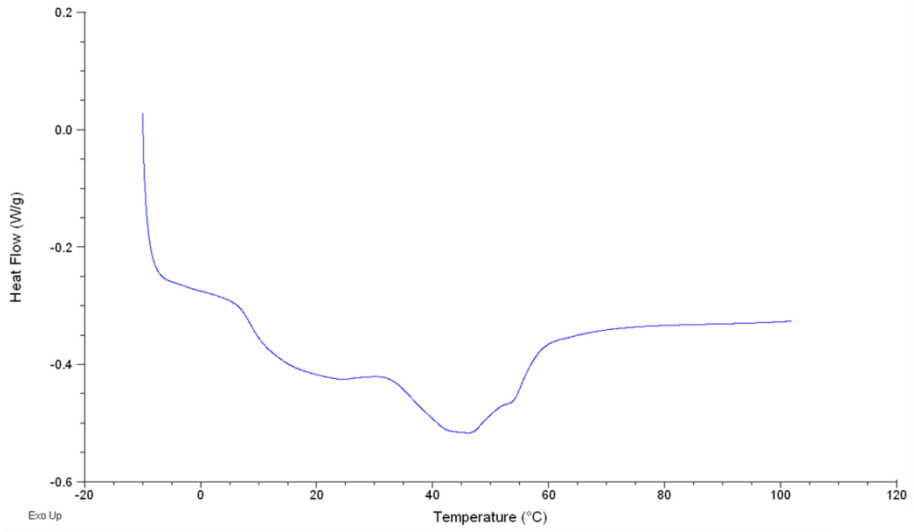


Figure 3-12 DSC thermogram of crude maize stover wax.

3.3.2 Supercritical fractionation of maize stover wax

A well-known concept that can be incorporated in a supercritical extraction process, in order to improve extract selectivity and obtain products of higher value, is fractional separation.[208] With conventional solvent extraction, crude products contain unwanted families of compounds (that have varying solubilities and mass transfer resistances) and it is impossible to prevent co-extraction of these molecules. This is often the case as well when extracting compounds using $scCO_2$ at a single pressure and temperature. However, a significant advantage of $scCO_2$ over conventional organic solvents is the possibility of carrying out fractional separation of crude products, whereby compounds are precipitated out in successive steps, at decreasing pressures and temperatures.

Selection is achieved by decreasing the solubility of the desired compounds in the supercritical solvent by altering the CO_2 density. Supercritical extraction apparatus can contain a number of fractional separators in series having different pressures and temperatures resulting in a variation in the density of CO_2 and hence the solvation properties of $scCO_2$. Molecules that are only soluble at a specific CO_2 density range will precipitate out when passing through a fractional separator having a different CO_2 density, while other molecules which are soluble at that density will pass on to the next fractional separator. This results in fractions of molecules having similar solubilities. This concept is illustrated in Figure 3-13 below.

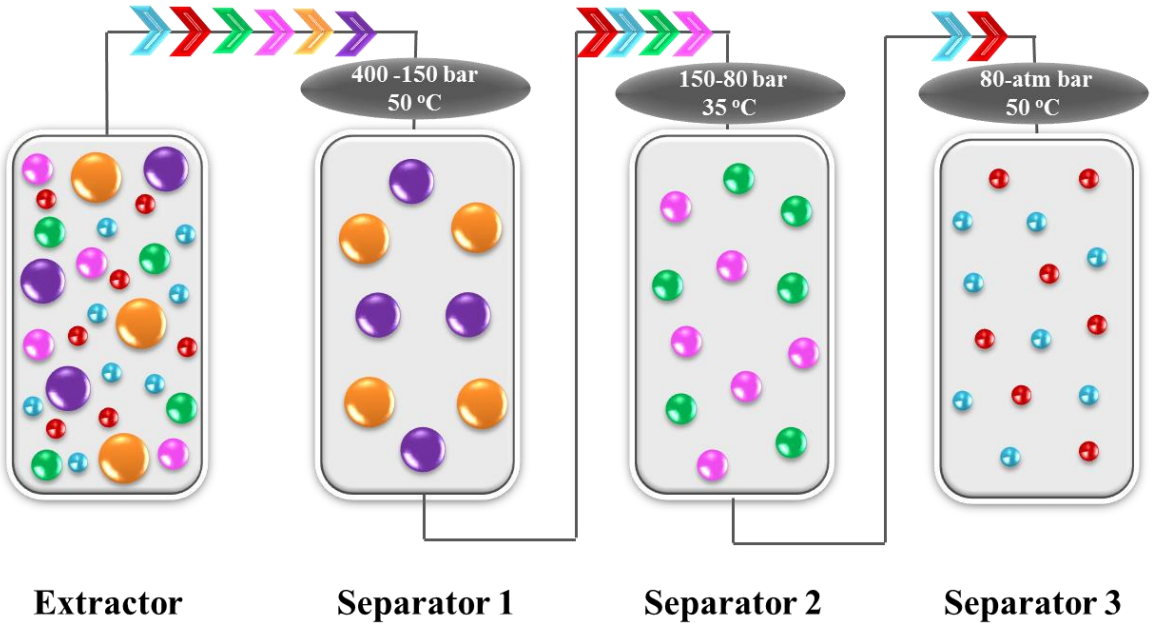


Figure 3-13 Graphical illustration of the fractionation of molecules with $scCO_2$.

Supercritical fractionation has been mainly studied for the isolation and purification of essential oils;[209-214] such as in the fractionation of oleoresin based on essential oil composition and antioxidant activity.[214] Limited literature describes the supercritical fractionation of epicuticular waxes. The majority of the work focuses on separating the epicuticular waxes as a whole from essential oils as these two families of compounds are normally co-extracted during the supercritical process.[210-212] Essential oils remain completely soluble in CO_2 at very low temperatures (-5 ^{o}C to 5 ^{o}C) while waxes are completely insoluble at these temperatures thus allowing for fractionation of the two to occur. Deswarte *et al.* looked into obtaining valuable wax fractions from wheat straw using $scCO_2$, however two separate extractions at different pressures and temperatures were carried out rather than a fractionation step.[54] Therefore, to the best of the author's knowledge, this is the first reported fractionation of epicuticular waxes by means of fractional separators. This was attempted on the maize stover to try and obtain wax fractions concentrated with families of compounds of similar solubilities. The fractionation was not an additional step post-$scCO_2$ extraction (i.e. not a fractionation of the crude wax). The fractionation occurred concurrently while extracting from the maize stover.

Fractional separation at three different pressures and temperatures was achieved at: 400 – 150 bar/50 ^{o}C (Fraction A); 150-80 bar/35 ^{o}C (Fraction B); and 80 bar-ATM/50 ^{o}C (Fraction C) resulting in three wax fractions having different chemical compositions,

textures and crucially melting point ranges (Figure 3-14 (i) and Figure 3-14 (ii)), opening doors to multiple applications.

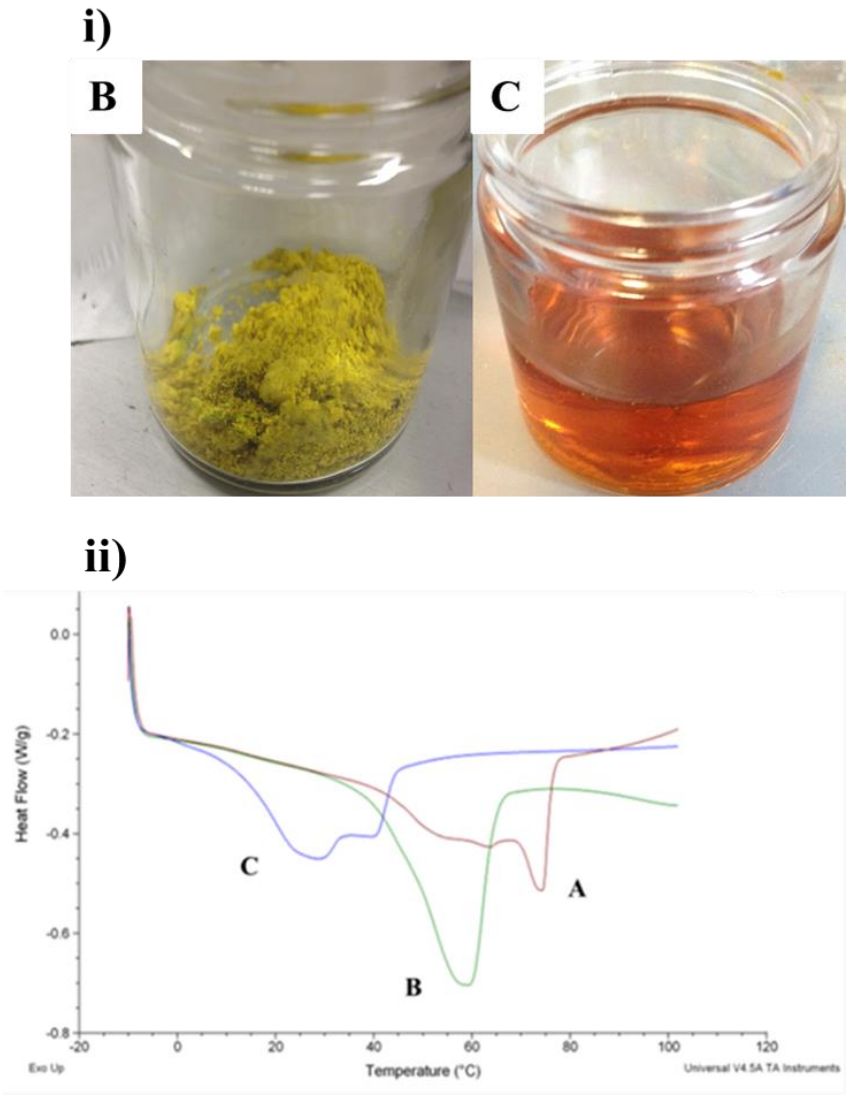


Figure 3-14 i) Textures of A) Fraction B at 50 ^{o}C (solid) B) Fraction C at 50 ^{o}C (liquid). ii) DSC plot illustrating the melting profiles for each wax fraction. Fraction A (400-150 bar/50 ^{o}C), B (150-80 bar/ 35 ^{o}C) and C (80 bar-ATM/50 ^{o}C)

Table 3-5 Quantities of different families of compounds in Fraction A, Fraction B and Fraction C in µg/g of plant.

Compound	Fraction A, 400 – 150 bar, 50 ^{o}C (µg/g of plant)	Fraction B, 150-80 bar, 35 ^{o}C (µg/g of plant)	Fraction C, 80 bar-ATM, 50 ^{o}C (µg/g of plant)
Hexanoic acid	-	-	1 ±0.06
Heptanoic acid	-	-	0.3 ±0.07
Octanoic acid	0.03 ±0.004	0.2 ±0.01	3.9 ±0.3

Nonanoic acid	0.1 ±0.004	0.6 ±0.06	2.3 ±0.2
Decanoic acid	0.2 ±0.004	0.2 ±0.01	3.7 ±0.1
Dodecanoic acid	1.9 ±0.05	1.6 ±0.04	10 ±0.4
Tetradecanoic acid	2.4 ±0.08	2.5 ±0.04	18.5 ±1
Pentadecanoic acid	0.8 ±0.1	0.5 ±0.02	3.9 ±0.04
Hexadecanoic acid	25.8 ±0.6	28.1 ±0.4	525.1 ±19.9
Heptadecanoic acid	0.5 ±0.2	0.5 ±0.02	12.5 ±0.4
Octadecanoic acid	7.5 ±0.2	12.6 ±0.4	186.1 ±10.2
Nonadecanoic acid	0.2 ±0.01	0.4 ±0.01	4.5 ±0.8
Eicosanoic acid	4 ±0.2	7 ±0.1	79.7 ±5.6
Heneicosanoic acid	0.7 ±0.2	0.9 ±0.2	9.8 ±1.2
Docosanoic acid	2.3 ±0.1	7.8 ±0.2	45.5 ±3.9
Tricosanoic acid	1.3 ±0.05	6.4 ±0.3	39 ±3.5
Tetracosanoic acid	3.4 ±0.3	19.7 ±2.5	53.7 ±5.5
Pentacosanoic acid	0.9 ±0.04	6.7 ±0.5	10.5 ±0.9
Hexacosanoic acid	2.3 ±0.1	15.3 ±1.3	21.2 ±2.6
Octacosanoic acid	0.5 ±0.03	-	5.8 ±0.8
Total saturated fatty acids	**54.8 ±2.3**	**111 ±6.1**	**1035.7 ±57.6**
9-hexadecenoic acid	-	-	56.5 ±1.8
C_{18} unsaturated fatty acids	19.8 ±0.5	79.9 ±2.5	1310.5 ±79
Total unsaturated fatty	**19.8 ±0.5**	**79.9 ±2.5**	**1367 ±80.8**

acids			
Hexacosanol	0.6 ±0.02	2.9 ±0.7	9.9 ±1
Octacosanol	0.7 ±0.01	2.9 ±0.3	21.6 ±3
Triacontanol	3.8 ±1.9	58.5 ±1.3	61.2 ±6.2
Dotriacontanol	3.6 ±0.2	63.5 ±6.5	17.6 ±1.6
Total fatty alcohols	**8.7 ±2.1**	**127.8 ±8.8**	**110.3 ±11.8**
Hexacosanal	2.3 ±0.4	17.2 ±1.4	43.8 ±4.6
Octacosanal	2.7 ±0.006	9.5 ±0.8	35.4 ±2
Triacontanal	1.5 ±0.1	50.3 ±6.5	21 ±1.6
Total fatty aldehydes	**6.5 ±0.5**	**77 ±8.7**	**100.2 ±8.2**
Pentacosane	0.4	-	1.8 ±0.1
Heptacosane	0.5 ±0.06	1.1 ±0.2	7.7 ±0.1
Nonacosane	0.6 ±0.1	1.5 ±0.1	22.6 ±0.7
Hentriacosane	0.9 ±0.03	4.3 ±0.3	44 ±3.9
Triatriacontane	1.4 ±0.2	29.6 ±0.8	17 ±0.6
Total alkanes	**3.8 ±0.4**	**36.5 ±1.4**	**93.1 ±5.4**
Campesterol	7.3 ±0.2	131.4 ±6.1	87.7 ±2.8
Stigmasterol	12.1 ±0.4	203.2 ±9.6	104.3 ±3.6
B-sitosterol	23.1 ±0.1	390 ±12.8	322.4 ±2.9
Stigmastanol	1.8 ±0.2	29.6 ±0.8	48.9 ±4.8
Total Sterols	**44.3 ±0.9**	**751 ±29.3**	**563.3 ±14.1**

Stigma-4-*en*-3-one	3.6 ±0.5	11.5 ±0.9	80.7 ±1.7
5α-stigmastan-3,6-dione	1.8 ±0.06	7.3 ±0.3	33.5 ±2.2
Total steroid ketones	**5.4 ±0.6**	**18.8 ±1.2**	**114.2 ±3.9**
Wax ester 40	0.3 ±0.04	1.7 ±0.3	11.9 ±0.7
Wax ester 42	1 ±0.1	5.9 ±0.5	18 ±0.9
Wax ester 43	0.3 ±0.06	1.1 ±0.2	-
Wax ester 44	2.7 ±0.06	16.2 ±6.2	10.2 ±0.2
Wax ester 45	0.4 ±0.07	1.6 ±0.6	-
Wax ester 46	1.9 ±0.1	14.6 ±7.6	6.9 ±0.1
Wax ester 47	0.3 ±0.01	1.1 ±0.7	-
Wax ester 48	2.8 ±0.1	8.7 ±4	1.5 ±0.1
Wax ester 49	0.5 ±0.02	1 ±0.4	-
Wax ester 50	4.4 ±0.4	5.8 ±1.7	-
Wax ester 51	0.8 ±0.1	-	-
Wax ester 52	5.9 ±0.5	-	-
Wax ester 53	0.8 ±0.1	-	-
Wax ester 54	5 ±0.5	-	-
Wax ester 55	0.05 ±0.05	-	-
Wax ester 56	2.9 ±0.4	-	-
Wax ester 58	1 ±0.06	-	-
Total Wax esters	**31.5 ±2.7**	**57.7 ±22.2**	**48.5 ±2**
Phytol	-		8.4 ±1.1
2-Pentadecanone-6,10,14-trimethyl	9.2 ±0.2	10.4 ±1.1	70.5 ±2.4
Total 'other' compounds	**9.2 ±0.2**	**10.4 ±1.1**	**78.9 ±3.5**

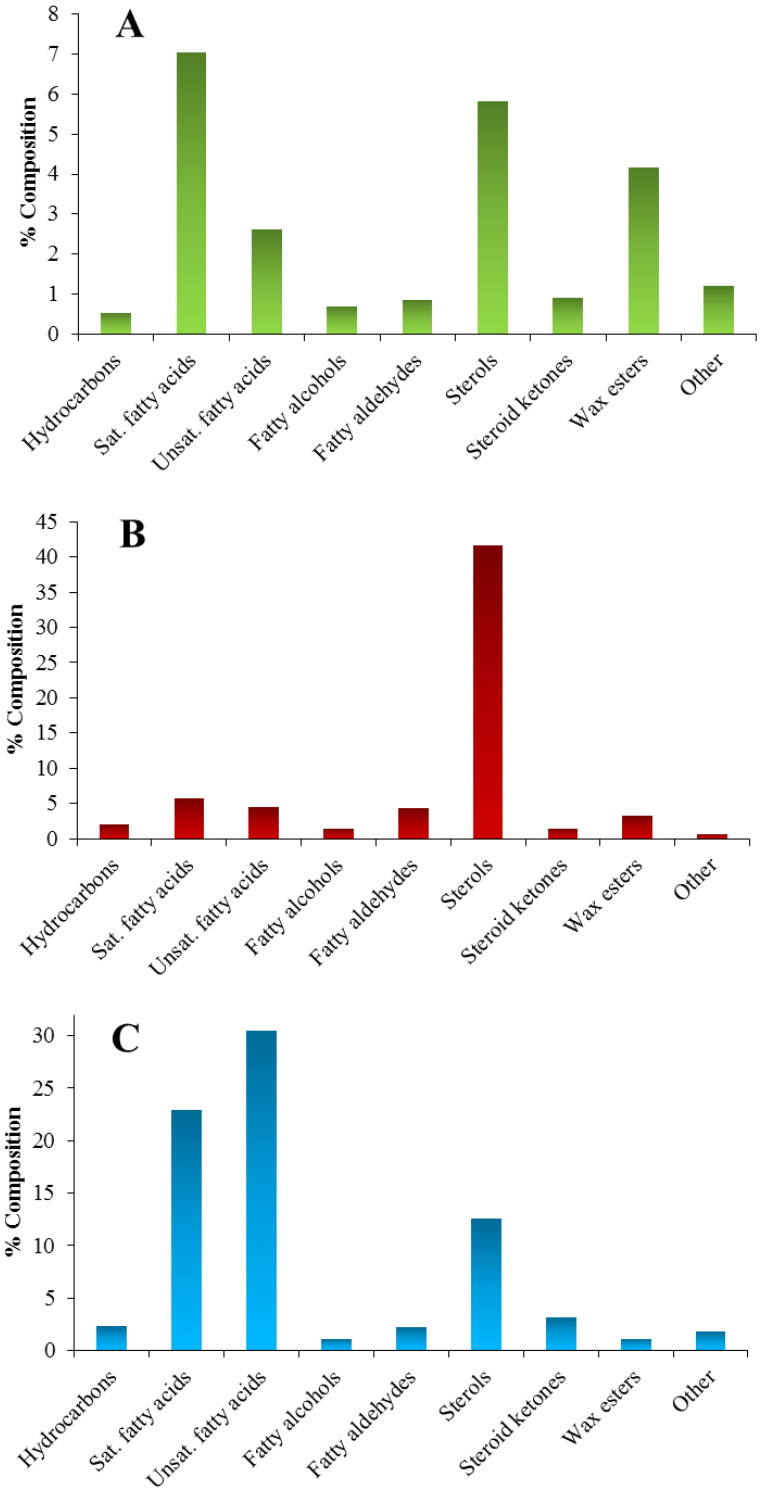


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Figure 3-15 % Composition of groups of compounds for wax Fraction A (400-150 bar/50 ^{o}C) B (150-80 bar/35 ^{o}C) and C (80-ATM/50 ^{o}C).

Wax fraction A has the highest melting profile with an endothermic minimum centred at around 74 ^{o}C, which could make it suitable for applications such as instrument and automobile polishes which require waxes with higher melting temperatures. This wax fraction has the largest quantities of wax esters (43 mg/g of wax). Only around 21% of

the wax Fraction A by composition was identified and quantified (Figure 3-15), which could suggest that the remaining composition is made up of high-molecular weight compounds such as phospholipids and triglycerides, which cannot be determined by GC. Therefore, further work could look into using other analytical techniques (gas-liquid chromatography, high performance-liquid chromatography, supercritical fluid chromatography, size-exclusion chromatography etc.) to identify and quantify these high-molecular weight compounds.

Interestingly, wax Fraction B is predominantly phytosterols (402 ±15.7 mg/g of wax) which, as mentioned in the previous chapter have significant nutraceutical properties. Solubility studies of phytosterols in $scCO_2$ have demonstrated limited solubility of β-sitosterol and stigmasterol close to the supercritical point (there is a massive increase in solubility of sterols with an increase in pressure and in temperature (once the cross-over pressure has been reached)) which could explain why the majority of the sterols crashed out in Fraction B (≈80 bar and 35 ^{o}C); while some passed through and crashed out in Fraction C.[203, 215] The substantial amount of phytosterols in this wax fraction (42% of wax fraction composition) could allow for relatively straightforward isolation and purification of these compounds for nutraceutical application of commercial interest. Wax Fraction C has a melting point profile ranging from 28 – 41 ^{o}C.

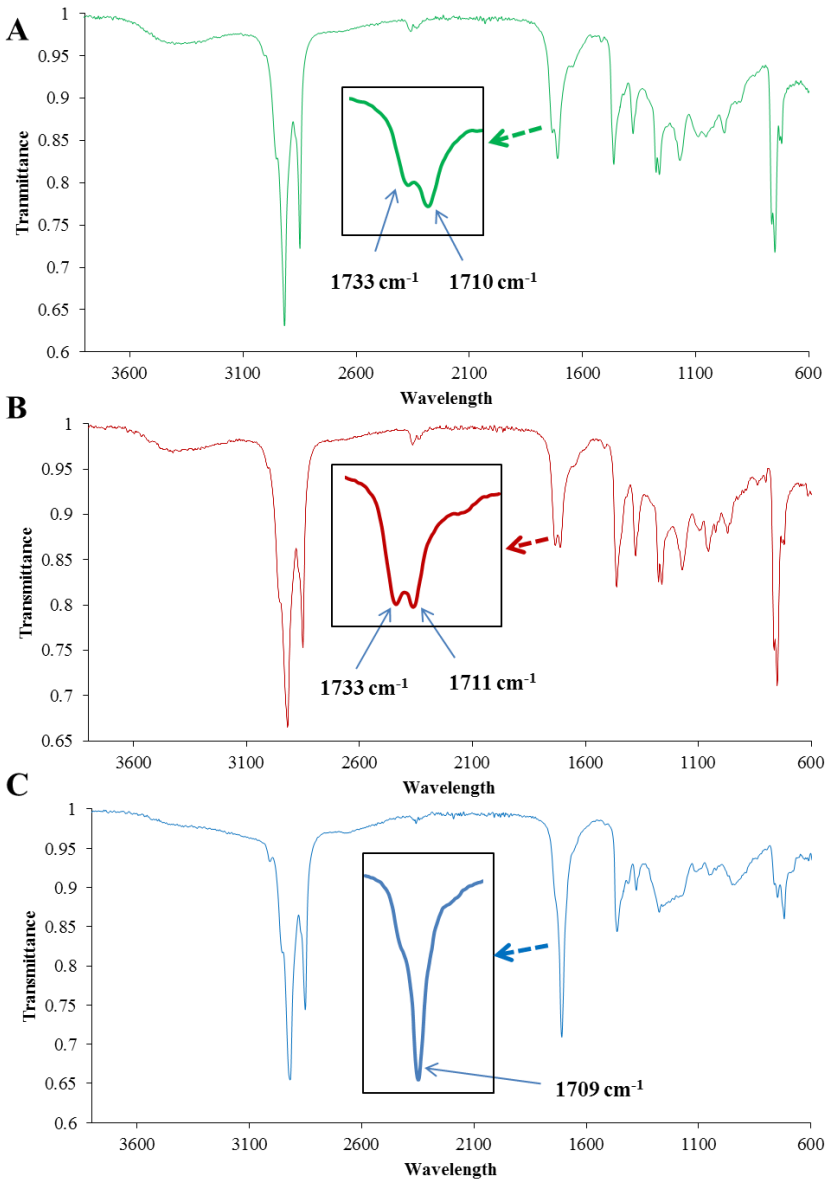


Figure 3-16 IR spectra of Fraction A, Fraction B and Fraction C.

Figure 3-16 illustrates the IR spectra for wax Fraction A, Fraction B and Fraction C. Fractions A and B have two bands appearing at 1733 cm^{-1} and 1710 cm^{-1}. These are attributed to the C=O stretching vibrations of the fatty acid and wax ester compounds respectively. In fraction A, there is just one intense band at 1709 cm^{-1} which corresponds to the fatty acids C=O stretching vibrations while no ester stretching vibrations are found in the spectrum. This, together with GC data, indicates a higher abundance of fatty acids (especially unsaturated fatty acids) and a significantly lower amount of wax esters in this fraction when compared to Fractions A and B. Previous studies have indicated that it is possible to separate saturated and unsaturated fatty acids from the triglyceride fraction using CO_2 densities of less than 700 kg/m^3.[216] Fatty acids have been shown to be soluble at low pressures and temperatures.[216, 217] However, this

work was carried out solely on the pure components and the intermolecular interactions in the liquid phase between the various components making up the system were not taken into consideration.[216]

The low melting point range of the wax Fraction C allowed for defoaming tests to be performed. Therefore the above method illustrates the benefits of applying $scCO_2$ extraction and fractionation over conventional organic extraction. Fractionation allowed for three very different wax fractions; one (Fraction A) providing wax esters and high-molecular weight compounds, one providing a steady source of phytosterols (Fraction B) and one having high amounts of saturated and unsaturated fatty acids (Fraction C).

3.3.3 Defoaming application of wax Fraction C.

The defoaming applications of wax fraction C were carried out in collaboration with Ecover, a Belgium-based green chemical manufacturing company. Two types of experiments were carried out: (i) foam measurements (lab-foam tests) and washing machine tests.

3.3.3.1 Foam measurements (lab-foam tests)

Foam measurements can provide important information about the defoaming capacity of waxes. Foam production and foam decay were measured with high resolution optical sensors (LED illumination and light detection). Figure 3-17 shows a significant defoaming effect produced by the maize stover wax on foam ability and foam stability (based on an average of 3 runs for each sample).

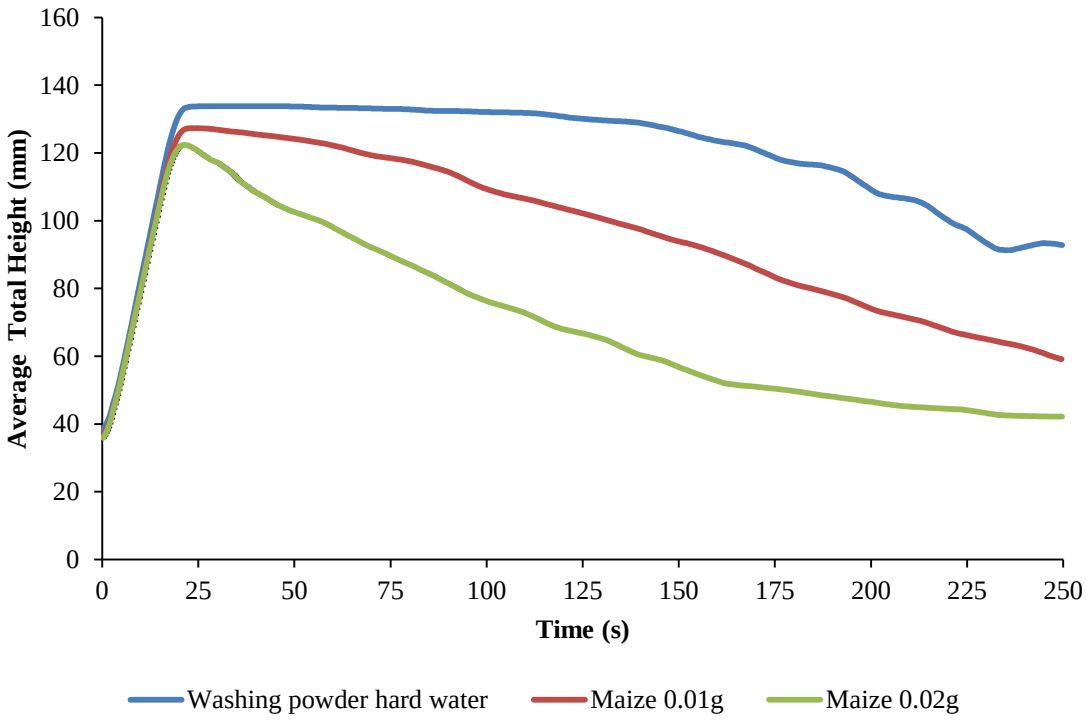


Figure 3-17 Average foam measurements of washing powder hard water, maize 0.01 g and maize 0.02 g.

The wax significantly reduced the quantity of foam generated as well as decreased the time taken for the foam to break up. The control (washing powder hard water) represents a sustainable antifoam currently used in washing powder formulations. The commercial available washing powder used contains soap and on addition of tap water (containing calcium and magnesium), salts with poor water solubility are generated and it is these which act as antifoams. Hence in Figure 3-17, the blue line represents the current technology (currently available green washing powder). It can be observed that addition of waxes gives rise to a substantial improvement in defoaming over the current strategy. The average foam height (average of 3 runs) measured after 250 s (approximately 42.2 mm) was close to the starting value when using 0.02 g of maize wax. The average foam height obtained for the control was 92.7 mm at the same time. Maize stover wax was observed to reduce the foam height faster than the control. The above results illustrate the defoaming performance of the maize wax showing its potential as a defoaming agent.

3.3.3.2 Washing machine tests

These defoaming characteristics were further investigated by mimicking a real-life situation whereby the wax was incorporated in washing machine runs in order to see

whether the defoaming effects observed during the foam measurements also occurred during a washing run. Washing machine tests were carried out whereby the wax was added to the detergent formulation, together with a number of towels, dish towels and soil ballast sheet and a typical washing machine run programme was set. A number of runs were carried out in order to ensure that repeatability was achieved.

The detergent formulation was synthesised in the laboratory consisting of the compounds found in Table 3-6 and is based on a standard formulation that is used to assess the cleaning performance of washing machine formulations (IEC A reference detergent as described by the European Ecolabel).[218]

Table 3-6 Compounds that are found in the detergent formulation prepared.

Compound	Mass (g)
Zeolite	21
SKS 6 (Sodium disilicate)	17
Sodium percarbonate	26
Ufarol TCT/90A	17.5
Sodium bicarbonate	9
Sodium Citrate	4
Polypeptide Donlar	4
FAMEE (Fatty acid methyl ester ethoxylates)	5
TAED (Tetraacetylethylenediamine)	3

Anti-foaming agents typically constitute 0.8 – 4% by weight of the total detergent formulation[219] and therefore the washing machine tests were carried out using 3 g (≈2.7% of total detergent composition) and 1.5 g (≈1.4% of total detergent composition) of maize stover wax.

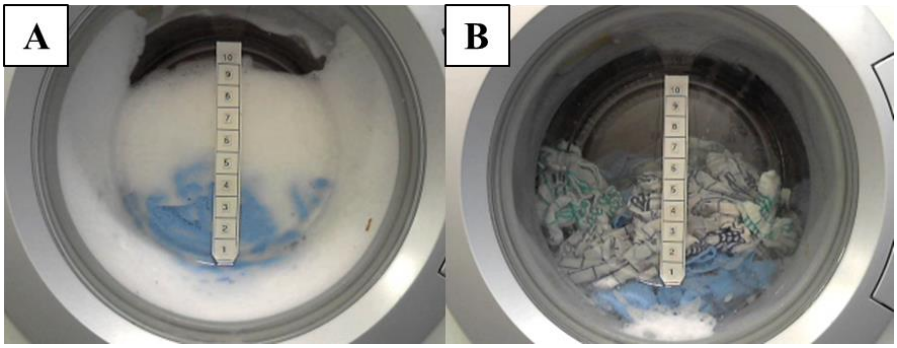


Figure 3-18 Washing machine tests illustrating foam generation A) Control Run B) Run with 1.5 g wax.

Figure 3-18 is a representative washing machine run carried out without wax (A), i.e. a blank run and a washing machine run where the wax was added (B). There is a significant difference in the amount of foam generated between the two runs, where there was on-average, a significant reduction in foam height in the washing machine when 1.5 g of maize stover wax was incorporated in the formulation. The height of the foam was measured every 5 minutes (using the measuring units found on the washing machine door, Figure 3-19) in order to investigate the efficiency of the wax as a defoaming agent. The results are summarised in Figure 3-20 below

Figure 3-19 Washing machine set-up.

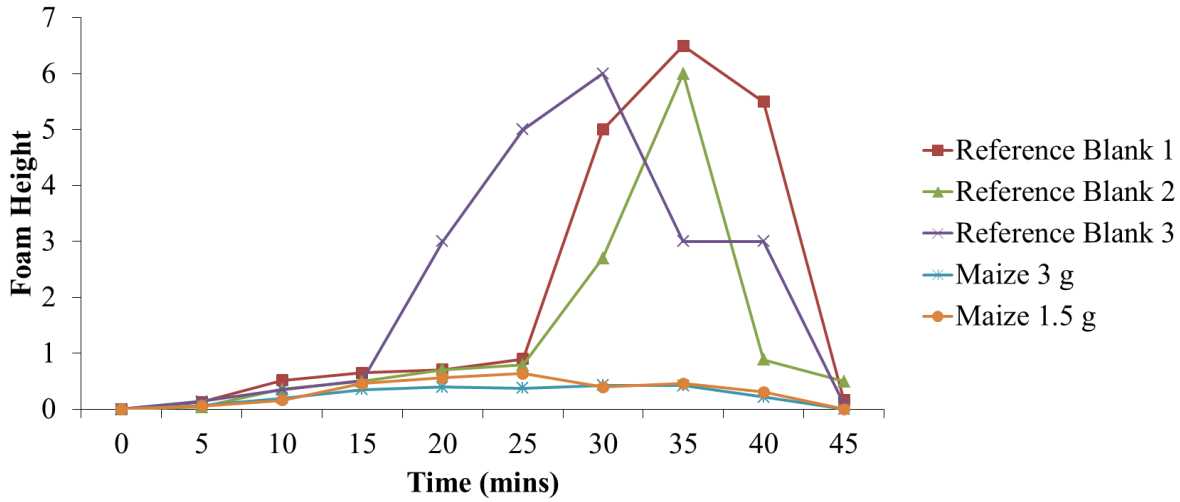


Figure 3-20 Rough measurements of average foam height during the washing machine run (average of 2 runs for 3 g of wax and 1.5 g of wax).

This provides further evidence that there is a defoaming effect by the wax sample. No lipid residues were found in the washing machine after the washing run, indicating that the wax was successfully expelled with the detergent solution.

3.3.3.3 Performance measurements

It is important to ensure that the wax does not affect the performance of the detergent formulation. Tests were carried out in order to see whether there was performance loss due to emulsification of the waxes by the surfactants in the formula. This was done by means of carrier type monitors (shown in Figure 3-21), which are sheets containing a number of reference stains (shown in Table 3-7) that are typically found on clothes.

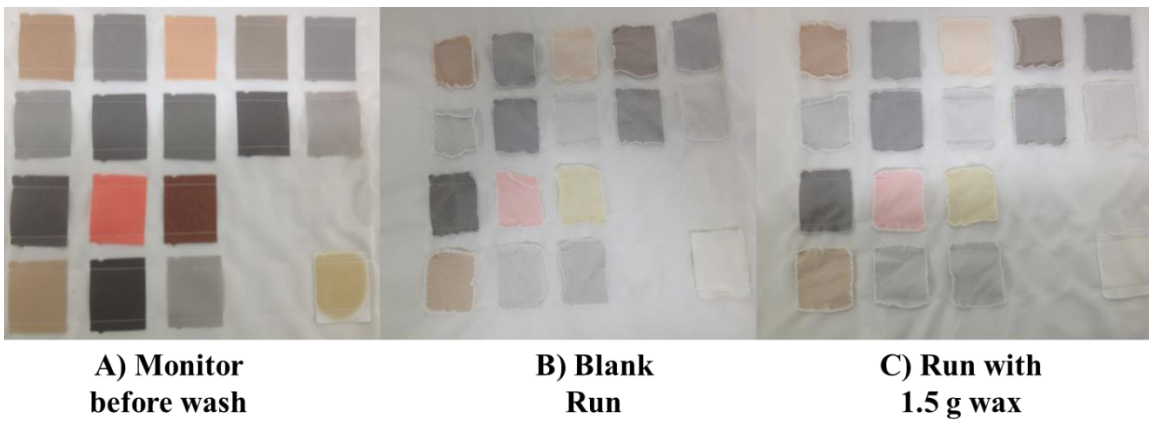


Figure 3-21 Carrier type monitor A) Before wash B) Blank run (without wax) C) Run with 1.5 g of wax.

Table 3-7 Type of stains found on the carrier type monitor.

Reference code	Type of stain
CS-44	Chocolate drink, pure
C-01	Mineral oil/soot
CS-17	Fluid make-up
C-03	Chocolate milk/soot
C-11	Milk with carbon black
WP-10PF	Vegetable fat/pigment
E-123	Low temperature test
E-125	Detergent/Wetting agent
CS-05S	Mayonnaise, carbon black
C-132	High Discriminative Sebum BEY with carbon black
CS-64	Beef fat with soot
CS-216	Lipstick diluted, red
CS-75	Blood with beef fat
E-165	Chocolate pudding
CS-37	Full egg, with pigment
CS-06	Salad dressing, with natural black
KC-H015	Used frying fat

Following the washing machine runs, an SP62 Portable Sphere Spectrophotometer was used for precise colour measurement of the stains for the blank run and the run containing the maize wax. The results are shown in Figure 3-22.

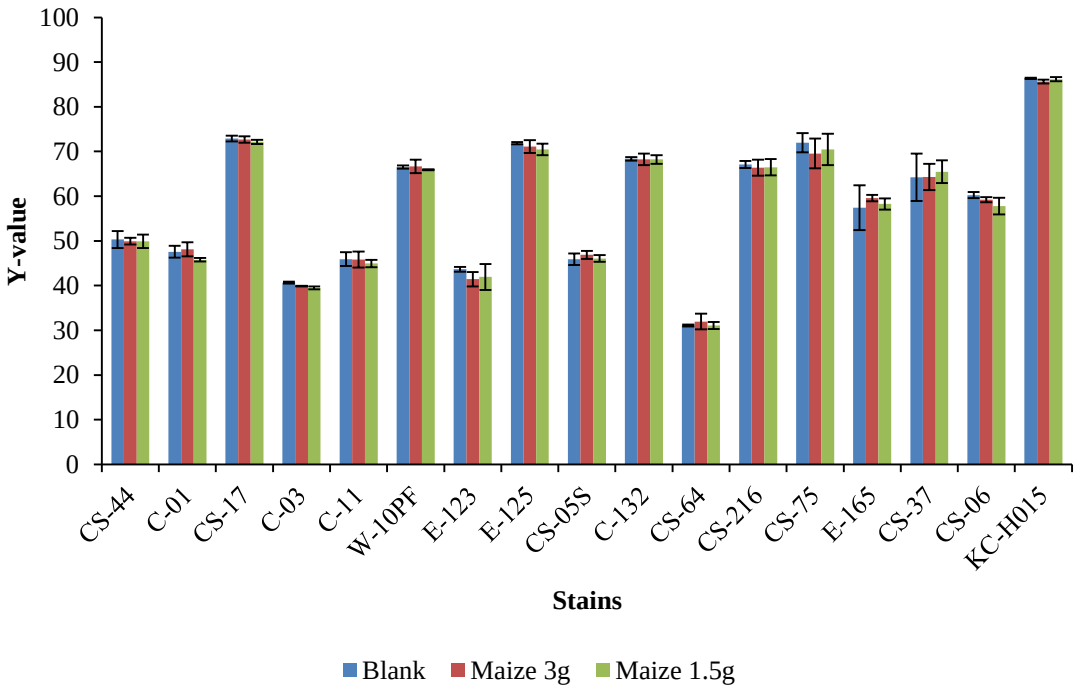


Figure 3-22 Y-values of the CIE tristimulus colour to study the effect of the waxes on the detergent performance.

The Y-value of the CIE tristimulus colour values represents the luminance. The higher the Y-value, the lighter the stain is so the better it is removed. The results indicate that there is no significant difference in luminance between the control runs and the runs containing the maize wax. This suggests that there is no significant loss in detergent performance indicating that the maize wax could be utilised as a defoaming agent.

Therefore, these promising results indicate the potential of utilising the wax extracted from maize stover in washing machine detergent formulations as a renewable defoaming agent.

3.4 Pre-treatment and hydrolysis

Supercritical pre-treatment of maize stover leads to the extraction of high-value waxes that can potentially be incorporated into a host of applications. However, the extraction of wax from maize stover only utilises around 1% of the total maize stover leaving 99% of the biomass unutilised. In order to have a systemic view of a maize stover processing scenario where $scCO_2$ extraction is integrated into a biorefinery, the supercritical extracted maize stover (MA $scCO_2$) was subjected to hydrolysis and fermentation and the results were compared to non-treated maize stover (MA).

$ScCO_2$-extracted maize stover and non-treated maize stover were subjected to a mild hydrothermal pre-treatment. Sugar yields after enzymatic hydrolysis were determined in both cases and were performed in collaboration with Processum in Sweden.

Figure 3-23 shows the carbohydrate composition of the material, as well as the yield of sugars after enzymatic hydrolysis for both $scCO_2$-extracted and non-treated maize stover. The level of xylan in the pre-treated material was relatively high (149-175 g/kgTS) (Figure 3-23A), indicating a low severity pre-treatment. This is consistent with a low sugar yield after enzymatic hydrolysis (Figure 3-23B). Since the pre-treatment and hydrolysis conditions only partially released the sugar present in the stover, subsequent fermentation gives an indication of the fermentability of the slurry and by no means a value of the potential yield in final products from maize stover. $ScCO_2$-extracted stover shows a small but significantly higher proportion of glucan.

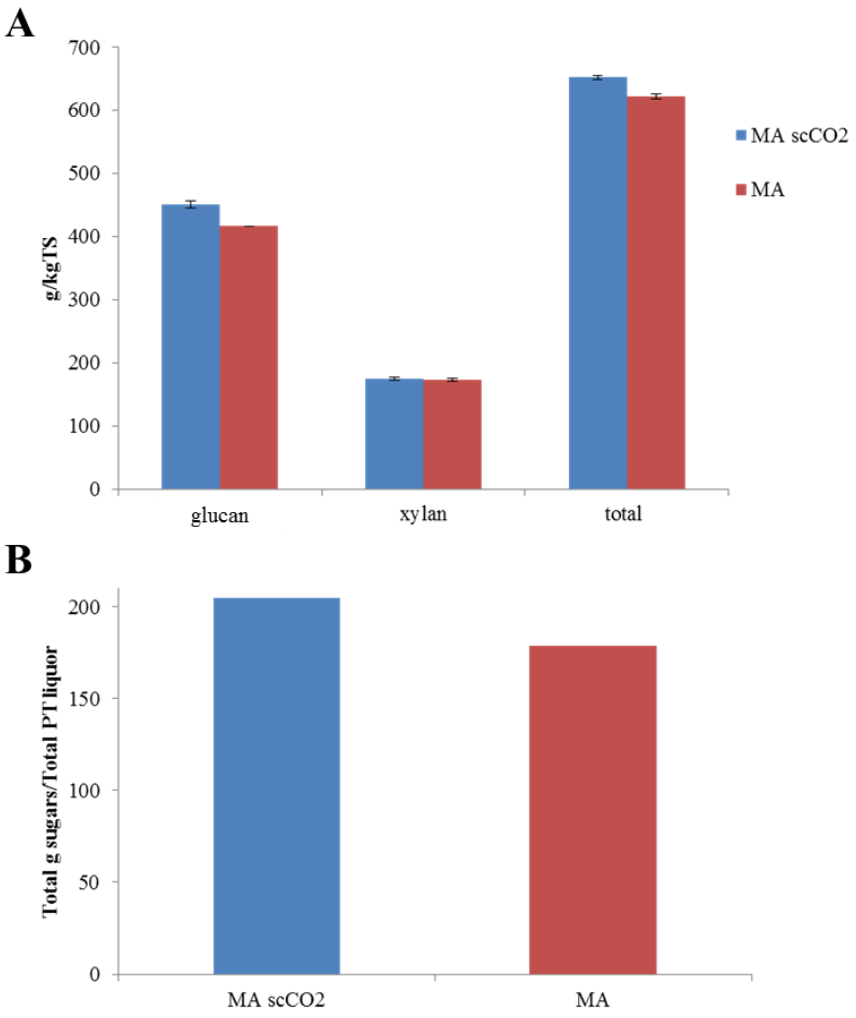


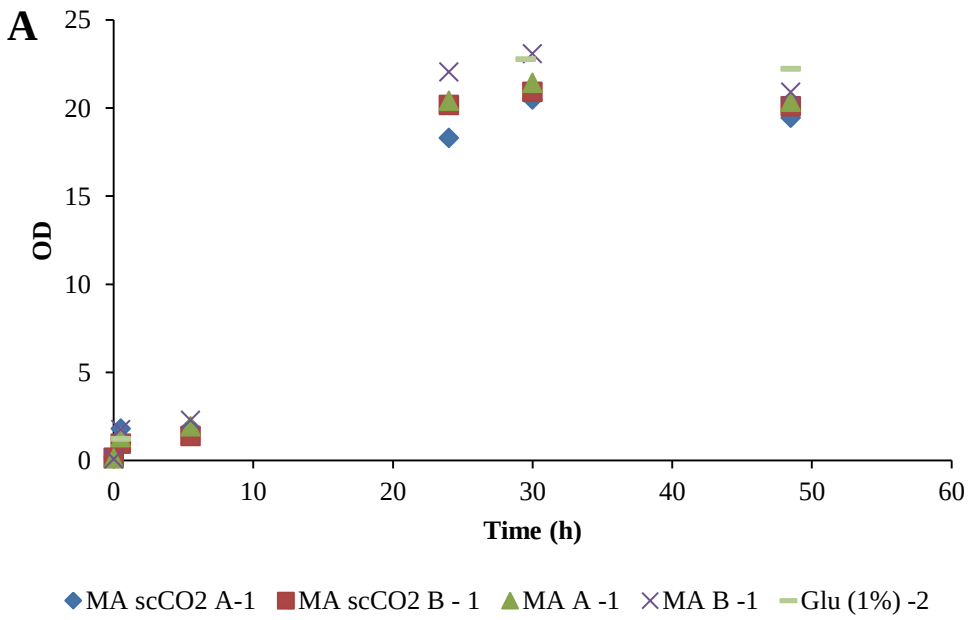
Figure 3-23 Composition of maize stover and its hydrolysate A) Polysaccharide content in raw material after pre-treatment with and without scCO2 B) Monosaccharide yield after enzymatic hydrolysis based on total dry weight prior to the enzymatic hydrolysis.

3.5 Fermentation

Fermentation experiments were carried out in collaboration with Ecover and Processum. Two types of fermentation were carried out; The first involved the use of *S. bombicola* for the production of sophorolipid surfactants from maize stover hydrolysate.[220] The second example of fermentation was carried out using *Saccharomyces cerevisiae Thermosacc.* for ethanol production.

3.5.1 Fermentation of biomass with *S. bombicola*

This was done in collaboration with Ecover. The growth and substrate consumption during shake flask experiments with *S. bombicola* is shown in Figure 3-24. Although a limited number of samples were taken, a lag phase, an exponential phase and stationary phase were observed. A decrease in substrate concentration occurred and no inhibition in growth was observed for any of the samples. In comparison with the reference, the same growth (OD) was reached.



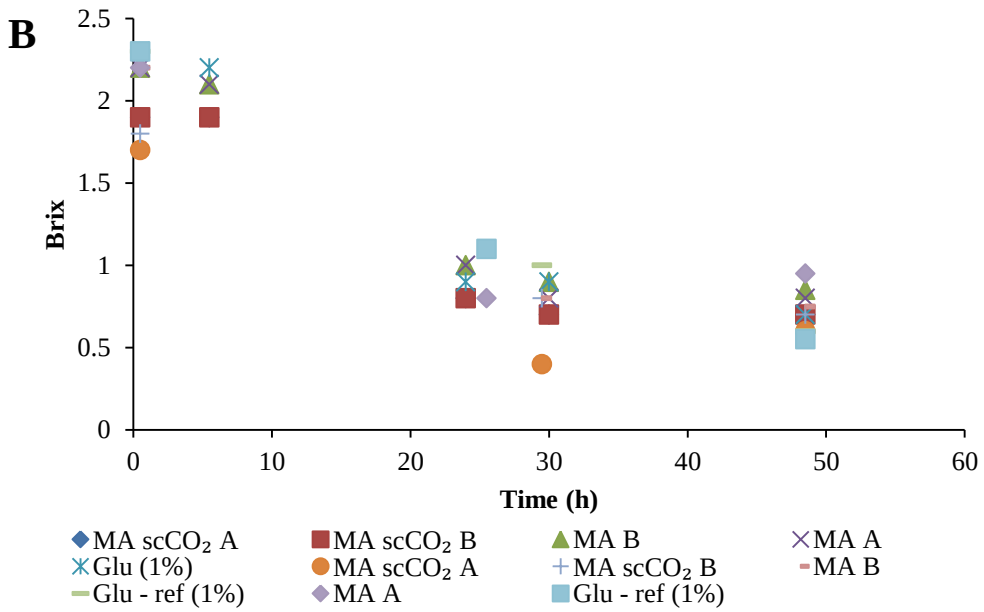


Figure 3-24 Growth of *Saccharomyces bombicola* on biomass hydrolysate A) OD indicates the growth of S. bombicola in function of time B) Brix value estimates the consumption of sugars *by S.bombicola* in function of time.

The substrate consumption and overall yield are shown in Figure 3-25 A and 3-25 B, where 2 repeats were carried out for the $scCO_2$-extracted (MA scCO2 A and MA $scCO_2$ B) and non-treated maize stover (MA A and MA B). Figure 3-25 A shows that there is on average, a 19% increase in glucose consumption with the $scCO_2$-extracted maize stover when compared to the non-treated maize stover. Furthermore, Figure 3-25 B indicates that, on average, substrate growth increased by 18% (Figure 3-25 B) with the $scCO_2$-treatment of maize stover. $ScCO_2$ has therefore an overall positive effect on fermentation for the production of sophorolipid surfactants in comparison with the non-$scCO_2$ extracted hydrolysates.

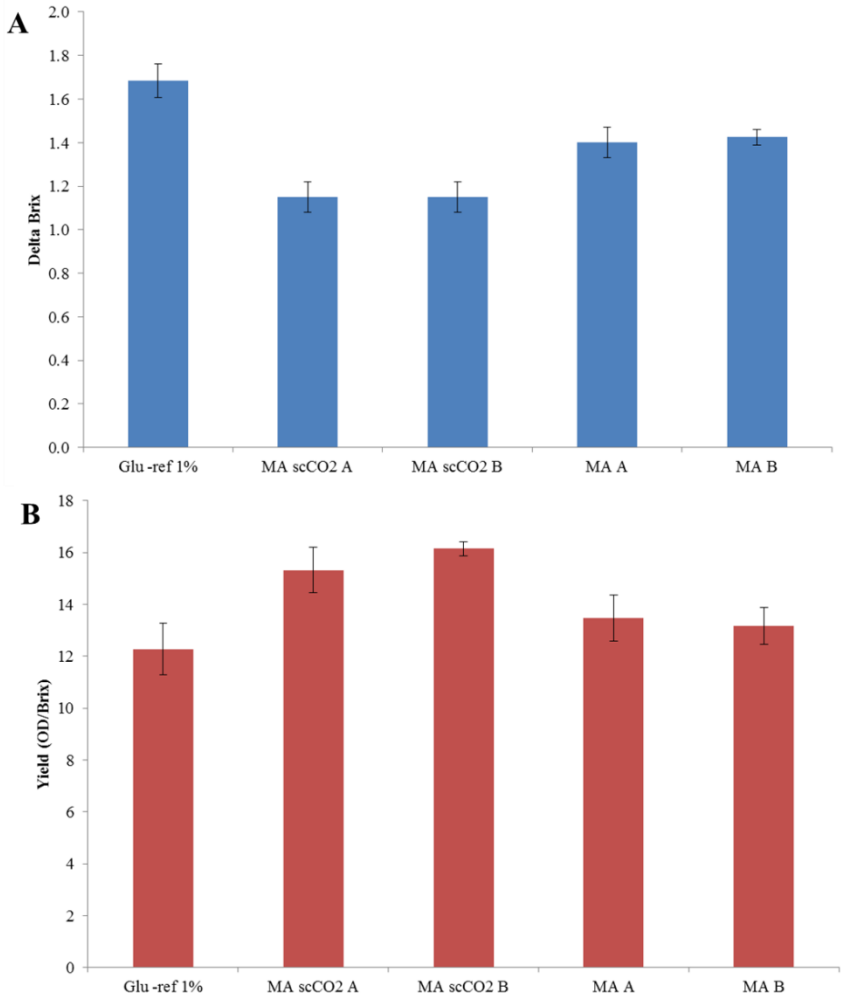


Figure 3-25 Utilisation of biomass derived sugars during fermentation A) Consumption of substrate when using scCO2 extraction (MA scCO2) and untreated maize stover (MA) (2 repeats where carried out for each – A and B) . B) Growth (Yield) when using scCO2 extracted and non-treated maize stover.

3.5.2 Fermentation of biomass with *S. cerevisiae*

Fermentation was carried out on $scCO_2$-extracted and untreated maize stover by *S. cerevisiae* for ethanol production. This was done in collaboration with the Swedish company Processum.

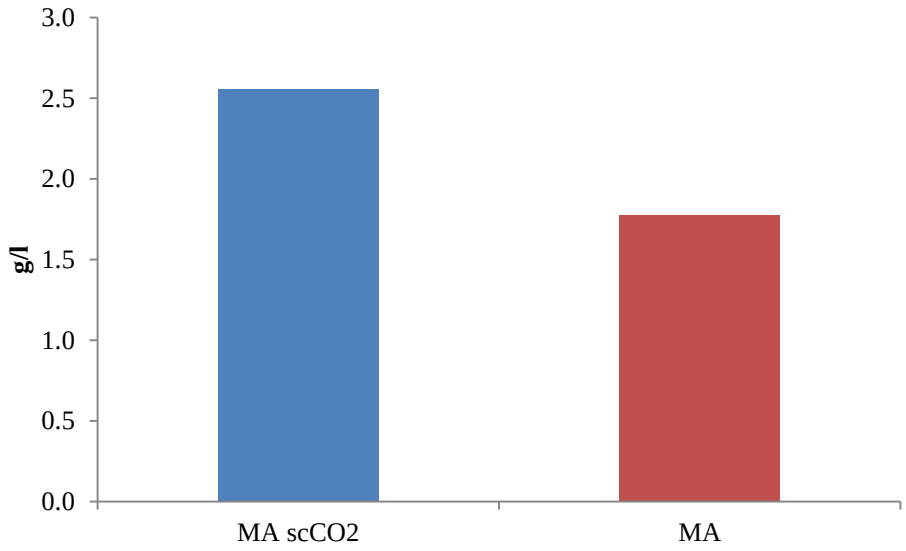


Figure 3-26 Ethanol production when using scCO2-extracted and untreated maize stover.

Extraction of stover $scCO_2$ led to a 40% increase in ethanol production when compared to the non-extracted maize stover (Figure 3-26). Figure 3-23 shows that the total sugars is higher in the $scCO_2$-extracted maize (higher glucan content) and therefore fermentation can occur at a higher rate due to more available substrate.

These results are consistent with observations found in the literature that indicate enhanced hydrolysis of biomass post-treatment in a supercritical reactor (in static mode).[221, 222] It is possible that the removal of waxy lipid layers from the plant improves the effectiveness of the hydrothermal pre-treatment and enzyme access to polysaccharides during downstream processing of the biomass. It has been shown that cuticular waxes of C_4 biomass are critical inhibitors of fermentation.[223] Crucially, within this current study, by conducting this pre-treatment process as an extraction (in dynamic flow rather than in static mode) there is an enhanced downstream effect on hydrolysis and fermentation, whilst also providing a source of valuable waxes.

The techno-economic assessment of the holistic maize stover biorefinery shows that, with the integration of $scCO_2$-extraction, the production costs of ethanol are 35% lower when compared with the non-$scCO_2$ extracted maize stover. Even though equipment costs and thus the total capital investment of the biorefinery are increased with the inclusion of the $scCO_2$ pre-treatment, these are outweighed by the increased product rates which result in lower production costs.

Thus $scCO_2$ would be an effective extraction step in a maize stover biorefinery leading to the extraction of high-value waxes as well as enhancing the downstream processing

of the maize stover biomass. The following schematic (Figure 3-27) is proposed for the development of a holistic maize stover biorefinery, which leads to the production of waxes, surfactants and fuels (ethanol and solid fuels for power generation). From a systemic viewpoint, it could also be possible to produce silicates solutions and recovery metals from the ashes generated in combusting any biomass residues.[224-226]

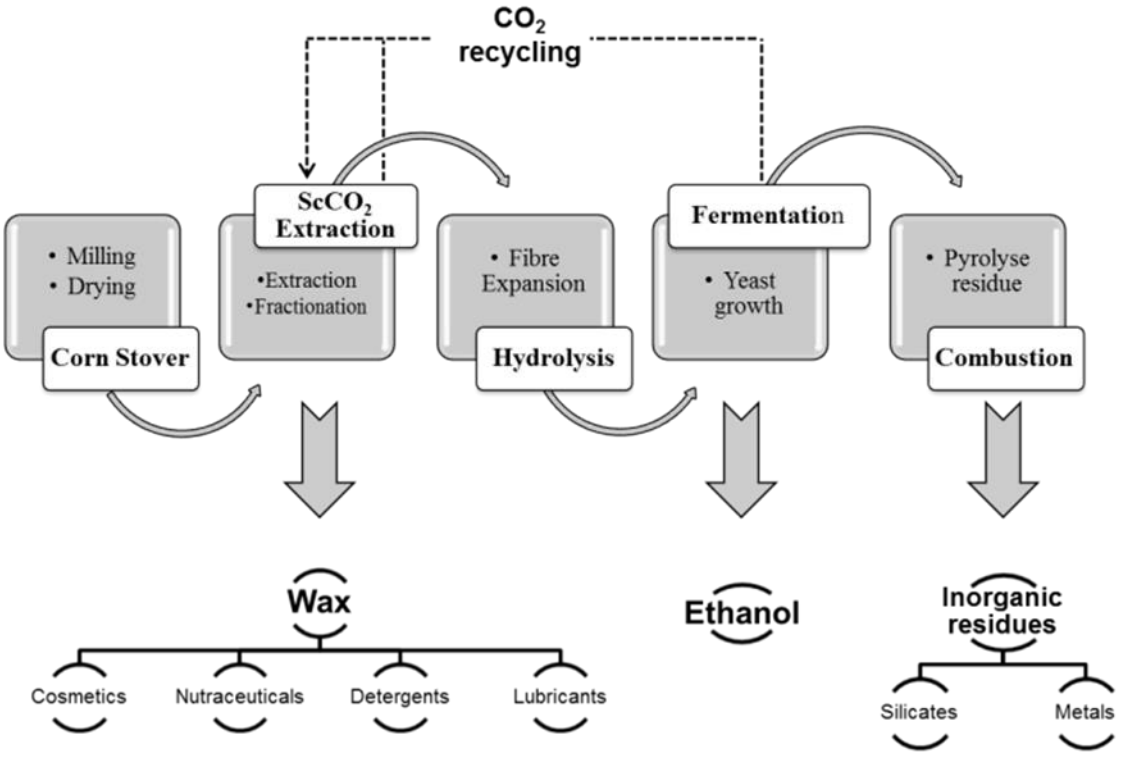


Figure 3-27 Concept of holistic maize stover biorefinery with $scCO_2$ as a pre-treatment step.

3.6 Conclusion

A full characterisation and quantification of the hydrophobic compounds found in corn stover (stems, husks and leaves) was carried out. % yields indicate that the highest amount of wax was extracted from the leaves followed by the husks and stems. Results show that, although there is a similarity in the type of compounds extracted from the different parts of the plant, the abundance of each family of compounds differs. This difference was correlated to the melting point profiles of the lipophilic extractives.

The second part of the chapter illustrates the numerous benefits of integrating supercritical extraction for maize stover in a holistic biorefinery. $ScCO_2$ extraction and fractionation of wax from maize stover resulted in waxy fractions

containing different compositions and melting points enabling their utilisation in different applications. One of the fractions was demonstrated as an effective de-foaming substance which can be implemented in detergents in place of non-renewable defoaming agents. Furthermore, $scCO_2$ had a positive effect on downstream processing of the maize stover biomass. In the first fermentation for surfactant production, results show that there was a higher glucose consumption (19%) and greater growth (18%) for the $scCO_2$-extracted maize stover when compared to non-treated maize stover. The second type of fermentation showed a 40% increase in overall ethanol production for the $scCO_2$-extracted maize stover when compared to the non-treated stover. Techno-economic assessment of the maize stover biorefinery showed that the integration of $scCO_2$ extraction decreases ethanol production costs by 35%. Therefore this shows that $scCO_2$ extraction is ideal for the generation of valuable waxy compounds in a holistic biorefinery, enhancing fermentation processes and the economic viability of the whole biorefinery.

Further work includes investigating (in more detail) the mechanism by which defoaming occurs. It is proposed that the (large concentration of) saturated and unsaturated fatty acids present in this wax fraction become saponified in the aqueous washing powder solution, and form salts due to the presence of calcium and magnesium present in the hard water; and it is this that works as the de-foamer. However it would be interesting to identify the exact compounds which bring about the defoaming effect by means of standards (of the compounds constituting the wax). By comparing the defoaming results of the standards with that of the wax as a whole it would be possible to conclude whether the defoaming effect is brought about by individual groups of compounds or through a synergistic effect by the different groups of compounds constituting the wax. Furthermore, the amount of maize stover wax used in the washing machine runs was 3 g and 1.5 g, making up ≈2.7% and ≈1.4% of the total detergent formulation respectively. It would be interesting to carry out further washing machine tests using smaller amounts of wax (since antifoam could make up 0.8 – 4 % of the total detergent formulation) to see whether the same defoaming effect is observed with less wax. Reducing the amount of wax utilised for each washing machine run would have significant positive effects from an economical viewpoint.

Approximately 21% of wax Fraction A by composition was identified and quantified which could suggest that the remaining composition is made up of high-molecular

weight compounds such as phospholipids and triglycerides, which cannot be determined by GC. Therefore, further work could investigate the use of other analytical techniques (gas-liquid chromatography, high performance-liquid chromatography, supercritical fluid chromatography, size-exclusion chromatography etc.) to identify and quantify these high-molecular weight compounds. Finally, it would also be interesting to conduct application testing on the other maize stover fractions (Fraction A and Fraction B).

Chapter 4

Supercritical extraction of lipophilic molecules from miscanthus and its effects on the downstream processing of miscanthus.

4 Chapter 4

4.1 Introduction

Miscanthus is a perennial grass that exhibits C_4 photosynthesis. It shows high photosynthetic activity and has a high rate of carbon dioxide fixation, high radiation and high water use efficiencies, which result in rapid growth and excellent productivity.[10, 13-15] Experiments have shown the possibility of utilising miscanthus as a solid biofuel and construction material (pressed particle-boards).[20] There is also the possibility of producing chemicals from miscanthus such as activated carbon, adhesives, ethanol, methylcellulose and carboxymethylcellulose by organosolv fractionation of its main constituents (i.e. cellulose, hemicellulose and lignin).[21-26]

Miscanthus side-streams could be exploited for wax applications. Limited work has been carried out on the extraction of chemicals from *Miscanthus x. giganteus* and the solvent utilised was DCM.[106] No supercritical extraction of waxes from miscanthus species has been investigated.

This chapter illustrates the benefits of introducing supercritical extraction as a pre-treatment step in a miscanthus biorefinery. $ScCO_2$ extraction of waxes from two species of miscanthus was carried out; *Miscanthus x giganteus* and *Miscanthus sinensis*. The metabolic profile of the leaves and stems of each was characterised and compared in order to obtain high-quality compounds which could potentially add value to this waste biomass. Furthermore, the effect that $scCO_2$ has on the downstream processing of miscanthus was investigated by carrying out saccharification on untreated, ethanol-treated (conventional solvent) and $scCO_2$-treated miscanthus in order to investigate whether there is any beneficial effect of using $scCO_2$ extraction as a pre-treatment step.

4.2 Supercritical yields of waxes from stems and leaves of *Miscanthus x. giganteus* and *M. sinensis*

In this study two species of miscanthus were investigated; *M. x giganteus* (MG) and *M. sinensis* (MS). The leaves and stems of each were separated and analysed individually. Figure 4-1 illustrates the total % yield of crude extract, obtained by supercritical extraction, for the stems and leaves of each species.

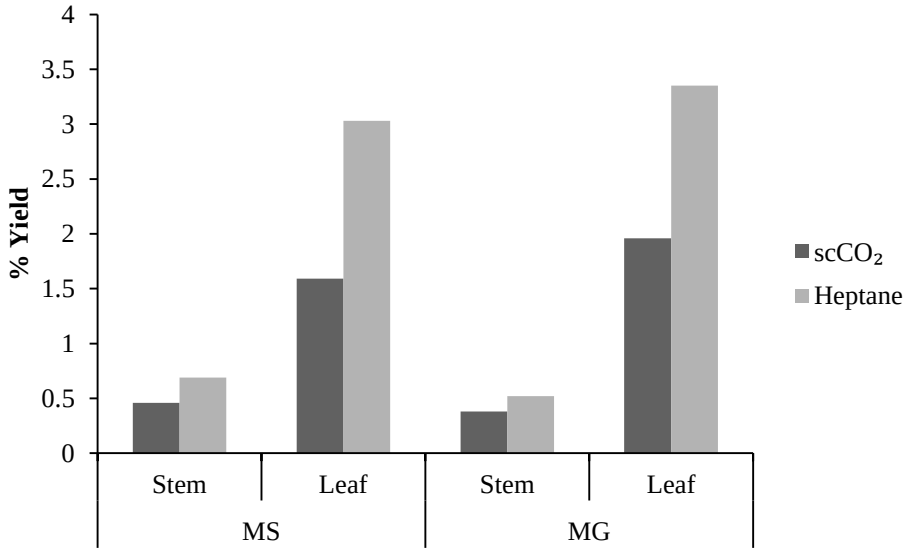


Figure 4-1 % Extraction yields of wax from the stems and leaves of the various miscanthus samples.

For all genotypes, a much higher % yield of wax was obtained from the leaves than the stems. For the leaf extractions, the highest % yield of wax was extracted from the leaves of MG (1.96%), while the lowest % yield was obtained from leaves of MS (1.4%). In contrast, the highest % yield of wax extracted from the stems occurred with MS (0.46%), while the lowest was obtained from the stems of MG (0.38%).

The results also show the same trend for both types of extraction ($scCO_2$ and soxhlet extraction), in which the highest % yield of wax extracted occurred with leaves of MG (3.35% for soxhlet extraction, 1.96% for $scCO_2$ extraction) while the lowest yield of wax occurred with the stems of MG (0.52% for Soxhlet extraction, 0.38% for $scCO_2$ extraction).

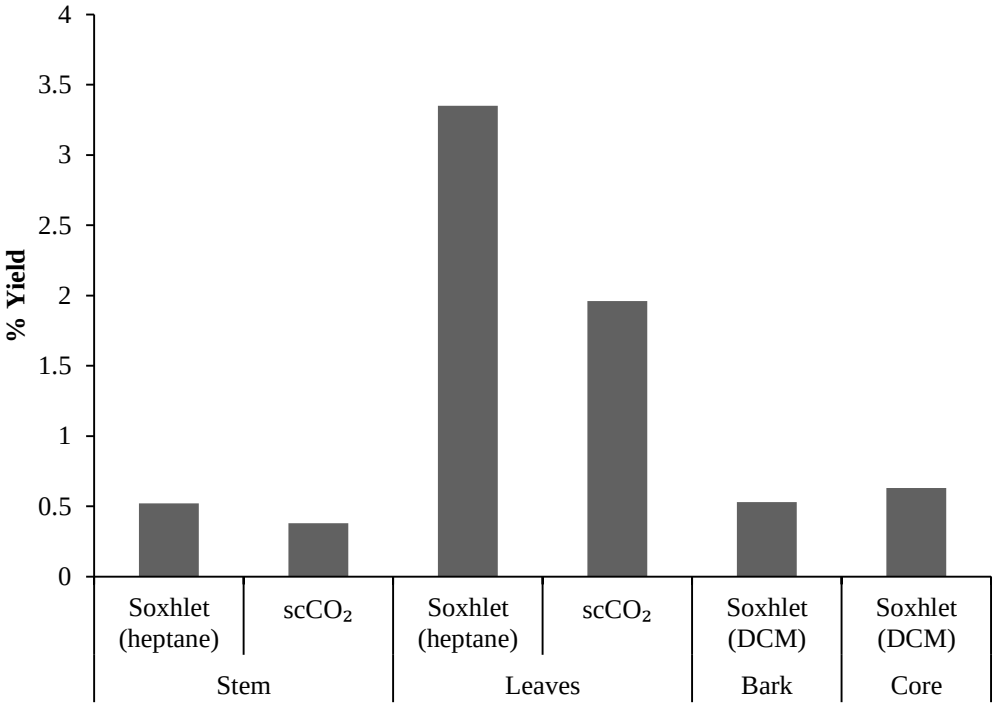


Figure 4-2 Comparison of % yield of lipophilic extractives of stem, leaves, bark and core of *miscanthus x. giganteus*.[106]

Villaverde *et al.* investigated the chemical composition of the lipophilic fraction of the bark and core of *Miscanthus giganteus*.[106] The % yield of lipophilic extractives obtained for the bark and core by soxhlet extractions with DCM for 8 hours, was found to be 0.53% and 0.63% respectively. Figure 4-2 compares the % yield of crude lipophilic extractives of the stem, leaves (obtained in this investigation), core and bark. The % yields obtained by Villeverde *et al.* for the bark (0.53%) and core (0.63%) are comparable with the % yield of crude lipophilic extract obtained for the stem (0.52%) using Soxhlet extraction with heptane.[106]

4.3 Characterisation and quantification of waxes from stems and leaves of miscanthus genotypes.

Analysis of the wax and derivative wax samples was carried out by GC and GC-MS whereby high-temperature methods were developed for the elution and identification of high-molecular weight molecules such as sterols, steroid ketones and wax esters.

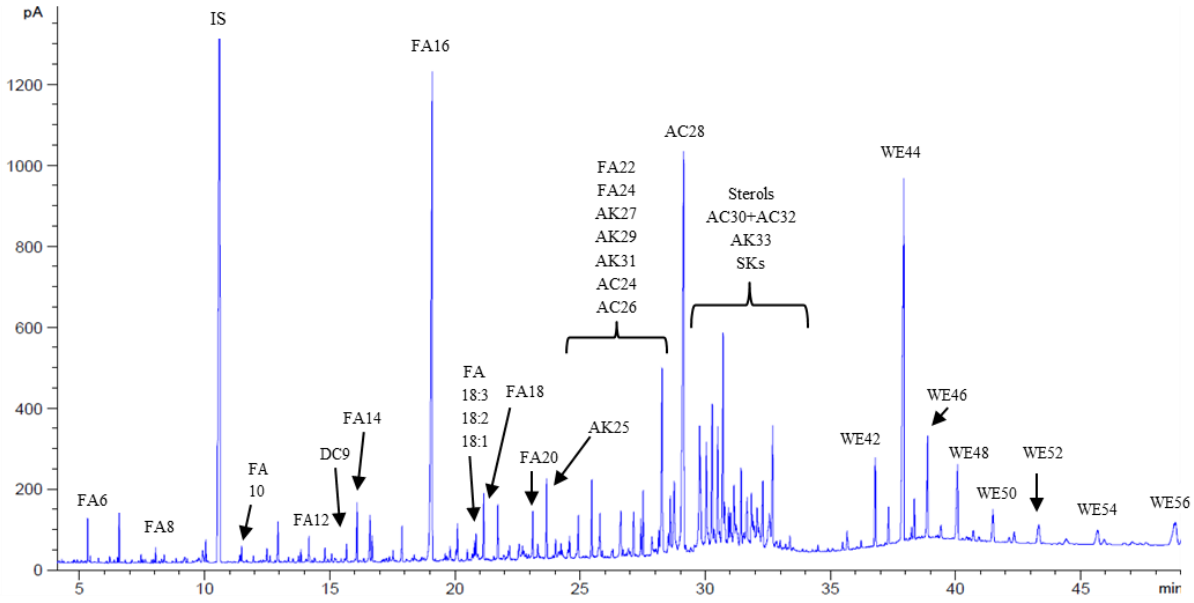


Figure 4-3 GC chromatogram illustrating some of the hydrophobic compounds found in the silylated lipophilic extractive from MS stems. FA (Fatty acid), DC (Difatty acid), AK (Alkane), AC (Alcohol), AD (Aldehyde), SKs (Steroid ketones) and WE (Wax esters).

The different hydrophobic components constituting the waxes from the leaves and stems of both species are indicated in Table 4-1. The results indicate that the waxes found in each species consist of a complex mixture of compounds having a very large range of molecular weights and structures. The major groups of compounds determined in the waxes were found to be long-chain saturated and unsaturated fatty acids, fatty alcohols, fatty aldehydes, *n*-alkanes, sterols, steroid ketones and wax esters.

GC-MS data has shown that there is a similarity among the types of compounds found in the extracts from the two species. However, quantification data shows that the abundance of certain compounds and their respective families varies among each species.

Table 4-1 Composition of compounds found in lipophilic extractives from MS and MG (in µg/g of biomass) for $ScCO_2$.

Compounds	MS $ScCO_2$ (µg/g of plant)		MG $ScCO_2$ (µg/g of plant)		MS Heptane (µg/g of plant)		MG Heptane (µg/g of plant)	
	Stems	Leaves	Stems	Leaves	Stems	Leaves	Stems	Leaves
Pentanoic acid	0.8 ±0.3	2.3 ±0.3	0.4	3.2 ±0.7	3.6 ±0.2	3.9 ±0.3	3.4	4.5 ±0.1
Hexanoic acid	5.1 ±0.3	5.1 ±0.3	7.6	12.8 ±1.8	18.2 ±0.2	9 ±0.3	10.6 ±0.3	11.2 ±0.4
Heptanoic acid	0.9 ±0.2	1 ±0.3	1.2 ±0.1	2 ±0.8	2.1	1.7	1.3 ±0.2	2.1 ±0.1
Octanoic acid	2.6 ±0.8	7 ±0.7	2.4 ±0.1	8.3 ±1.1	5.5 ±0.2	10.6 ±0.5	1.7 ±0.1	7.7 ±0.3
Nonanoic acid	2.8 ±0.4	4.3 ±1.1	4.2 ±0.1	10.4 ±1.1	5.6 ±0.2	5.7 ±0.3	2.7 ±0.1	6 ±0.2
Decanoic acid	2.4 ±0.9	18.5 ±1.7	1.7	15.9 ±2.1	2.8 ±0.05	26.4 ±0.2	1.2 ±0.1	17.7 ±0.6
Dodecanoic acid	9.2 ±0.6	128.1 ±12.3	5.1 ±0.2	124.4 ±13.8	20.1 ±0.5	191.8 ±5.7	6.6 ±0.3	172.2 ±5.7
Tetradecanoic acid	7.4 ±0.5	74.9 ±4.7	5.2	86.4 ±7.5	16.2 ±0.3	132.5 ±2.4	6.9 ±1	133.3 ±4.3

Pentadecanoic acid	5.9 ±1.2	7.5 ±0.9	8.5 ±0.2	16.9 ±2.1	13 ±0.6	18.8 ±1	9.5 ±1.7	20.1 ±0.6
Hexadecanoic acid	165.5 ±5.7	307.5 ±35.7	169 ±1.6	464.6 ±47.6	331 ±6.5	591.5 ±22.8	210.7 ±23.6	755.3 ±24.8
Heptadecanoic acid	4.2 ±0.3	15.5 ±7.9	8.7 ±0.1	13.4 ±0.4	14.5 ±0.7	32 ±14.3	10.6 ±1.8	25.2 ±0.8
Octadecanoic acid	18.6 ±1.2	94.3 ±13.1	15.4 ±0.1	88.5 ±7.6	42.7 ±1.2	175.9 ±6	27.6 ±4.8	155.9 ±5.1
Nonanoic acid	3.3 ±1.1	18 ±2.4	2.4	19.1 ±3.4	3.7 ±1.2	14.5 ±0.1	1.1	9.1 ±0.3
Eicosanoic acid	11 ±1	99.9 ±16.3	12.8 ±0.5	85.3 ±6.4	22 ±4.8	139 ±0.7	13.3 ±0.9	143.6 ±4.7
Heneicosanoic acid	3.7 ±0.4	8.1 ±0.7	7.1 ±0.1	10.6 ±2.1	6.1	7.8 ±0.2	2.3 ±0.3	9 ±0.3
Docosanoic acid	9.1 ±3.3	39.1 ±1.8	16.8 ±0.1	44.3 ±7.4	24.5 ±1.7	68.2 ±6.5	15.8 ±2.3	85.5 ±2.9
Tricosanoic acid	7.1 ±2	25.8 ±2.3	20.8 ±0.2	35.5 ±6.4	19.1 ±0.8	53.6 ±2.8	16.9 ±2.8	55.2 ±1.8
Tetracosanoic acid	7.4 ±2.7	35.3 ±5.8	29.5 ±0.4	60.7 ±12.6	26 ±0.3	85.1 ±1	18.4 ±0.5	139.7 ±4.6
Pentacosanoic acid	2.7 ±0.5	13.9 ±1.8	16.8 ±0.1	24.4 ±2.6	7.3 ±0.1	28.2 ±1.2	10.1 ±0.1	64.7 ±2.1
Hexacosanoic acid	17.8 ±0.7	10.4 ±2.5	2.9 ±0.1	69.4 ±27.2	9.5 ±0.2	49.5 ±0.02	17 ±1.3	149.8 ±56.1

Octacosanoic acid	TR*	TR	TR	TR	TR	TR	TR	TR
Tricontanoic acid	TR	TR	TR	TR	TR	TR	TR	TR
Dotriacontanoic acid	TR	TR	TR	TR	TR	TR	TR	TR
Total saturated fatty acids	**287.5 ±24.1**	**916.5 ±112.6**	**338.5 ±4**	**1196.1 ±154.7**	**593.5 ±19.7**	**1645.7 ±66.3**	**387.7 ±42.2**	**1967.8 ±115.8**
9-hexadecenoic acid	1.6 ±0.3	-	0.9	15.1 ±2.2	-	-	-	-
9-octadecenoic acid	6.6	4.6 ±2.2	1.1 ±0.1	8.7 ±1.4	12.7 ±3.2	58.9 ±5.4	1.4 ±0.4	-
9,12-Octadecadienoic acid	64.9 ±0.1	213.6 ±17.4	1.8 ±0.1	48.2 ±1.8	22 ±5.4	134 ±17.9	0.5 ±0.1	-
9,12,15-Octadecatrienoic acid	90.1	87 ±10.1	1.7 ±0.1	27.1 ±4.9	7.5 ±2.2	17.6 ±0.3	1.2 ±0.3	-
Total unsaturated fatty acids	**160.7 ±0.1**	**305.2 ±29.7**	**4.6 ±0.3**	**99.1 ±8.1**	**42.2 ±10.8**	**210.5 ±23.6**	**3.1 ±0.8**	**-**
Heptanedioic acid	-	-	1.2 ±0.1	-	-	-	-	-

Octanedioic acid	0.9 ±0.2	-	0.9 ±0.1	3 ±0.2	5.8 ±0.2	2.8 ±1	4.8 ±0.2	5.8 ±0.2
Nonanedioic acid	10.2 ±4	5.1 ±1.7	21.5 ±0.5	26 ±7.7	27.9 ±1.3	13.6 ±2.4	16.4 ±1.5	31.2 ±1.4
Decanedioic acid	1 ±0.3	-	1.5	-	-	-	-	-
Total saturated difatty acids	**12.1 ±4.5**	**5.1 ±1.7**	**25.1 ±0.7**	**29 ±7.9**	**33.7 ±1.5**	**16.4 ±3.4**	**21.2 ±1.7**	**37 ±1.6**
Docosanol	1.7 ±0.1	12 ±2.6	0.9	6.5 ±2.5	4 ±0.7	11.6 ±4.3	0.9 ±0.2	5.8 ±1.4
Tetracosanol	1.4 ±0.1	9.1 ±3.7	2.3 ±0.2	13.6 ±3.7	2.2 ±0.7	10.1 ±0.5	1.7 ±0.9	16.7 ±3.3
Hexacosanol	6.3 ±0.4	16.2 ±0.1	12.7 ±0.1	31.5 ±7.4	9.3 ±0.1	32.7 ±1.6	22.4 ±0.1	44.3 ±1.5
Octacosanol	26.4 ±2.4	54.2 ±1.6	210.2 ±1.4	227.7 ±9.6	46.9 ±0.1	230.2 ±0.8	279.4 ±8.7	457.5 ±19.9
Triacontanol	84.3 ±0.8	138.5 ±23.6	36.6 ±0.8	157.8 ±20.5	66.3 ±0.6	195 ±4.5	67.8 ±2.5	212.6 ±9.9
Dotriacontanol	17.1 ±0.9	319.4 ±45.6	29.3 ±0.4	356 ±119.3	38.2 ±0.3	824.6 ±32	27.8 ±0.2	795.7 ±37.3
Total saturated fatty	**137.2 ±4.7**	**549.4 ±77.2**	**292 ±2.9**	**793.1 ±163**	**166.9 ±2.5**	**1304.2 ±43.7**	**400 ±12.6**	**1532.6 ±73.3**

alcohols								
Tetracosanal	-	14.3 ±3.4	1.8 ±0.1	7.2 ±1.2	7.6 ±0.8	19.4 ±6.5	5 ±0.1	109.2 ±1.6
Hexacosanal	11.6 ±0.8	24.7 ±1	18.2 ±0.3	50.8 ±12.5	15.8 ±1.1	80.9 ±7.9	29.7 ±2.8	107.2 ±10.9
Octacosanal	49.2 ±8.1	121.2 ±12.3	206.7 ±0.1	498.3 ±55.6	99.7 ±3.8	538.2 ±7	365 ±28.7	1251.1 ±58.9
Triacontanal	64 ±3.4	124.9 ±61	35.4 ±1.3	311.7 ±71.5	85.9 ±2.4	326.5 ±13.9	296.9 ±67.2	1124.4 ±54.3
Total saturated fatty aldehydes	**124.8 ±12.3**	**285.1 ±77.7**	**262.1 ±1.8**	**868 ±140.8**	**209 ±8.1**	**965 ±35.3**	**696.6 ±98.8**	**2591.9 ±125.7**
Pentacosane	4.9 ±0.4	25.8 ±4.5	13.6 ±0.2	34.4 ±1.1	12.7 ±1.9	39.6 ±0.2	12.6 ±0.5	53.1 ±2.2
Heptacosane	11.2 ±0.1	82 ±5.1	15.8 ±0.2	175.7 ±10.1	18.2 ±0.8	122.5 ±1.6	16.1 ±0.9	217.2 ±8.2
Octacosane	-	14.2 ±2.4	2.9 ±0.2	20 ±2.4	2.6 ±0.3	4.1 ±0.6	4.2 ±0.4	3.2 ±0.3

Nonacosane	16.7 ±0.9	120.7 ±5.9	8.1 ±0.1	215.9 ±16.9	20.2 ±0.8	187.1 ±14.5	10.5 ±0.5	263.5 ±7
Hentriacontane	17.9 ±2.2	158.4 ±0.7	21.9 ±0.7	169.8 ±6.7	60.9 ±0.1	367 ±18.9	22.1 ±3.9	291.8 ±6.8
Triatriacontane	6.1 ±1.3	63.1 ±1.4	7.7 ±0.7	63.8 ±11.7	12.9 ±0.4	116.4 ±3.8	5.7 ±1.5	95.6 ±0.6
Total hydrocarbons	**56.8 ±4.9**	**464.2 ±20**	**70 ±2.1**	**679.6 ±48.9**	**127.5 ±4.2**	**836.7 ±39.6**	**71.2 ±7.7**	**924.4 ±25.1**
Campesterol	73.5 ±1.2	117.9 ±19.1	25.7 ±0.5	165.3 ±15	71.3 ±0.4	255.3 ±24	95.3 ±4.4	391.4 ±5.9
Stigmasterol	78.3 ±2.3	73.4 ±20.7	45 ±0.4	135.6 ±12.6	87.8 ±2	344 ±6	62.1 ±13.6	573.4 ±19.2
β-Sitosterol	140.2 ±5	387.7 ±45.7	57.8 ±0.6	311.3 ±21.5	220.9 ±2.5	410 ±20.7	152.6 ±13.4	874.1 ±14.4
Total sterols	**292 ±8.5**	**579 ±85.5**	**128.5 ±1.5**	**612.2 ±49.1**	**380 ±4.9**	**1009.3 ±50.7**	**310 ±31.4**	**1838.9 ±39.5**
Stigmast-4-*en*-3-one	37.3 ±2.8	85.7 ±2.9	21.6 ±0.6	55 ±12.2	86.6 ±1.5	168.7 ±3.6	50 ±3	144.3 ±4.7
5α-Stigmastan-3,6-dione	24 ±3.3	67.4 ±20.4	22.6 ±0.9	81.1 ±27.6	50.8 ±0.8	130.8 ±32.5	31.2 ±4	83.2 ±3.1

Total steroid ketones	**61.3 ±6.1**	**153.1 ±23.3**	**44.2 ±1.5**	**136.1 ±39.8**	**137.4 ±2.3**	**299.5 ±36.1**	**81.2 ±7**	**227.5 ±7.8**
Wax ester 38	3 ±0.2	64.6 ±20.4	1.4	4.5 ±0.3	3.4 ±0.3	8.3 ±1.3	0.9 ±0.05	11 ±0.2
Wax ester 40	9.8 ±0.9	36.3 ±7.3	5.2	11.8 ±2.8	9.2 ±0.3	9.4 ±0.8	3.7 ±0.2	9.7 ±0.2
Wax ester 41	0.9 ±1.5	-	0.6 ±0.2	4.5 ±1.1	1.7	8.6 ±1.8	1.1 ±0.1	4.2 ±0.4
Wax ester 42	31 ±2.3	23 ±5.3	24.4 ±0.2	24.2 ±1.2	21.3 ±0.9	26 ±1.8	15.7 ±1	29.3 ±2.2
Wax ester 43	6.9 ±0.6	14.9 ±4.3	10.7	16.6 ±5.8	4.8 ±0.1	9.8 ±0.3	6.8 ±0.4	13.6 ±0.4
Wax ester 44	83.8 ±11.2	28.7 ±1.6	143.1 ±0.3	72.2 ±2.6	51.9 ±1.6	76 ±0.7	94.6 ±5.6	94.4 ±5.3
Wax ester 45	8.4 ±1.2	14.3 ±0.3	9.4 ±0.1	14.5 ±0.4	7.7 ±3.2	17.8 ±5.9	6.7 ±0.1	17.4 ±3.3
Wax ester 46	36.4 ±7.1	60.8 ±9.4	28 ±0.1	50.5 ±7.3	28.7 ±1	68.2 ±1.2	21.2 ±3	75.7 ±6.1
Wax ester 47	5.7 ±1	12.9 ±0.9	4.1	12.6 ±2.1	7.9 ±0.2	21.4 ±0.8	3.5 ±0.1	21.6 ±1.4
Wax ester 48	37.8 ±9.2	110.7 ±1	27.7	76.1 ±6.4	28.8 ±0.9	157.8 ±1.5	23.9 ±1.8	112.1 ±9.5

Wax ester 49	5 ±1.5	11.9 ±1.5	3.8	10.4 ±1.5	6.3 ±0.3	17.7 ±0.4	3.4 ±0.3	16.4 ±1.1
Wax ester 50	19.8 ±6.2	65.3 ±9	12.5	34.7 ±4.4	20.7 ±0.8	122.9 ±13.7	14.3 ±1	53.4 ±6.7
Wax ester 51	3.9 ±1.6	8.6 ±2.8	4.1	7.8 ±2.1	2.8 ±0.2	18.2 ±0.2	5.1 ±0.2	15.2 ±1.5
Wax ester 52	13.7 ±5.2	46 ±25.4	9.6 ±0.1	31 ±9.1	11.3 ±0.4	120.9 ±3.5	15.9 ±0.7	67.4 ±6.8
Wax ester 53	TR	3.6 ±6.2	2.5	TR	1.8 ±0.1	21.6 ±0.9	2.8 ±0.1	16.7 ±2.1
Wax ester 54	7.4 ±3.1	24.1 ±13.1	7.9	16.7 ±11	8.4 ±0.2	81.9 ±5.7	18.4 ±0.3	50.7 ±1.9
Wax ester 55	TR	TR	-	TR	-	23.6 ±1.2	3 ±0.1	8.2 ±0.7
Wax ester 56	11.5 ±1.1	31.4 ±10.4	11.5 ±1.7	19.6 ±8.5	8.9 ±0.4	91.3 ±2.6	46.8 ±2.1	32.2 ±1.9
Wax ester 58	TR	17 ±1.4	TR	19.8 ±4.2	-	12.5 ±2.5	5 ±0.7	13.7 ±2.6
Wax ester 60	-	TR	-	TR	-	56.3 ±1.2	-	26.2 ±0.3
Total wax esters	**285 ±53.9**	**574.1 ±120.3**	**306.5 ±2.7**	**427.5 ±75.7**	**225.6 ±10.9**	**1015.6 ±63.5**	**292.8 ±17.9**	**689.1 ±54.6**

2-Pentadecanone-6,10,14-trimethyl	49 ±18.3	264.2 ±124.7	19.3 ±0.9	597.6 ±60.1	77.9 ±1.3	453.7 ±22.7	23.9 ±1.3	444.7
Phytol	2.2 ±1.4	359.5 ±15.2	3.3	26.5 ±4.9	-	110.4 ±15.7	-	74.8
Total 'other' compounds	**75.2 ±19.7**	**623.7 ±139.9**	**21.3 ±0.9**	**624.1 ±65**	**77.9 ±1.3**	**564.1 ±38.4**	**23.9 ±1.3**	**519.5**

TR***=** *trace* amounts**.**

4.3.1 Saturated fatty acids

The stem and leaf extracts from both species demonstrated saturated fatty acids having chain lengths ranging from C_5 to C_{32} with the even-chain length predominating. Odd-chain fatty acids were detected however in considerably lower amounts. In all extracts, palmitic acid (C_{16} acid) was the major fatty acid. Other major fatty acids included lauric acid (C_{12} acid), myristic acid (C_{14} acid) and stearic acid (C_{18} acid). In the leaf waxes of both species, there was an unusually high amount of C_{12} acid present when compared to the stems (for both MS and MG, C_{12} was found to be the second most abundant fatty acid). When looking at the wax composition (and not taking % yield into account), it was found that MG had the largest amount of fatty acids.

In the study carried out by Villeverde *et al.* a number of saturated fatty acids were found in the bark and core of MG, when extracting with DCM, ranging from C_6 to C_{30}.[106] Similar results were obtained in this investigation, where saturated fatty acids having chain lengths of up to C_{26} were quantified while trace amounts of C_{28}, C_{30} and C_{32} were detected in the stem and the leaves. In their study, palmitic acid was the most abundant saturated fatty acid in the core of MG (which is consistent with this study) while it is the second most abundant saturated fatty acid following stearic acid in the bark.[106]

4.3.2 Saturated dicarboxylic acids

Small quantities of dicarboxylic acids were detected in the wax extracts. The type and amount varied among the different samples. The main dicarboxylic acid identified, found in all of the extracts, was azelaic acid (nonanedioic acid). The widest distribution of dicarboxylic acids was found in the stem extracts of MG, with chain lengths ranging from C_7 – $C_{10.}$ No saturated dicarboxylic acids have been previously reported for miscanthus in the literature.[106]

4.3.3 Unsaturated fatty acids

Unsaturated fatty acids having two chain lengths were identified in the waxes: one C_{16} unsaturated acid ($C_{16:1}$) and three C_{18} unsaturated acids ($C_{18:3}$, $C_{18:2}$ and $C_{18:1}$). Interestingly, there is a significant difference in the abundance of unsaturated fatty acids between the MS $scCO_2$ extracts (35.3 ±0.04 mg/g of wax for the stem, 17 ±1.6 mg/g of wax for the leaf) and the MG $scCO_2$ extracts (1.2 ±0.01 mg/g of wax for the stem, 5.1 ±0.1 mg/g of wax for the leaf), with the latter having significantly lower quantities of unsaturated fatty acids in both the stem and the leaf extracts (Figure 4-4).

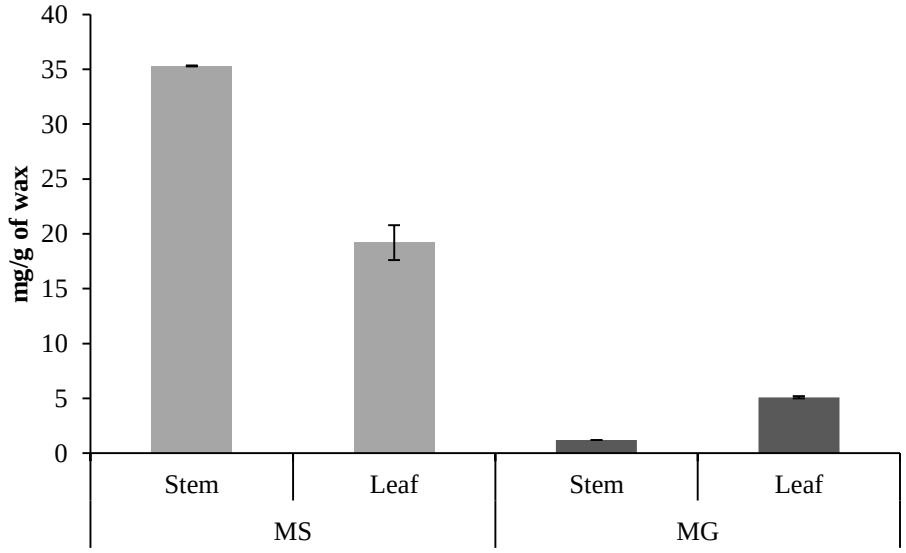


Figure 4-4 Unsaturated fatty acid distribution in the stem and leaf extractives of the various miscanthus samples.

Villeverde *et al.* reported the presence of linoleic acid, oleic acid and palmitoleic acid in the bark and core of *Miscanthus giganteus* samples but no linolenic acid was found.[106]

4.3.4 Fatty alcohols

A number of even-chained policosanols, having chain lengths of C_{22} to C_{32} were detected in the stem and leaf extracts of both miscanthus species.

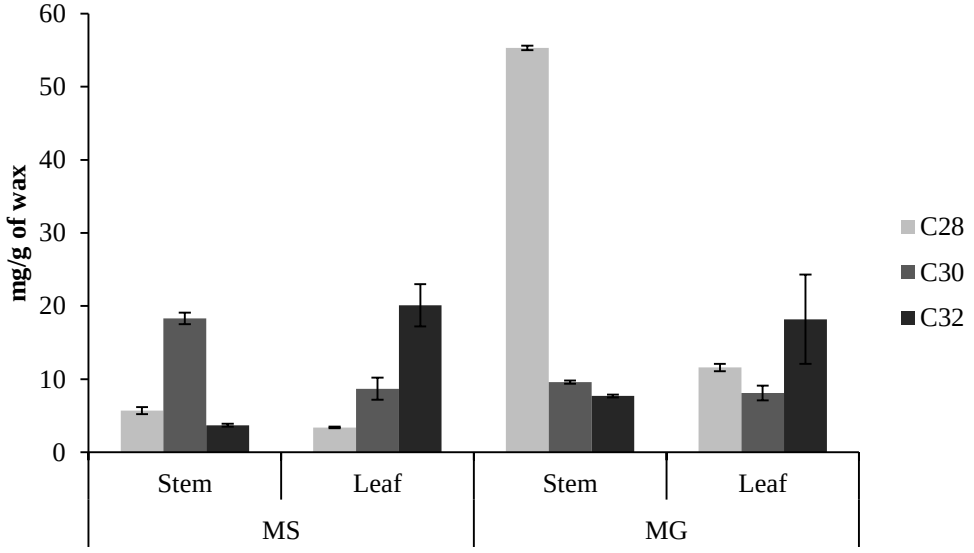


Figure 4-5 Distribution of major fatty alcohols in miscanthus species.

The three main fatty alcohols found in the extracts were found to be 1-octacosanol (C_{28}), 1-triacontanol (C30) and 1-dotriacontanol (C_{32}). The relative quantities of each differed among the stem and leaf extracts for both species (Figure 4-5). In the stem wax

from MS, 1-triacontanol predominated followed by 1-octacosanol. In contrast, in the stem wax from MG, there is a significantly higher abundance of 1-octacosanol (55.3 ±0.2 mg/g of wax) when compared to the stems from the MS (5.7 ±0.5 mg/g of wax). In all stem extracts, 1-dotriacontanol was found in relatively smaller quantities. On the other hand, in all leaf extracts in both miscanthus species, 1-dotriacontanol was found to be the dominant alcohol chain length.

Villeverde *et al.* found four different types of fatty alcohols in the bark and core, however 1-octacosanol was the only fatty alcohol that was found in reasonable quantities (81 mg/kg of dry plant in the bark and 25 mg/kg of dry plant in the core) similar to the stem wax of MG in this study. The other fatty alcohols (1-hexacosanol, 1-heptacosanol and pentacosan-1,2-diol) were found in quantities less than 7 mg/kg of dry plant.[106]

4.3.5 Fatty aldehydes

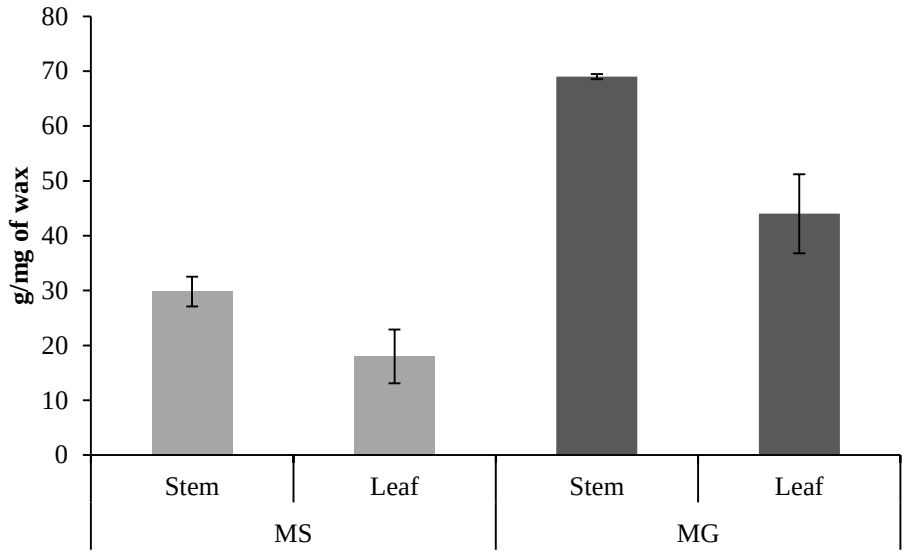


Figure 4-6 Fatty aldehyde distribution in the stem and leaf extractives of the various miscanthus samples.

Long-chain fatty aldehydes having chain-lengths ranging from C_{24} to C_{30} were also found. In the stem and leaf waxes of MS, the dominant aldehyde was found to be triacontanal (C_{30}), while in the MG samples (stems and leaves) octacosanal (C_{28}) predominated. Significantly larger quantities of aldehydes were found in the MG wax samples (69 ±0.48 mg/g of wax for the stem, 44 ±7.2 mg/g of wax for the leaf) when compared to the MS wax samples (29.8 ±2.7 mg/g of wax for the stem, 18 ±4.9 mg/g of

wax for the leaf). Octacosanal was the sole aldehyde identified in the bark and core of MG in other studies.[106]

4.3.6 *n*-Alkanes

All leaf and stem extracts from miscanthus demonstrated *n*-alkanes with chain lengths ranging from C_{25} to C_{33}. One even-chained alkane (C_{28}) was detected albeit in minimal quantities. Quantification data indicates that nonacosane (C_{29}) and hentriacontane (C_{31}) were the two most abundant hydrocarbons in all wax extracts. The published long-chain hydrocarbon pattern in miscanthus is limited. In the study carried out by Villeverde *et al.* only one long-chain alkane was found in the bark and core of MG when extracting with DCM and in very small amounts.[106] Heptacosane was found in concentrations of 11 mg/kg in the bark and 2 mg/kg in the core. Therefore a wider range of long-chain hydrocarbons were extracted from the leaves and stems in this study with both heptane and $scCO_2$.[106]

4.3.7 Phytosterols

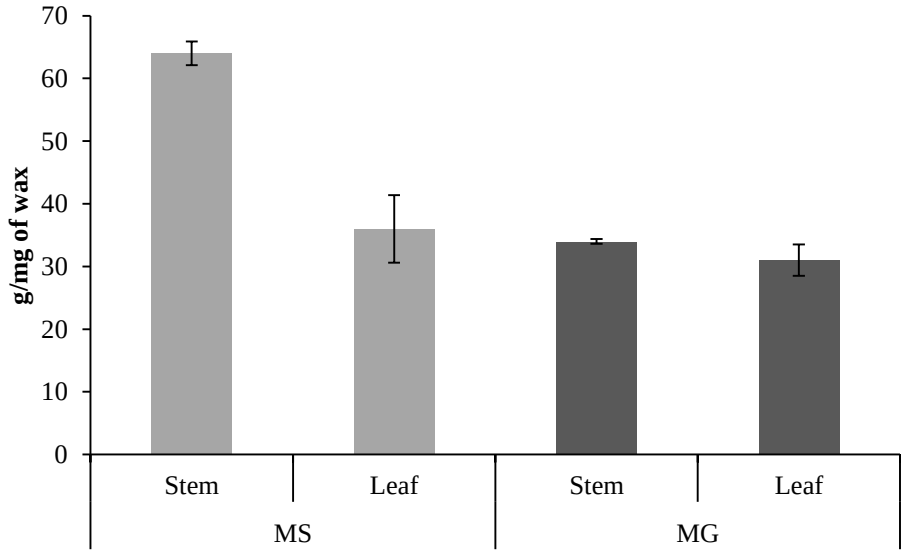


Figure 4-7 Phytosterol distribution in the stem and leaf extractives of the various miscanthus samples.

Three phytosterols were identified in the extracts: β-sitosterol, stigmasterol and campesterol; with β-sitosterol predominating. In the stem wax extracts of MS and MG, there is a higher amount of sterols (64 ±1.9 mg/g of wax for MS, 34 ±0.4 mg/g of wax for MG) when compared to the leaf wax extracts (36 ±5.4 mg/g of wax for MS, 31 ±2.5 mg/g of wax for MG). The phytosterol results are in good correlation with those obtained in other studies where β-sitosterol was found in the largest quantities in the bark and core of MG.[106]

4.3.8 Steroid ketones

Two steroid ketones were identified in all lipophilic extractives: stigmast-4-*en*-3-one and 5α-stigmastan-3,6-dione; with stigmast-4-*en*-3-one predominating. Stigmast-4-*en*-3-one was detected in the DCM extracts from MG bark and core in literature along with stigmasta-3,5-dien-7-one. No 5α-stigmastan-3,6-dione was detected.[106]

4.3.9 Wax esters

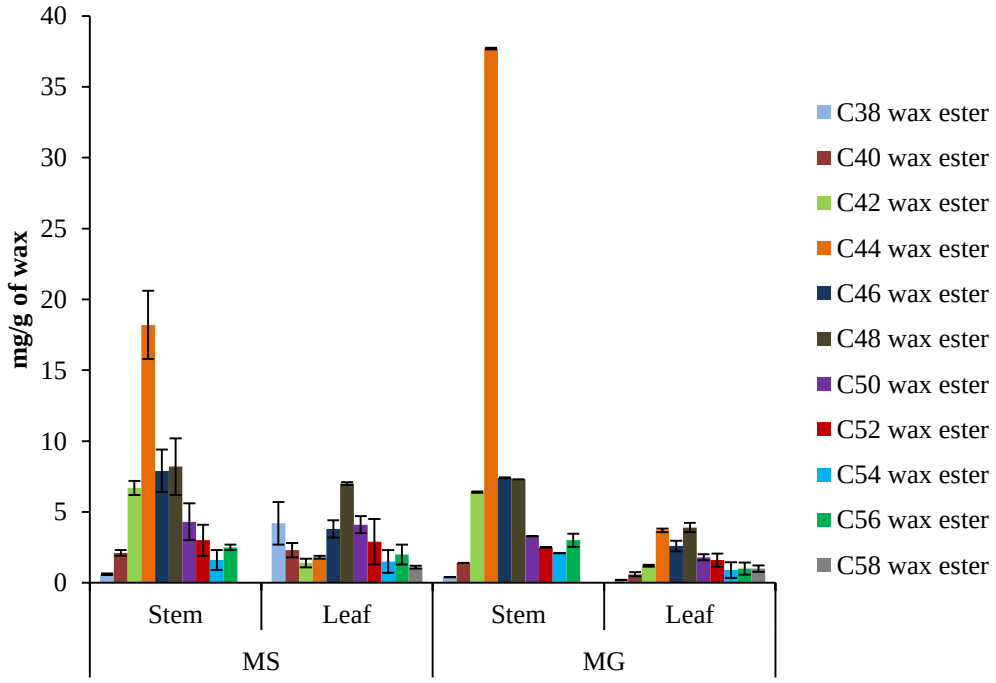


Figure 4-8 Wax ester composition of different miscanthus samples

High-molecular weight wax esters, with an even-over-odd predominance, were abundant in the wax extracts (Figure 4-8). The stem waxes for both miscanthus samples had relatively higher quantities of wax esters (55 ±10 mg/g of wax for MS, 71 ±0.6 mg/g of wax for MG) when compared to the leaf extracts (32 ±6 mg/g of wax for MS, 18 ±3 mg/g of wax for MG), with a maximum chain length of C_{58} detected. The predominant chain length in the stem extracts was found to be C_{44}, particularly in the MG stem extract where C_{44} was found to constitute around 53% of the total wax ester composition (37.7 ±0.1 mg/g of wax), as can be seen in Figure 4-8. On the other hand, trace amounts of C_{60} wax ester were detected in the wax extracts from the leaves and the predominant wax ester (in all leaf wax extracts) was found to be C_{48}. No wax esters were indicated in the study carried out by Villaverde *et al.*[106]

4.3.10 Other compounds in extracts.

Other compounds such as 2-pentadecanone-6,10,14-trimethyl and phytol were identified in the extracts. For all miscanthus wax extracts, there is a larger amount of 2-pentadecanone-6,10-14-trimethyl in the leaf extracts than the stem. In the MS samples, the leaf extract had considerably larger amounts of phytol than the stem extract. There are significantly lower amounts of phytol in MG extracts.

4.3.11 Melting profiles of MG and MS wax extracts.

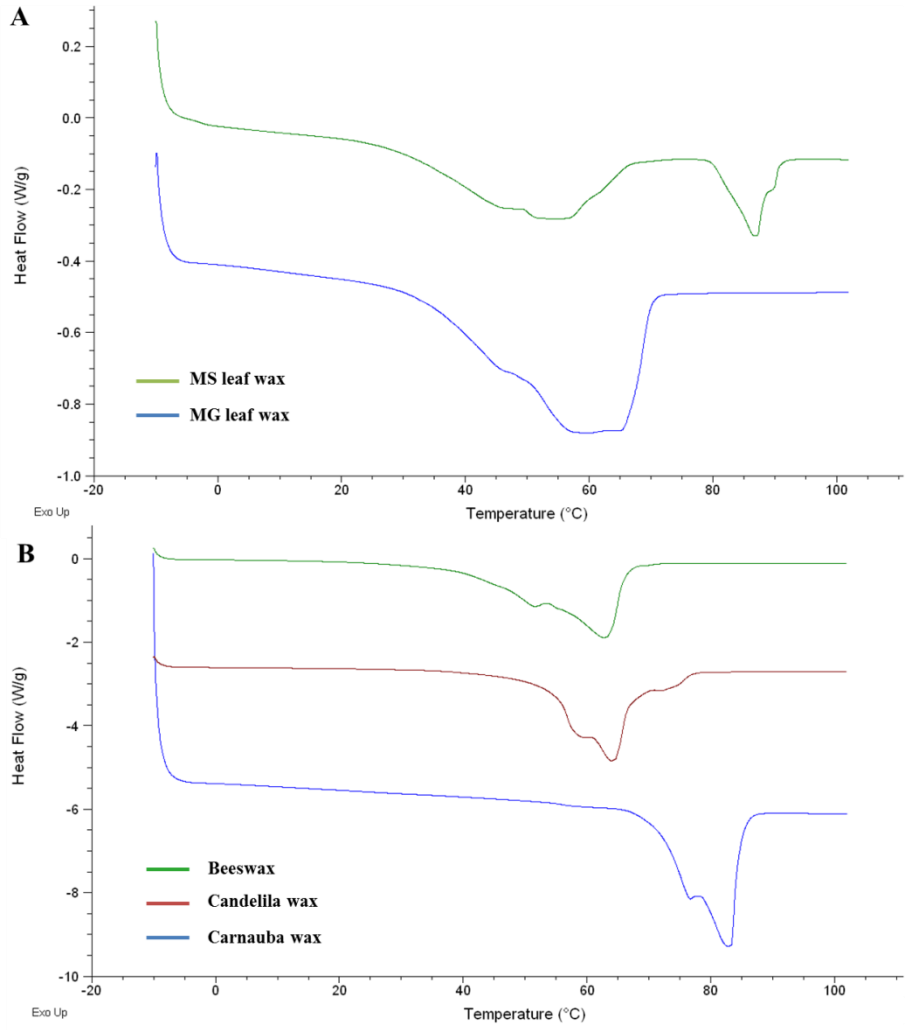


Figure 4-9 DSC thermograms of: A) MS and MG leaf wax B) Commercial waxes: Beeswax wax, candelila wax and carnauba wax.

Figure 4-9A shows the DSC thermograms of $scCO_2$ extracted MS and MG leaf waxes while Figure 4-9B illustrates the DSC thermograms of some commercially available waxes (candelila wax, beeswax and carnauba wax). A significant difference between the thermograms of each wax sample can be observed, including MG and MS waxes. The stem and leaf waxes of MG were similar, exhibiting melting point profiles with maximum peaks at 68 ^{o}C and 66 ^{o}C respectively similar to the melting point profile of

candelila wax (64 ^{o}C). The DSC thermograms of the MS stem and leaf waxes are particularly interesting as, in contrast to what is observed in the MG wax samples, they show two distinct melting regions. The first melting region had a melting maximum at 60 ^{o}C and 67 ^{o}C for the stem and leaf extracts respectively. The higher second melting point range showed a sharper peak with an endothermic minimum at 87 ^{o}C for the stem and leaf extracts. This peak was found to be higher even than that of carnauba wax (83 ^{o}C).

The difference in melting temperatures among the lipophilic extracts shows that waxes from the two species could be utilised for different applications. The high melting-point waxy constituents in the MS stems and leaves could be ideal for polishes such as automobile and instrument polishes with melting point temperatures higher even than that of carnauba wax. Further work is required to isolate and fractionate the high melting-point waxy constituents for application testing. The lower melting point waxy fraction of MS stems and leaves as well as the MG stem and leaf waxes could be utilised in other applications such as cosmetics, food and pharmaceuticals.

4.4 Comparison with organic solvent – heptane

The soxhlet extractions were carried out with heptane in this study rather than with hexane as heptane is considered to be a safer solvent.[194]

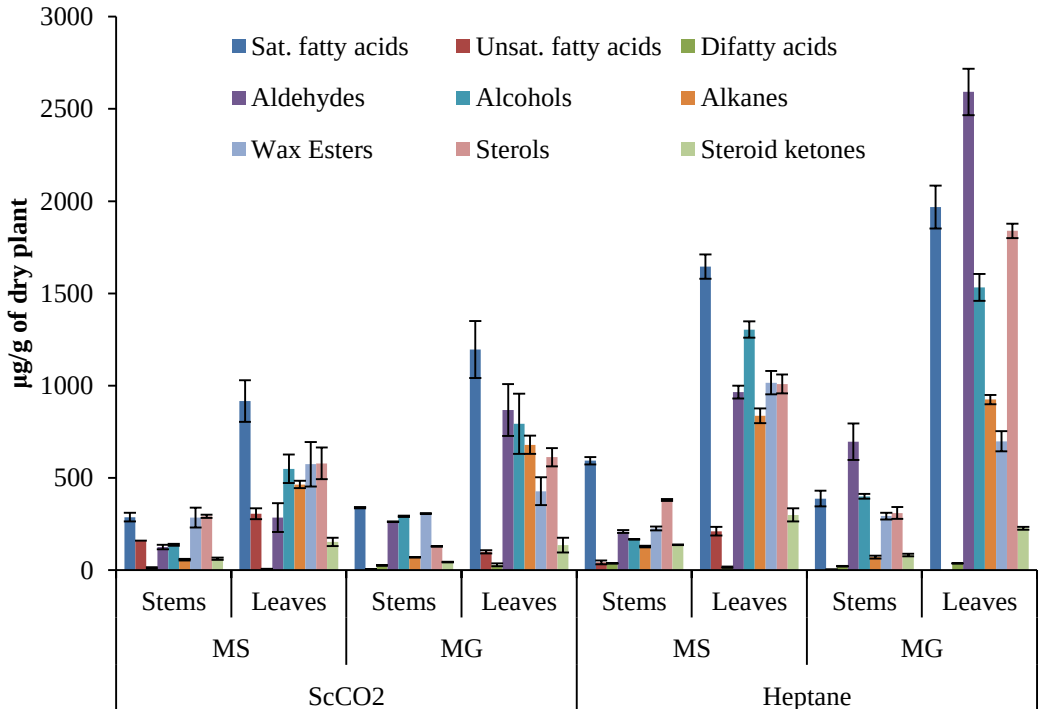


Figure 4-10 Distribution of family of compounds in all lipophilic extractives of miscanthus; $scCO_2$ and heptane.

Figure 4-10 indicates the total amount of each family of compounds (in µg/g of dry plant) in the stem and leaf waxes of MS and MG extracted with $scCO_2$ and heptane. It can be seen that a larger concentration of compounds was extracted using heptane than $scCO_2$, with the exception of total unsaturated fatty acids. As explained in Chapter 1, a reason for the higher amount of total unsaturated fatty acids in $scCO_2$ could be the protection of the unsaturated fatty acids against oxidation.

It is not clear as to why heptane extracted larger amounts of compounds compared to $scCO_2$. No explanations were found in literature, as the conventional organic solvent chosen for extraction of waxes is hexane. One possible explanation is that the high boiling point of heptane (98 ^{o}C) could melt the wax enhancing its solubility (the temperature at which the $scCO_2$ extractions were carried out was 50 ^{o}C).

Although the extraction yields are higher when using heptane, other factors have to be taken into consideration. It would not be practical to switch from hexane to heptane as an extraction solvent. The cost of heptane is significantly higher than hexane and the relatively high boiling point of heptane means that more energy is required to remove it.[181] Therefore, in terms of costs and energy consumption, a switch to heptane would not be viable. The advantage of utilising $scCO_2$ is that no solvent residues remain so the product can be collected straight after extraction. Furthermore, as shown in the previous chapter, $scCO_2$ has the added advantage of being able to fractionate the wax into

different groups of compounds during the extraction process. Therefore, wax fractions of higher purity can be obtained straight after the extraction whereas more extensive purification steps have to be carried out with the solvent-extracted wax.

Finally, as will be shown in section 4.6 and as seen in the previous chapter, $scCO_2$ extraction has the added benefit of having a positive effect on the downstream processing of the biomass. The extraction of waxes should be part of a holistic biorefinery (as an added-value pre-treatment step) not stand-alone and therefore the extraction technique implemented should not have a negative effect on the next stage of biomass processing.

4.5 Optimisation of $scCO_2$ extraction of waxes from miscanthus

A 2x2 factorial experimental design was also carried out for the leaves of MG (as this gave rise to the highest yield of waxes).

Table 4-2 % Extraction yields obtained at different pressures and temperatures for MG leaves.

Experiment	Temperature (^oC)	Pressure (bar)	Extraction Yield (%)
1	35	80	0.43
2	65	80	0.014
3	50	240	1.27
4	50	240	1.28
5	50	240	1.25
6	35	400	1.32
7	50	325	1.70
8	65	400	1.74
9	50	350	1.96
10	50	350	1.93

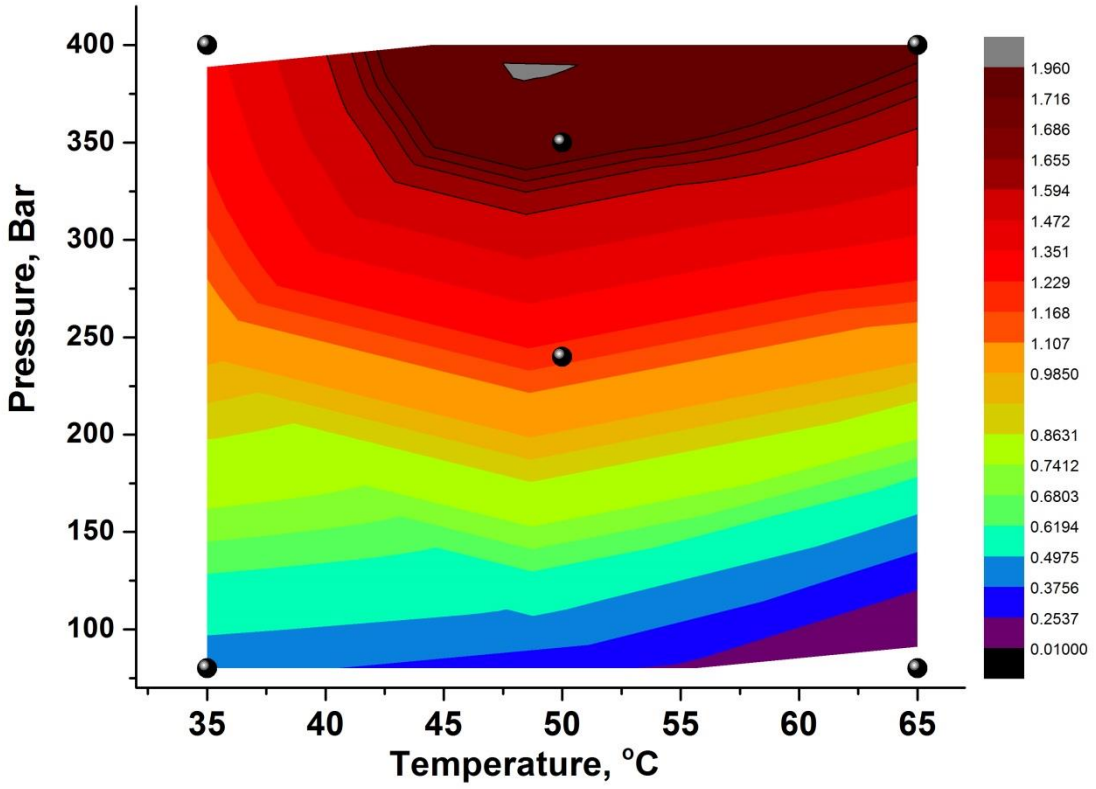


Figure 4-11 % crude yield of MG waxes from supercritical extraction.

The extraction yields for the different experiments are summarised in Table 4-2. These results have shown that the highest extraction yield was obtained when a pressure of 350 bar and temperature of 50 ^{o}C were implemented. The density of CO_2 at this temperature and pressure is higher than the extraction that took place at an elevated temperature and pressure (400 bar, 65 ^{o}C). Therefore, there is an optimal temperature and pressure which give a density of 0.899 g/cm^3, which result in the highest extraction yields of wax from MG leaves. Contrary to what was seen in the optimisation of the maize leaves, density appears to play a more important role in the extraction process than temperature. This suggests that there is a specific density of CO_2 that is essential for the extraction of waxes from MG. Very low yields of wax were extracted when carrying out the extractions at low pressure.

4.6 Saccharification of *Miscanthus x. giganteus*

In order to determine whether scCO_2 would be an effective pre-treatment step in a miscanthus biorefinery, it is important to see what effect the supercritical extraction has on the downstream processing of the biomass. Miscanthus, particularly *miscanthus x.*

giganteus, is widely seen as being a prime candidate as an energy resource for releasing high amounts of fermentable glucose for ethanol production as a result of its high cellulose content.[227] The process leading to ethanol production involves hydrolysing the cellulose into glucose followed by fermentation of the glucose into ethanol by yeast or bacteria. Acid hydrolysis, alkaline hydrolysis and enzyme degradation are standard techniques for breaking down the cellulose content.[228]

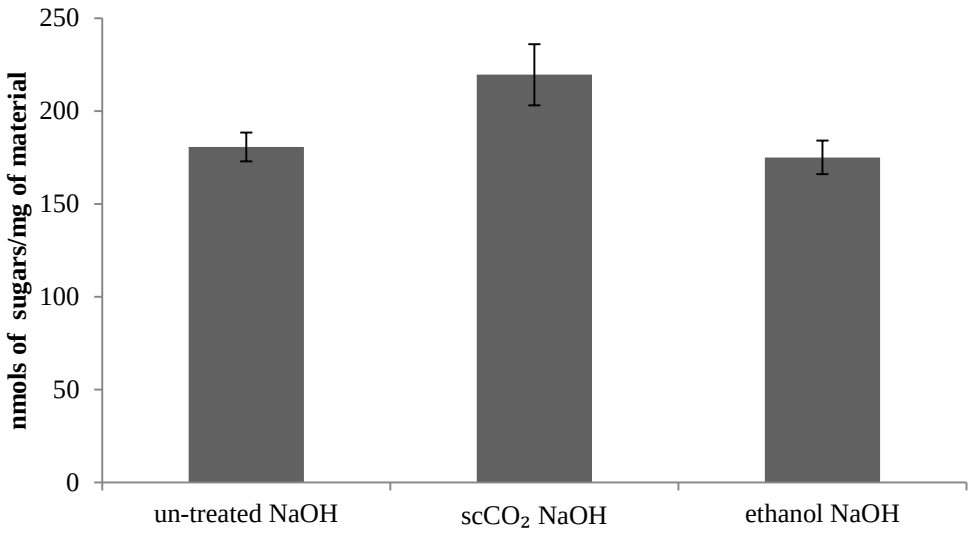


Figure 4-12 Different pre-extractions compared to optimise release for saccharification.

Saccharification of MG leaves was conducted in collaboration with Dr. Richard Gammons (University of York). Saccharification was carried out on the $scCO_2$ extracted MG leaves and compared to a conventional solvent (ethanol) and non-treated MG leaves. The optimised conditions (shown in section 4.5) were used in the $scCO_2$ extraction of the MG leaves, i.e. a pressure of 350 bar and 50 ^{o}C. Each sample of biomass was pre-treated with 0.1 M NaOH before enzyme hydrolysis to show sugar release.

Results, shown in Figure 4-12, indicate that the $scCO_2$ pre-treatment (wash) step improved the saccharification efficiency of the plant material significantly (220 nmols of sugars/mg of material) compared to none (181 nmols of sugars/mg of material) or ethanol wash (175 nmols of sugars/mg of material). Therefore, there is approximately 22% increase in sugar release after $scCO_2$-pretreatment compared to none and approximately a 26% increase in sugar release when compared to the ethanol wash. Therefore the results obtained here suggest that, although the % yields of wax extracted from $scCO_2$ may not be as high as other organic solvents (such as heptane), the effect

that $scCO_2$ extraction has on the downstream processing of the biomass is more beneficial than conventional solvent extraction.

As explained in the previous chapter ($scCO_2$ extraction as a pre-treatment step in a holistic maize stover biorefinery), $scCO_2$ has already been shown as an effective pre-treatment step for lignocellulosic biomass, enhancing sugar release.[221, 229] However, in all the previous studies, $scCO_2$ was run in static mode rather than dynamic mode meaning that no added-value products are obtained as a result of the pre-treatment process. The results obtained here are similar to the results obtained in the previous chapter for maize stover which further strengthens the idea that $scCO_2$ extraction (run in dynamic mode) is useful as the first step in a holistic biorefinery for extracting high-value waxes from the plant surface while improving saccharification efficiency for the production of biofuels.

It is important to fully demonstrate this method as an economically viable pre-treatment process in a miscanthus biorefinery. The next chapter will look into the economic costs associated with the supercritical extraction of waxes from miscanthus and maize stover biomass.

4.7 Conclusion

The extraction of waxes using $scCO_2$ from leaf and stem samples from two miscanthus species (MS and MG) has been carried out. The largest amount of wax was extracted from the leaves (1.96% for MG, 1.59% for MS) followed by the stems (0.38% for MG, 0.46% for MS). There were appreciable differences in the complex lipid mixtures of long-chain fatty acids, policosanols, fatty aldehydes, hydrocarbons, sterols and wax esters among other compounds between the two species. Significantly larger amounts of unsaturated fatty acids were found in the MS samples. Greater amounts of long-chain fatty aldehydes were present in the MG samples. Furthermore, there is also a difference in composition between the leaves and stems in of the same plant. The stem samples had waxes with considerably larger contents of phytosterols and wax esters when compared to the leaf samples. These compounds could be used in a wide variety of applications ranging from food and nutraceuticals to polishes, detergents and cosmetics. The difference in melting temperature of the extracts indicates that waxes from the two species could be utilised in different applications. Finally, $scCO_2$ extraction also demonstrated to be effective as a pre-treatment prior to saccharification of miscanthus,

enhancing the digestibility of the plant when compared to non-treated and ethanol-treated miscanthus.

Chapter 5

Economical assessment of the industrial-scale supercritical extraction of waxes from miscanthus and maize stover.

5 Chapter 5

5.1 Introduction

Several methods exist for the extraction of high-value molecules from natural matrices including conventional organic solvent extraction, hydrodistillation, low-pressure solvent extraction and maceration.[230] However, there has been significant focus on developing clean, 'greener' technologies as a result of public-health requirements and consumer demands.[230]

Supercritical fluid extraction (SFE) has been shown to be a technically viable process for the extraction of high-quality compounds from natural matrices due to its high solvation power, ability to produce extracts that are free of organic residues and high mass transfer rates at relatively low temperatures, preventing degradation of thermosensitive molecules.[230-233]

The high manufacturing costs associated with SFE, which are the result of high initial investments (associated with high pressure operation/equipment cost) used to be a major stumbling block preventing its use in industrial processes.[230, 231, 234, 235] However, there has been significant development of industrial scale units, leading to lower equipment costs associated with SFE processes.[235, 236] It is therefore necessary to look at the extraction of compounds by SFE from an economical perspective.

In previous chapters, the extraction of hydrophobic compounds from C4 plant waste (maize, miscanthus and sugarcane) and optimisation of the process using SFE have been investigated. However, this work cannot be developed further unless an economic evaluation of the manufacturing process is taken into account. Therefore this chapter aims to assess the cost of SFE of waxes from miscanthus and maize on an industrial scale using a methodology proposed by Turton *et al.*.[237] A number of assumptions need to be considered when using this methodology which will be highlighted when appropriate. This methodology has been employed in studies associated with SFE economics and has found to be an effective and appropriate method for evaluating costs of SFE processes.[230, 231, 233, 235, 238] To the author's knowledge, this method has not been employed to calculate costs of SFE of epicuticular waxes from plant matrices.

It should be stated that the supercritical extraction of waxes will be an initial pre-treatment step as part of a biorefinery plant and therefore some costs will not be solely attributed to the SFE extraction but to the biorefinery as a whole.

5.2 Estimation of the costs of supercritical extraction of waxes from miscanthus biomass

5.2.1 Cost of Manufacturing (COM)

When calculating manufacturing costs of a chemical product (such as extractives), three main types of expenses are involved:

(i) **Direct Costs (DC)**: Direct costs deal with the operational costs. They are dependent on the production (manufacturing) rate and include raw material costs, operational labour, utilities among others.

(ii) **Fixed costs (FC)**: These are not dependent on production rate and include territorial taxes, insurance, depreciation and so on. They are charged at constant rates even when the plant is not operational.

(iii) **General expenses (GE):** The general expenses cover business maintenance and consist of management, administrative sales, research and development costs etc.[237]

$$COM = DC + FC + GE$$

Therefore, the cost of manufacturing (COM) is the sum of direct costs, fixed costs and general expenses. These three components of the COM are estimated in terms of five main costs:

1) **Fixed capital investment (FCI)**
2) **Cost of operational labour (C_{OL})**
3) **Cost of utilities (C_{UT})**
4) **Cost of waste treatment (C_{WT})**
5) **Cost of raw materials (C_{RM})**

Table 5-1 Data given to estimate individual cost items.[237]

Cost Item	Typical Range of Multiplying Factors	Value used in Text
1) Direct Costs		
A. Raw Materials	C_{RM}	
B. Waste treatment	C_{WT}	
C. Utilities	C_{UT}	

D. Operating labour	C_{OL}	
E. Direct supervisory and clerical labour	$(0.1 – 0.25)C_{OL}$	$0.18C_{OL}$
F. Maintenance and repairs	(0.02 – 0.1)FCI	0.06FCI
G. Operating supplies	(0.1 – 0.2)Line1.F	0.009FCI
H. Laboratory charges	$(0.1 – 0.2)C_{OL}$	$0.15\ C_{OL}$
I. Patents and royalties	$(0 – 0.06)C_{OM}$	$0.03C_{OM}$
Total Direct Costs	$\mathbf{C_{RM} + C_{WT} + C_{UT} + 1.33C_{OL} + 0.03COM + 0.069FCI}$	
2) Fixed Costs		
A. Depreciation	0.1FCI	0.1FCI
B. Local taxes and insurance	(0.014 – 0.05)FCI	0.032FCI
C. Plant overhead costs	(0.5 – 0.7)(Line 1.D. + Line 1.E + Line 1.F)	$0.708C_{OL}$ + 0.036FCI
Total Fixed Costs	$\mathbf{0.708C_{OL} + 0.068FCI + depreciation}$	
3) General Expenses		
A. Administration costs	(0.15)(Line 1.D. + Line 1.E + Line 1.F)	$0.177C_{OL}$ + 0.009FCI
B. Distribution and selling costs	(0.02 – 0.2)COM	0.11COM
C. Research and development	0.05COM	0.05COM
Total General Expenses	$\mathbf{0.177C_{OL} + 0.009FCI + 0.16COM}$	
Total Costs (COM) : $\mathbf{C_{RM} + C_{WT} + C_{UT} + 2.215\ C_{OL} + 0.190COM + 0.146FCI +depreciation}$		

Other individual items can be calculated using equations that are displayed in Table 5-1. There is a typical range for constants (multiplication factors) for each equation, which are needed to estimate each of these individual costs. The mid-point value for each range is used for assessing the costs where no available information is given.

When utilising the midpoint values shown in Table 5-1, three equations can be generated for each category:

$$DC = C_{RM} + C_{WT} + C_{UT} + 1.33C_{OL} + 0.069FCI + 0.03COM$$

$$FC = 0.708C_{OL} + 0.068FCI + depreciation$$

$$GE = 0.177C_{OL} + 0.009FCI + 0.16COM$$

Addition of these categories and solving for COM gives the total COM such that:

$$COM = 0.180FCI + 2.73C_{OL} + 1.23(C_{RM} + C_{WT} + C_{UT})$$

The above equation is the COM without depreciation. In order to calculate the COM with depreciation the final equation is:

$$COM = 0.280FCI + 2.73C_{OL} + 1.23(C_{RM} + C_{WT} + C_{UT})$$

1.3.1.1 Fixed capital investment FCI

A typical industrial supercritical extraction unit, which is used in the extraction of spices, natural pigments, nutraceuticals etc., is composed of two 0.4 m^3 extractors, a series of flash tanks (for fractionation), a CO_2 reservoir, a CO_2 pump (for compression of the solvent) and a CO_2 heater. The cost of the industrial scale unit is around **€ 1,400,000**.[235, 238] On a yearly basis, the fraction of investment is calculated by multiplying the total investment by the depreciation rate.

The depreciation rate is assumed to be 10% per year and is used in the calculation of the COM. Another part of the investment is the initial quantity of CO_2 that is required to fill the CO_2 reservoir; however this cost is generally negligible when comparing it to the extraction unit cost.

5.2.1.1 Operational labour costs (C_{OL})

In terms of man-hour per operation-hour, the total $\mathbf{C_{OL}}$ is estimated by using tables which are presented by Ulrich (1984). The total time when the extraction columns are under operation was taken to be 330 days per year of continuous 24 hour per day shift which corresponds to 7920 h of continuous extraction. It is assumed that, in the

industrial SFE unit there will be two operators per shift and the **C_{OL}** was taken to be **€ 3.00/h**. This value is solely attributed to the work that the operators will carry out on the SFE of waxes. The operators will have other duties within the biorefinery and their overall wage would therefore be higher.

5.2.1.2 Raw Material Costs (C_{RM})

When looking at the raw material costs for SFE, all the materials directly related to the production must be taken into account. These include: (i) **The solid substrate** containing the solute to be extracted (ii) **Carbon dioxide** that is lost in the extraction process.

The cost of the former includes the price of the biomass itself as well as all the cost of all the pre-processing steps leading to the final biomass product used in the extraction such as drying, comminution and cleaning.

An extensive study by Smeets *et al.* analysed and projected the economic performance of miscanthus production and supply chain in the European Union for the years 2004 and 2030.[239] They investigated five regions in five countries (Figure 2) which are promising producers of miscanthus biomass: Poland – Lubelski, Hungary – Del Dunantal, United Kingdom – Devon, Italy – Lombardia and Lithuania.[239]

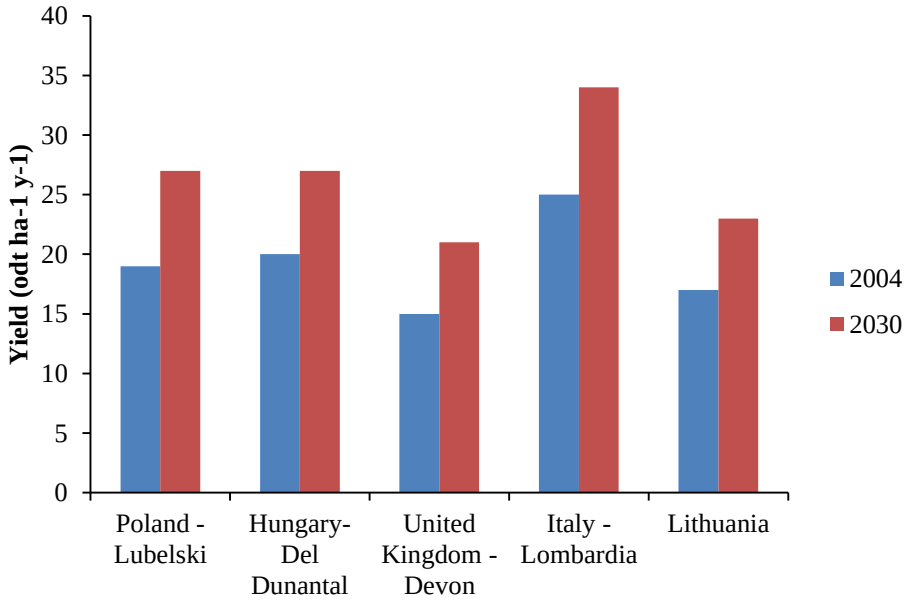


Figure 5-1 Yield of miscanthus in five regions in the years 2004 and 2030.[239]

When looking at product cost of materials for miscanthus the following have to be considered:

(i) Land rent

(ii) Rhizome costs

(iii) Agricultural machinery

(iv) Weeding

(v) Fertilising

(vi) Harvesting

(vii) Disease and insect control

(viii) Storing

(ix) Milling/Pelletising[239]

5.2.1.2.1 Agricultural Machinery

Agricultural machinery costs can be split into capital, fuel, repair and maintenance, labour, lubrication, storage and insurance.[239] Table 5-2 summarises the costs involved. The data displayed assumes a typical 50 ha farm, where 10 – 20% of the land is used to cultivate perennial grasses (the rest is used for conventional agricultural crops). This 10–20% estimation is used in accordance with the agricultural land availability for production of energy crops projected for the year 2030.[239] Labour and diesel prices (in € h^{-1}, including taxes and social security expenditures) are taken from the Eurostat database in the year 2004.[240] It can be assumed that the price of diesel doubles in certain countries between the years 2004 and 2030.[239]

Figure 5-2 illustrates the cost of labour and diesel for crop production in 2004 and predicted costs for 2030 (€ h^{-1}).

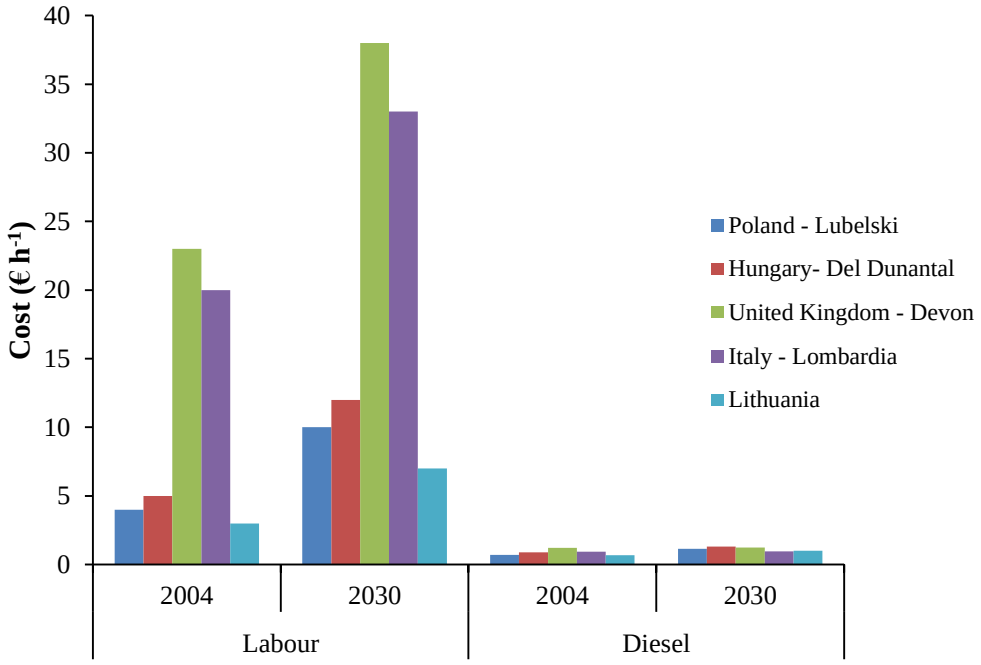


Figure 5-2 Cost of labour and diesel for production of miscanthus.[239]

5.2.1.2.2 Land Renting

Table 5-3 indicates the cost of land rent for 2004 and predicted values for 2030 (€ ha^{-1} y^{-1}). It is assumed that there is a proportional increase of cropland cost with crop value produced from the land. [241]

Table 5-2 Cost of land rent for 2004 and predicted values for 2030. [239, 241]

Country	**Land (€ ha^{-1} y^{-1})**	
	2004	**2030**
Poland - Lubelski	50	63
United Kingdom - Devon	201	253
Hungary Del - Dunantal	45	57
Italy - Lombardia	232	292
Lithuania	10	13

Table 5-3 Data on agricultural machinery costs for miscanthus production.[239, 242-244]

	Purchase Price (k€)	Use	Depreciation	Repair coefficients		Lubrication ($1h^{-1}$)	Fuel use ($1\ h^{-1}$)	Insurance (€ h^{-1})	Storage ((€ h^{-1})	General Costs (€ h^{-1})
				RF 1	RF2					
Tractor 60KW	53	800	12	0.003	2	0.06	13	0.3	1.2	2.0
Tractor 75KW	72	800	12	0.003	2	0.07	17	0.4	1.6	2.7
Tractor 100KW	75	800	12	0.003	2	0.08	22	0.5	1.6	2.8
Power harrower	11	70	20	0.230	1.4	n/a	n/a	0.8	2.9	4.9
Plough	27	70	20	0.290	1.8	n/a	n/a	1.9	6.7	11.6
Roll	13	70	20	0.160	1.3	n/a	n/a	0.9	3.2	5.4
Drill	16	70	20	0.320	2.1	n/a	n/a	1.1	3.9	6.7
Rhizomes planter	18	150	10	0.320	2.1	n/a	n/a	0.6	2.1	3.5
Weed cultivator	5.3	100	10	0.23	1.4	n/a	n/a	0.3	0.9	1.6
Fertiliser- spreader	4.4	50	10	0.63	1.3	n/a	n/a	0.4	1.5	2.6
Sprayer	14	100	10	0.41	1.3	n/a	n/a	0.7	2.5	4.3

Mounted big- baler	123	400	5	0	5.4	9.2	1.5	103	10	1.08
Mower	12	70	20	0.18	1.6	n/a	n/a	0.9	3	5.2
Self-propelled chopper	185	500	5	0.03	2	0.07	18	1.8	6.5	11.1
Trailer	25	250	12	0.19	1.3	n/a	n/a	0.5	1.7	3.0
Fork	3	250	12	0.007	2	n/a	n/a	0.1	0.2	0.4
Rotary cultivator	10	40	12	0.27	1.4	n/a	n/a	1.3	4.5	7.8

5.2.1.2.3 Rhizome costs

Miscanthus is produced by vegetative propagation from rhizomes. In literature there is a variation in the cost of miscanthus rhizomes from **0.04 €** to more than **0.45 €.**[242, 245] 20000 stems per hectare are needed for the establishment. In 2004, it was estimated that **0.16 €** per rhizome was required. It is estimated that this cost will decrease to **0.08 €** per rhizome by 2030.[239]

5.2.1.2.4 Herbicide application (Weeding)

Herbicide application to control weeds is also required in the cultivation of miscanthus. General costs for herbicide application (taken from literature) are shown in Table 5-4.[239]

Table 5-4 Costs of herbicide application.[239]

Herbicide	**Application rate (kg ha^{-1})**	**Cost (€ kg^{-1})**	**Overall Cost (€ ha^{-1})**
Glycophosphate	2.5	6.2	16
Bromosynil/ioxynil/fluoxypyr	2.0	32	64
Trifloex-tra	7.7	7.6	59

5.2.1.2.5 Storage

There are several methods to store miscanthus. On average miscanthus is stored on the farm for 6 months. The available methods of storage include:

(i) Storing it without cover in open air.
(ii) Covering it with plastic sheeting and storing it in open air.
(iii) Covering it with organic material and storing in open air.
(iv) Using existing farm buildings to store it.
(v) Using new farm buildings to store it.[246]

The most frequently used and cheapest method to store miscanthus is the second option, i.e. using plastic sheeting.[243, 246] The first option is not ideal due to the loss of biomass as a result of decay.[246] Storage costs are summarised for chopped miscanthus and summarised in Table 5-5.

Table 5-5 Cost for plastic covering and use of tractors (75 KW), labour and diesel fuel for chopped miscanthus in euros.[239]

Biomass	Plastic Sheeting (cover) €	Diesel consumption €	Use of tractor (75 KW) and labour €	Dry matter loss (%) €
Chopped miscanthus	1.6	1.5	0.11	2.0

5.2.1.2.6 Pelletising

According to Smeets *et al.*, the cost for pelletising miscanthus was **26 – 29 € odt^{-1}** (oven dried tonne) in 2004 and **31 – 36 € odt^{-1}** in 2030. However pelletising data is very limited and there are wide variations in results. Pelletising costs are sensitive to assumptions that have been made on electricity and natural gas prices for the process as well as moisture content of the biomass.[239]

5.2.1.2.7 Transportation

It is assumed that the harvested miscanthus will travel a maximum of 100 km. This is adequate for the majority of biomass processing facilities if (i) 15% of agricultural land is used for miscanthus cultivation (ii) the miscanthus originates from a circular production area around the processing facility. For the five regions mentioned above the transportation costs were calculated to be around **13 – 20 € odt^{-1}** in 2004. Costs are higher for the UK and Italy when compared to the other three regions due to high labour costs. It is estimated that the transportation costs increase by 8 – 31% between 2004 and 2030 as a result of increased fuel costs and labour costs.[239]

5.2.1.2.8 Overall C_{RM}

When looking at all of the costs mentioned above, Smeets *et al.* calculated that the production, storage and transportation costs (100 km) for miscanthus in 2004 for the five countries varied from **55 – 106 € odt^{-1}**. It is estimated that miscanthus cost will decrease between 2004 and 2030 to **47 – 99 € odt^{-1}** as a result of improved technology leading to higher yields and decreased planting costs.[239] The reason for this variation in costs is the difference in land rental, labour costs and fuel costs amongst the five different countries. For this study the values estimated for the year 2004 will be used (since the values between 2004 and 2030 do not vary significantly) and since a range of

55 – 106 € odt^{-1} was calculated, the mid-point between this range was selected, i.e. it is assumed in this study that the cost of raw material C_{RM} is **€ 80.5 per odt^{-1} (80.5 ±25.5 per odt^{-1}).**

5.2.1.3 Cost of waste (C_{WT})

Since in an industrial SFE unit, the CO_2 is recycled, the only waste that is involved in the process is CO_2 which leaks from the system and the exhausted solid. The former is negligible while the exhausted miscanthus and maize biomass can be utilised further downstream as part of a biorefinery process (or incorporated back into the soil for the uptake of nutrients) and therefore it can be assumed that little or no waste is generated during the extraction process. Therefore the C_{WT} can be ignored (or has a value of € 0).

5.2.1.4 Cost of utilities (C_{UT})

A number of factors need to be taken into consideration when looking at utility costs namely: (i) Costs associated with the electric power used in the CO_2 pump. (ii) Costs associated with the CO_2 heater. (iii) Costs associated with refrigeration.

5.2.1.4.1 Costs associated with the electric power used in the CO_2 pump

In order to calculate electric power costs for the CO_2 pump it is important to determine: (i) The pressure and temperature applied during the extraction process (ii) The extraction time. From data obtained previously from the optimisation of miscanthus wax extraction using $scCO_2$, it was found that the highest yields were obtained using a pressure of **350 bar** and **50 ^{o}C**.

Therefore these conditions would be implemented during the extraction of waxes from miscanthus. The pressure and temperature utilised in the extraction process are required in order to give the specific enthalpy, from which the total energy used in the extraction process can be obtained by multiplying the variation of specific enthalpy by the extraction time and the CO_2 mass flow rate. In the case of miscanthus, the specific enthalpy of carbon dioxide using a pressure of 350 bar and 50 ^{o}C is **287.70 kJ/kg**.

Furthermore, apart from the data shown above, it is also important to know the extraction time, the amount of wax that can be extracted from the biomass during this time as well as kinetic behaviour of the extraction process of miscanthus.

Supercritical extraction (SFE) kinetics were carried out on miscanthus using a laboratory-scale supercritical unit. Therefore another assumption made is that the performance of the industrial scale unit should be the same or very similar to that of the laboratory supercritical unit. It is assumed that this should not be a problem if the bed

density, particle size and the ratio between the mass of the solid and the CO_2 flow rate are kept constant.

The supercritical extraction of miscanthus was carried using a 500 ml extractor containing 107.70 g of milled miscanthus biomass. The CO_2 flow rate was 40 g/min (or **6.7×10^{-4} kg/s)** and the extraction was carried out for 4 hours, collecting samples at specific time intervals in order to look at the extraction kinetics. Figure 5-3 illustrates the extraction curve obtained for the extraction of wax from miscanthus.

When SFE is carried out on solid substratum, the system (consisting of biomass and CO_2) can be seen as constituting three components:

(i) The solvent
(ii) The desired solute
(iii) The cellulosic structure.

Although the cellulosic structure is totally inert to the supercritical solvent, there is a strong interaction between the cellulosic structure and the extract (desired solute). In this particular instance, the extract is the epicuticular wax consisting of a wide range of hydrophobic organic compounds.

Typically, there are three scenarios in an SFE process that could exist. The first is a system consisting solely of the CO_2 solvent and the pure component solute. The second scenario is when, instead of a pure component, the solute consists of a multi-component mixture such as epicuticular waxes found in plant biomass. This is referred to as a pseudo-binary system. The third scenario is when the system comprises of the CO_2 solvent, the multi-component mixture (solute) and the inert cellulosic structure, which can loosely be referred to as a pseudo-ternary system.

Typically, in an SFE process there are three linear regions in the extraction curve profiles (Figure 5-3). The first line is referred to as the constant extraction rate (CER) which corresponds to the extraction of solute molecules that are easily accessible and therefore convection in the solvent film surrounding the biomass particles dominates the mass transport. The second line represents the falling rate period (FER), and in this instance both convection and diffusion effects play a role in mass transport. The third line corresponds to a process that is entirely diffusion-controlled, *i.e.* when all of the easily accessible solute has been extracted and mass transfer is solely reliant on the diffusion of particles from inside of the biomass and as such extraction rates are

typically low. Figure 5-3 illustrates the results obtained when extracting waxes from miscanthus, with the three regions highlighted.

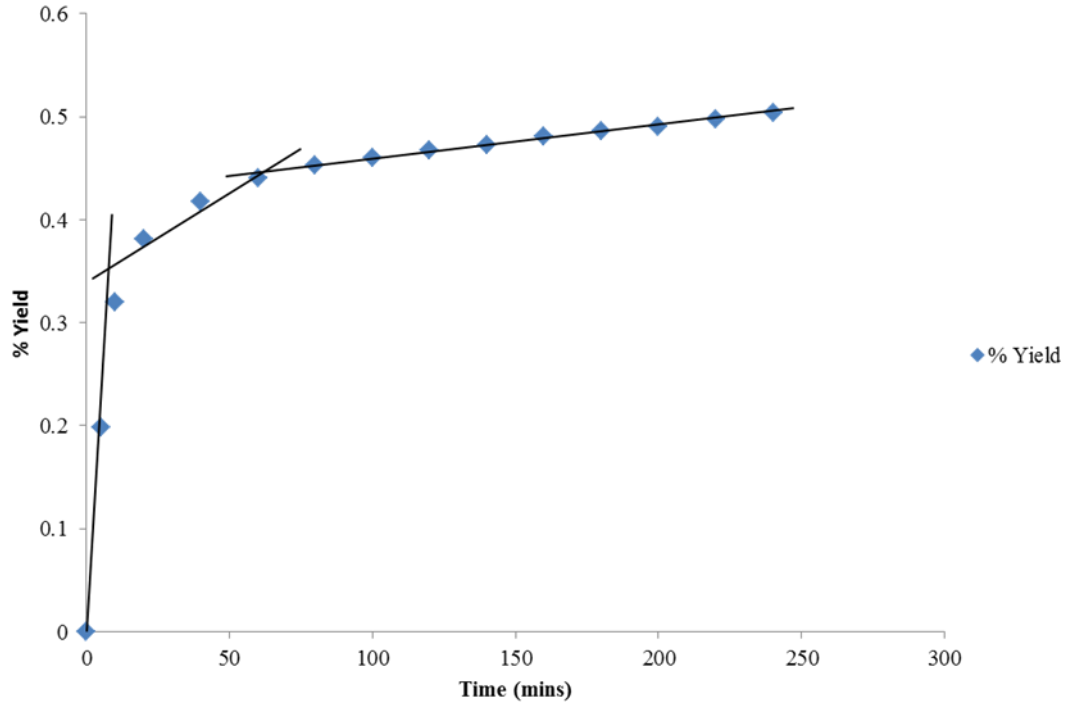


Figure 5-3 Extraction curve for miscanthus showing the three regions; CER, FER and diffusion-controlled region.

Since maximum extraction rates are normally observed in the CER region it is necessary, from an economical perspective, to identify this region for extraction of solutes from miscanthus. It was found that, when using a flow rate of 40 g/min, over 50% of the total wax extracted after 4 hours was obtained within the first 10 minutes of the extraction. This illustrates that there is no need for long-extraction times which highlights the advantage of $scCO_2$ over conventional organic solvents which often require much longer extraction times.[52] 40 minute extractions give approximately 83% of the total wax yield that can be extracted from miscanthus while 1 hour gives around 87%. When analysing the cost of raw materials and total wax which could be extracted per day, it was found that it is more profitable to carry out 40 minute extractions (giving a higher overall wax yield per day and reducing the overall costs significantly) when compared to 1 hour extractions.

The experimental bed density of the miscanthus biomass was found to be **0.32 g/cm^3**. Therefore it was calculated that for each extraction on the industrial scale unit, **128.4 kg** of miscanthus biomass can be loaded into the extractor.

Since the CO_2 flow rate in the laboratory-scale extraction was 6.7 x 10^{-4} kg/s, the CO_2 mass flow rate required for the industrial-scale unit would be approximately **2875.1**

kg/hr or **22.35 kg CO_2 kg^{-1} misc/h^{-1}**. The cost of electricity was assumed to be €0.112/kwh.[247] As such, the costs associated with the pumping CO_2 can be calculated as follows;

$$Pure\ CO_2\ enthalpy\ at\ 350\ bar, 50^oC = 287.70\ kJ/kg\ CO_2$$

$$1\ kg\ of\ CO_2 = 287.70\ kJ\ CO_2$$

$$2875.1\ kg\ of\ CO_2 = 827{,}166.27\ kJ\ CO_2$$

$$P_{(kw)} = {}^{E_{(kJ)}}/_{t_s}$$

$$P_{(kw)} = {}^{827166.7}/_{3600} = 229.768\ Kwh$$

$$Cost\ of\ electricity\ (UK) = €0.112\ per\ Kwh$$

$$Cost\ of\ electricity\ = 229.768\ Kwh\ \times 0.112\ €Kwh^{-1}\ =\ €25.73/h$$

The costs associated with the CO_2 pump were calculated to be **€25.73/h**.

5.2.1.4.2 Costs associated with the CO_2 heater

In order to look at the costs associated with the CO_2 heater, the energy associated with the heating process needs to be estimated. It is assumed that the heaters have 50% efficiency. This was done using the following equation:

$$Q = MC_p\Delta T$$

Where Q is the energy needed, M is the mass of CO_2, Cp is the specific heat capacity of CO_2 and ΔT is the change in temperature. The CO_2 has to be heated from 4 ^oC (temperature in the pumps) to 50 ^oC. The mass of CO_2 used per hour is **2875.1 kg,** the C_p at 50 ^oC is **0.871 kJ kg^{-1} k^{-1}** and the ΔT is 46 ^oC.

$$Q = MC_p\Delta T$$

$$Q = 2875\ \times 0.871 \times 46$$

$$Q = 115000\ KJ$$

$$Assume\ 50\%\ efficiency$$

$$Q = 230{,}000\ KJ\ per\ hour\ (230\ MJ)$$

Therefore, the energy that is required is **230 MJ per hour**. This energy can be obtained by burning the residual miscanthus biomass post extraction. The energy that is given off when burning dry miscanthus is **17 MJ kg^{-1}**, thus the system requires the burning of

13.5 kg per hour or 10% of the biomass extracted. As such, the costs associated with heating the extractors are negligible.

5.2.1.4.3 Costs associated with refrigeration

A typical refrigeration cycle comprises of a working fluid circulated around a loop consisting of a compressor, evaporator, expansion valve or turbine and condenser. Refrigeration is more expensive than heating since it requires electrical power. The water has to be cooled from 20 ^{o}C (around room temperature) to 4 ^{o}C. In order to determine the refrigeration costs the energy required for refrigeration must be determined by calculating the coefficient of performance, COP.

Using the same equation used to calculate the energy required for heating:

$$Q = MC_p\Delta T$$

$$Q = 2875 \times 0.846 \times 16$$

$$Q = 38916\ kJ$$

$$COP\ 20^oC = 0.08$$

$$COP\ 4^oC = 0.15$$

$$Q = 38916\ kJ \times \frac{0.15}{0.08} = 72967.5$$

$$P = \frac{72967.5}{3600} = 20.27\ Kwh^{-1}$$

$$= 20.27\ Kwh^{-1} \times 0.112\ €Kwh^{-1} = €2.27/h$$

The costs associated with refrigeration are **€2.27 per hour of extraction**, therefore it is assumed that the total utility costs, C_{UT} are **€28 per hour.**

5.2.1.5 Total COM calculation

COM for the supercritical extraction of waxes from miscanthus can be calculated with the full equation:

$$COM = 0.280FCI + 2.73C_{OL} + 1.23(C_{RM} + C_{WT} + C_{UT})$$

$$COM = 0.280(1{,}400{,}000) + 2.73(47{,}250) + 1.23(122839.2 + 0 + 221786.2)$$

$$COM = 392000 + 129729.6 + 423889.2$$

$$COM = \frac{€945618.8/year}{1525.37\ tonne/year}$$

$$\boldsymbol{COM = €620/tonne\ of\ miscanthus}$$

$$\boldsymbol{COM = €148/kg\ of\ miscanthus\ wax}$$

The final COM has been calculated to be **€620 per tonne of miscanthus biomass** or **€148 per kg of wax.**

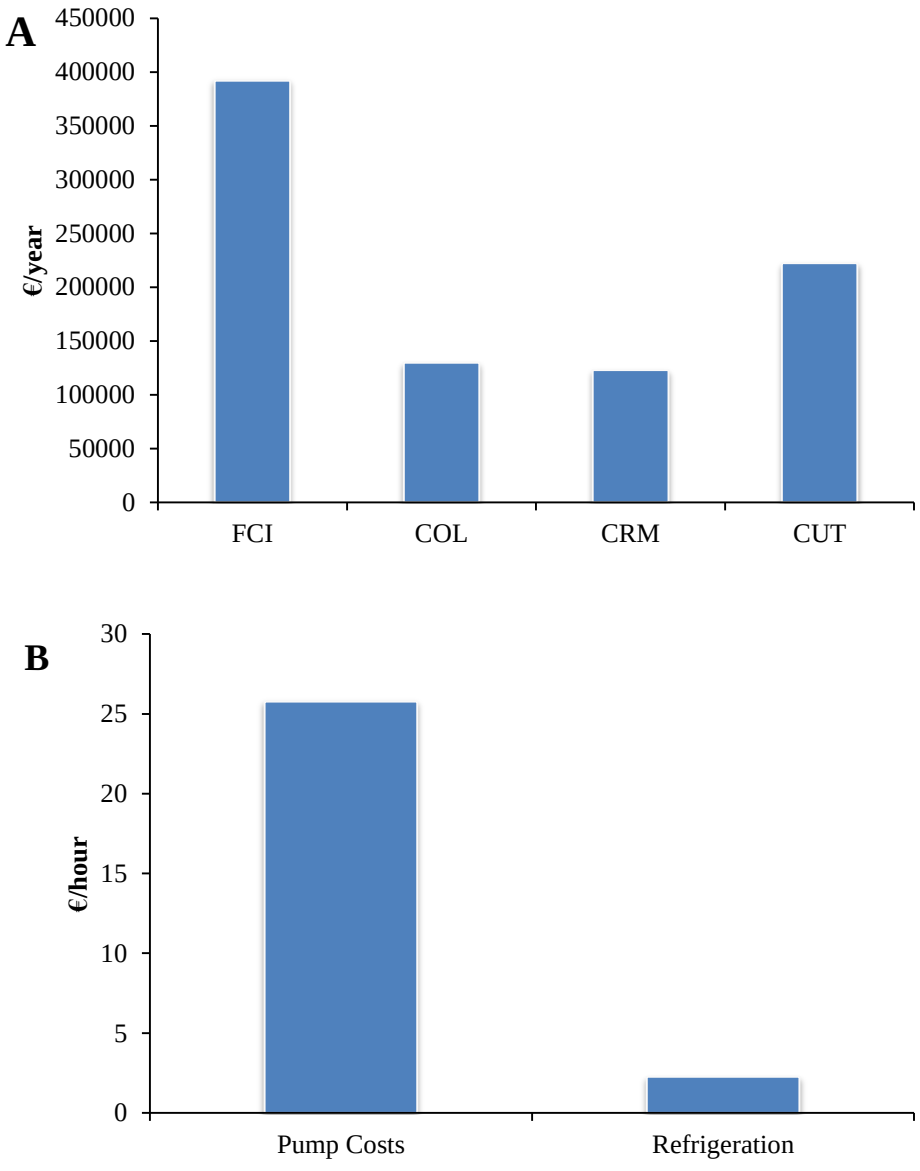


Figure 5-4 A) Distribution of costs B) Distribution of utility costs.

As can be seen from Figure 5-4, the main contributors to the COM are the fixed capital investment and the utility costs. The main cost for the C_{UT} is the electricity that is

required to pump the CO_2 at the required pressure and temperature, as shown in Figure 5-4 B. Raw material costs and labour costs contribute less to the COM.

This value is only an estimate and is based on a number of assumptions, as previously discussed. The costs can be improved by varying some of the parameters. First of all the figure for the amount of biomass that can be loaded into the supercritical extractor was based on milled biomass. In industry, biomass is normally received as pellets (pelletised) and this increases the biomass loading by threefold. This would result in the C_{RM} increasing by threefold. The C_{UT} would also increase as the CO_2 pumped through increases by threefold as well. The equation is therefore modified as follows:

$$COM = 0.280(1{,}400{,}000) + 2.73(47{,}250) + 1.23(368517.6 + 0 + 665358.3)$$

$$COM = 392000 + 129729.6 + 1271667.36$$

$$COM = \frac{€1793396.96/year}{4576.11\ tonne/year}$$

COM = €391.9/tonne of miscanthus

COM = €93.52/kg of miscanthus wax

Although the raw material costs and utility costs increase, if pelletised miscanthus was taken into account the total COM would be reduced to around **€391.9/tonne of biomass or €93.52/kg of miscanthus wax.** Furthermore, in this study miscanthus straw was used in the process, yielding 0.5% wax. Miscanthus leaves have a greater wax content than straw and are considered to be a waste resource. If the miscanthus leaves were used, then the wax yield is almost 4 times as much (1.96%) compared to that of the straw.

$$COM = 0.280(1{,}400{,}000) + 2.73(47{,}250) + 1.23(368517.6 + 0 + 630511.2)$$

$$COM = 392000 + 129729.6 + 1228805.42$$

$$COM = \frac{€1793396.96/year}{89693.05\ kg\ wax/year}$$

COM = €19.99/kg of miscanthus wax

This would lead to more wax extracted per run resulting in a much lower COM, of around **€19.99/ kg of wax.**

Finally, it was assumed in the calculations that the cost of raw materials (C_{RM}) is solely for the supercritical extraction. As stated previously, the supercritical extraction is only the first step (pre-treatment step) in a biorefinery and thus the biomass will be passed on within the biorefinery for further processing. The cost of raw materials must also be

shared throughout the entire processes within the biorefinery. The C_{RM} value would therefore be lower leading to lower overall manufacturing costs (COM).

5.2.1.5.1 Utilisation of miscanthus biomass for electricity generation

As stated above, since the supercritical extraction of waxes would be carried out as part of a biorefinery set-up, the miscanthus biomass collected after the extraction would be passed on to the next stage of the biorefinery process (electricity generation) and hence further lower the COM of the wax. Herein, cost estimations for electricity generation were carried out.

Different technologies have different energy conversion efficiencies from biomass. However, intense development is occurring within this area and a number of highly efficient technologies are emerging. The greatest efficiency was found to be 43%,[248] while the average of all available technologies is 27%.[249] Therefore two calculations were carried out: one based on the technology with the greatest efficiency while the other based on the average efficiency of all technologies.

1. **Calculation assuming use of most efficient technology (combustion)**

$$Mass\ of\ milled\ miscanthus\ per\ extraction\ (40\ mins): 128.4\ kg$$

$$Mass\ of\ pelletised\ miscanthus\ per\ extraction: 128.4 \times 3 = 385.2\ kg$$

$$Mass\ of\ wax\ extracted\ (assuming\ 1.96\%\ yield): 385.2 \times 0.0196 = 7.55\ kg$$

Therefore miscanthus biomass after each extraction:

$$385.2\ kg - 7.55\ kg = 377.65\ kg\ of\ miscanthus$$

When heating the extractors, 40.67 kg of miscanthus is required for each extraction (pelletised miscanthus requires more CO_2 to flow through and therefore three times the amount of biomass to cover costs of heating CO_2 pumps):

$$377.65\ kg - 40.67\ kg = 336.98\ kg\ of\ available\ miscanthus\ per\ extraction$$

Energy of combustion for miscanthus:

$$1\ kg\ of\ miscanthus = 17\ MJ$$

$$17\ MJ \times 336.98 = 5728.66\ MJ/extraction\ (40\ mins)$$

$$= 8592.99\ MJ/hour$$

Assuming 43% efficiency:

$$P_{(kw)} = {}^{E_{(kJ)}}/_{t_s}$$

$$Assume\ 43\%\ efficiency:$$

$$P_{(kw)} = \frac{(8592990)}{3600} \times 0.43 = 1026.38\ Kwh$$

$$Cost\ of\ electricity\ (UK) = €0.112\ per\ Kwh$$

$$Value\ of\ electricity\ generated\ per\ extraction$$
$$= 1026.38\ Kwh \times 0.112\ €\ Kwh^{-1} = €114.95$$

$$Value\ of\ electricity\ generated\ per\ tonne\ of\ miscanthus = €114.95 \times \frac{1000}{385.2}$$

$$= €298.4\ per\ tonne\ of\ miscanthus$$

$$= €15.23\ per\ kg\ of\ wax$$

Therefore when subtracted from the cost of wax production:

$$Total\ COM\ cost = 19.99 - 15.23 = €4.76\ per\ kg\ of\ wax$$

2. Calculation assuming average efficiency of all technologies

When carrying out the same calculation above using the average efficiency of all technologies (27% efficiency), the total COM per kg of wax was found to be €10.47 per kg of wax. Therefore if the biomass was to be utilised after the extraction for electricity production, the COM of the miscanthus wax would decrease to around **€4.77 per kg of wax** if the best available technology was utilised (43% efficiency) and **€10.87 per kg of wax** when taking the average energy efficiency of all available technologies (27%).

5.3 Estimation of the Supercritical extraction costs of wax from Maize Stover

The same model was also used in order to estimate the costs of supercritical extraction from maize stover since there is also potential to utilise this biomass within an integrated biorefinery. Since the values for FCI, C_{WT} and C_{OL} are the same as those that were utilised in the miscanthus calculation; they will not be mentioned again in this section.

5.3.1 Cost of Manufacturing (COM)

5.3.1.1 C_{RM}

Since maize is grown primarily for food production (production of grain), the raw material costs for growing and harvesting maize are irrelevant and are not considered in this study. Since wax extraction from maize comes from the waste following harvesting of the grain, *i.e.* from the corn stover (stalk, leaves, cob and husk tissues), the C_{RM} in this study focuses on the costs of harvesting and supplying corn stover to biorefineries.

Since maize stover has significant promise for the production of bioenergy, studies have been carried out on costs of corn stover. Studies have shown that under no till-conditions, two-thirds of the stover in certain maize-growing regions can be harvested on a sustainable basis without affecting the soil adversely.[43-45] In the United States alone, there is a significant amount of maize stover (as a by-product of maize production) with approximately 68,000,000 tonnes produced every year.[250] The harvesting of maize stover occurs once all the corn grain is harvested and is usually stored as round or square bales.[251] Typical problems during stover harvesting include a small harvesting window, weather disruptions, slow drying of fields, a low harvest efficiency and contamination of soil.[251] Besides the costs for harvesting (which involves equipment costs, fuel costs, labour costs and baling/chopping/pelletising of biomass) and storage, farmers also expect compensation costs in order to generate a profit as well as replace the nutrients lost when the stover is removed.[250, 252, 253]

It is challenging to estimate an appropriate C_{RM} for stover as an extensive literature search showed a large variation in the stover $C_{RM.}$ Perlack *et al*. estimated costs of $43.10–51.60/dry tonne which involved stover collection, packaging into bales using conventional baling equipment, storage and transport (trucks and flatbed trailers or tractors and bale wagons).[252] Farmer compensation was estimated to be around $10 per dry tonne.

Graham *et al.* assumed that around 30% of the stover is collected from fields at a price of around $33 per dry tonne of stover taking into account costs for nutrient replacement. The widely cited study by Humbird *et al.* estimated stover prices of $58.50/tonne of stover which involved $23.50/tonne to cover farmer costs and $35/tonne of stover to cover all the harvesting, processing, storage and transportation costs (from the farm to the plant). [253]

Sokhansanj *et al*. estimated stover prices based on different stover packaging formats-baled, chopped and pelletised. They calculated harvesting, storage and transportation

costs of around $73/dry tonne for baled stover, $84/dry tonne for chopped stover and $86/dry tonne for pelletised stover.[254]

A recent study carried out by Thompson *et al.* looked in to the harvesting and supplying costs of corn stover at varying stover prices. They estimated stover supply costs between $82.19-$100.56 per dry tonne of biomass.[250] They concluded that 33% removal of corn stover at a price of $88.14 per odt^{-1} would cover all costs as well as pay compensation to the farmers. Raw material costs included equipment costs, labour costs, fuel costs, replacement of nutrients and farmer compensation. They found that the largest contributor to the raw material costs was found to be nutrient replacement.[250]

Table 5-6 Estimates of maize stover costs found in literature.[43, 250-264]

Study	Cost of stover
Perlack *et al.*, 2003[252]	$43.10 - $56.10/dry metric tonne (Mid-point $49.60)
Eggeman *et al.*, 2005[255]	$35/dry metric tonne
Graham *et al.*, 2007[43]	$33/dry metric tonne
Sendich *et al.*, 2008[256]	$40/dry metric tonne
Dutta *et al.*, 2009[257]	$60.10/dry metric tonne
Sokhansanj *et al.*, 2010[254]	$74/dry metric tonne (baled), $84/dry metric tonne (chopped) and $86/dry metric tonne (pelletised) (assumed pelletised in this calculation)
Kazi *et al.*, 2010[258]	$83/dry tonne
Humbird *et al.*, 2011[253]	$58.50/dry tonne
Gonzalez *et al.*, 2012[259]	$80.3/dry tonne
Fiegel *et al.*, 2012[260]	$85.40/dry tonne
Vadas *et al.*, 2013[251]	$44.09/dry tonne (most expensive)

Tao *et al.*, 2013[262]	$58.50/dry tonne
Meyer *et al.*, 2013[261]	$58.50/dry tonne
Petrou *et al.*, 2014[263]	$58.50/dry tonne
Ou *et al.*, 2014[264]	$83/dry tonne
Thompson *et al*,2014.[250]	$88.19/dry tonne

Table 5-6 summarises all the literature found concerning the C_{RM} of corn stover. In order to determine the effect of the price of the biomass three different calculations based on three different C_{RM} values were carried out: the average C_{RM} obtained from all studies ($62.61/dry tonne equivalent to **€55.92 per odt^{-1}**, the highest C_{RM} ($88.19/dry tonne equivalent to **€78.76 per odt^{-1}**) and the lowest C_{RM} ($33/dry metric tonne equivalent **€29.47 per odt^{-1}**).

5.3.1.2 C_{UT}

5.3.1.2.1 Costs associated with the electric power used in the CO_2 pump

The 2x2 factorial experimental design carried out on the $scCO_2$ extraction of wax from the maize leaves indicated that the highest yields were obtained using a pressure of **400 bar** and **65 ^{o}C** (as demonstrated in Chapter 3). Therefore these conditions would be utilised to extract waxes from maize stover. The specific enthalpy of CO_2 with these conditions is **314.11 kJ/kg**.

SFE kinetics was also carried out on maize stover using the laboratory-scale supercritical unit. The 500 ml extractor was loaded with 44.21 g of milled maize stover, CO_2 was passed through at a flow rate of 40 g/min (or **6.7 x 10^{-4} kg/s)** and the extraction was carried out for 4 hours, collecting samples at specific time intervals. Figure 5-5 illustrates the extraction curve obtained for the extraction of wax from maize stover.

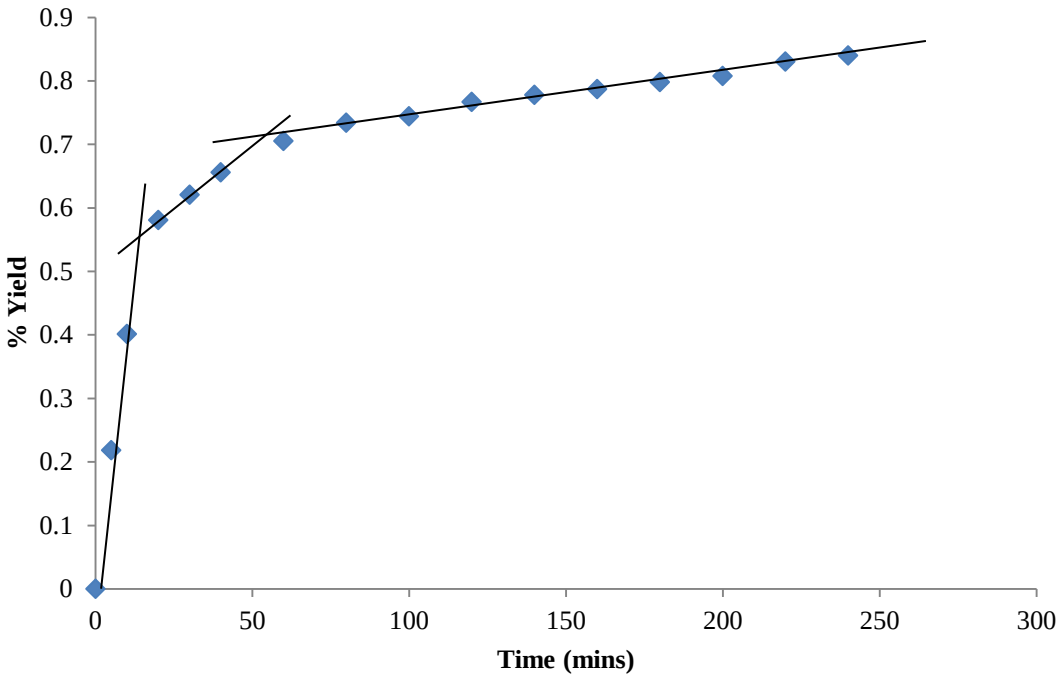


Figure 5-5 Extraction curve for scCO$_2$ extraction of maize stover.

Figure 5-5 shows that the scCO$_2$ extraction profile for the maize stover is very similar to that obtained for the miscanthus wax. The three regions (CER, FER and diffusion-controlled regions) are clearly visible. The total yield extracted after 4 hours was found to be 0.84%. After 40 minutes of extraction (the end of the FER region), 78% of the total wax is extracted and therefore, once again, this was selected as the time for each extraction.

The experimental bed density of the maize stover was found to be **0.33 g/cm^3**. Therefore it was calculated that for each extraction on the industrial scale unit, **132 kg** of maize stover biomass can be loaded into the extractor. Once again, the CO_2 mass flow rate required for the industrial-scale unit is approximately **2964.1 kg/hr** or **22.50 kg CO$_2$ kg^{-1} maize stover/h^{-1}** and the cost of electricity was assumed to be €0.112/kwh.[247]

$$Pure\ CO_2\ enthalpy\ at\ 400\ bar, 65^oC = 314.11\ kJ/kg\ CO_2$$

$$1\ kg\ of\ CO_2 = 314.11\ kJ\ CO_2$$

$$2964.1\ kg\ of\ CO_2 = 931{,}053.45\ kJ\ CO_2$$

$$P_{(kw)} = {}^{E_{(kJ)}}/_{t_s}$$

$$P_{(kw)} = {}^{903{,}097.66}/_{3600} = 258.6\ Kwh$$

$$Cost\ of\ electricity\ (UK) = €0.112\ per\ Kwh$$

$$Cost\ of\ electricity\ = 250.86\ \times 0.112 =\ €28.97/h$$

The costs associated with the CO_2 pump were calculated to be **€28.97/h**.

5.3.1.2.2 Costs associated with the CO_2 heater

The CO_2 has to be heated from 4 ^oC (temperature of CO_2 in the pumps) to 65 ^oC. The mass of carbon dioxide used per hour is **2875.1 kg,** the C_p of carbon dioxide at 65 ^oC is **0.88 kJ kg^{-1} k^{-1}** and the ΔT is 61 ^oC.

$$Q = MC_p\Delta T$$

$$Q = 2964.1\ \times 0.88 \times 61$$

$$Q =\ 159112.89\ kJ$$

$$Assume\ 50\%\ efficiency$$

$$Q =\ 318225.8\ KJ\ per\ hour\ (318.2\ MJ)$$

Thus the energy required is **318.2 MJ per hour**. A number of studies have looked into the calorific content of corn stover. An average value from these studies was taken and it is assumed that the energy that is given off when burning dry maize stover is **17.4 MJ kg^{-1}.**[265-269] Therefore the amount of maize stover that is required is 18.3 kg per hour which is only around 13.9% of the biomass that is used in each extraction. Therefore the energy which is required to heat the extractor may be obtained by burning 13.4% of the biomass that is loaded into the extractor and the costs that are associated with heating the extractors are thus negligible.

5.3.1.2.3 Costs associated with refrigeration

The costs associated with refrigeration are estimated to be around **€2.34 per hour of extraction.**

Therefore it is assumed that the total utility costs, C_{UT} are **€31.31 per hour.**

5.3.1.3 Total COM calculation

Taking all calculations into account the COM for the supercritical extraction of waxes from maize stover assuming the average C_{RM} of **€55.92 per odt^{-1}** are:

$$COM =\ 0.280FCI + 2.73C_{OL} + 1.23(C_{RM} +\ C_{WT} + C_{UT})$$

$$COM =\ 0.280(1{,}400{,}000) + 2.73(47{,}250) + 1.23(87691.51 +\ 0 + 247947.96)$$

$$COM =\ 392000 + 129729.6 + 412836.55$$

$$COM = \frac{€934566.2/year}{1568.16\ tonne/year}$$

COM = €596/tonne of maize stover

COM = €88.89/kg of maize stover wax

The final COM has been calculated to be **€596 per tonne of maize stover biomass** or **€88.89 per kg of wax.**

If the lowest C_{RM} was taken into account of **€29.47 per odt^{-1}:**

$$COM = 0.280FCI + 2.73C_{OL} + 1.23(C_{RM} + C_{WT} + C_{UT})$$

$$COM = 0.280(1{,}400{,}000) + 2.73(47{,}250) + 1.23(46213.68 + 0 + 247947.96)$$

$$COM = 392000 + 129729.6 + 361818.8$$

$$COM = \frac{€883548.4/year}{1568.16\ tonne/year}$$

COM = €563/tonne of maize stover

COM = €84.03/kg of maize stover wax

The final COM would come up to **€563 per tonne of maize stover biomass** or **€84.03 per kg of wax** if the lowest C_{RM} was used**.**

If the highest C_{RM} was used of **€78.76 per odt^{-1}:**

$$COM = 0.280FCI + 2.73C_{OL} + 1.23(C_{RM} + C_{WT} + C_{UT})$$

$$COM = 0.280(1{,}400{,}000) + 2.73(47{,}250) + 1.23(123508.3 + 0 + 247947.96)$$

$$COM = 392000 + 129729.6 + 456891.2$$

$$COM = \frac{€978620.8/year}{1568.16\ tonne/year}$$

COM = €624/tonne of maize stover

COM = €93.08/kg of maize stover wax

The final COM would come up to **€624 per tonne of maize stover biomass** or **€93.08 per kg of wax** if the highest C_{RM} was used**.**

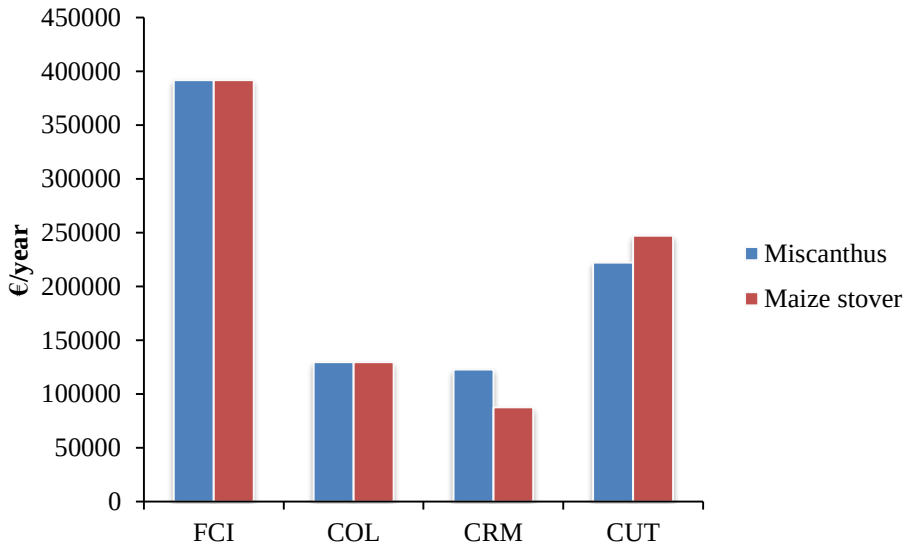


Figure 5-6 Comparison of distribution of costs between miscanthus and maize stover (assuming average C_{RM} for maize stover).

Therefore the cost per kg of wax is lower for the maize stover (€88.89/ kg of maize stover wax) when compared to miscanthus (€148/ kg of wax). Figure 5-6 compares the distribution of costs between miscanthus and maize stover. While the maize stover has slightly higher utility costs, as a result of the higher pressure and temperature (400 bar 65 ^{o}C for maize stover compared to 350 bar 50 ^{o}C for miscanthus), the C_{RM} of the maize stover is lower than that of the miscanthus. However, the main reason for such a difference in cost is the % yield of wax extracted from the maize (0.66%) which is significantly higher than that of miscanthus (0.45%) which leads to significant reductions in costs for the maize stover wax.

Once again, these calculations were based on a number of assumptions, such as loading the extractor with milled biomass. If pelletised biomass was used (and assuming an average C_{RM} of **€55.92 per odt^{-1}):**

$$COM = 0.280(1{,}400{,}000) + 2.73(47{,}250) + 1.23(263074.52 + 0 + 743843.88)$$

$$COM = 392000 + 129729.6 + 1238509.6$$

$$COM = \frac{€1760239.2/year}{4704.48\ tonne/year}$$

$$\boldsymbol{COM = €374.2/tonne\ of\ maize\ stover}$$

$$\boldsymbol{COM = €57.1/kg\ of\ maize\ stover\ wax}$$

Therefore, if pelletised maize stover was taken into account the overall COM would lower to around **€374.2/ tonne of maize stover** or **€57.1/kg of maize stover wax.**

The amount of wax extracted from the maize stover is around 0.8%. If the maize leaves were used, then the wax yield is almost 2.2 times as much (1.74%).

$$COM = 0.280(1{,}400{,}000) + 2.73(47{,}250) + 1.23(263074.52 + 0 + 706226.4)$$

$$COM = 392000 + 129729.6 + 1238509.6$$

$$COM = \frac{€1760239.2/year}{81857.952\ kg\ wax/year}$$

$$\textit{COM = €21.50/kg of maize leaf wax}$$

This would lead to more wax extracted per run resulting in a much lower COM, of around **€21.50/ kg of wax.**

5.3.1.3.1 Utilisation of maize biomass for electricity generation

1. **Calculation assuming use of most efficient technology (combustion)**

$$Mass\ of\ milled\ maize\ per\ exraction\ (40\ mins): 132\ kg$$

$$Mass\ of\ pelletised\ maize\ per\ extraction: 132 \times 3 = 396\ kg$$

$$Mass\ of\ wax\ extracted\ (assuming\ 1.74\%\ yield): 396 \times 0.0196 = 6.89\ kg$$

Therefore maize biomass after each extraction:

$$396\ kg - 6.89\ kg = 389.11\ kg\ of\ maize$$

When heating the extractors, 54.87 kg of maize is required for each extraction

$$389.11\ kg - 54.87\ kg = 334.24\ kg\ of\ available\ maize\ per\ extraction$$

Energy of combustion for maize:

$$1\ kg\ of\ maize = 17.4\ MJ$$

$$17.4\ MJ \times 334.24 = 5815.78\ MJ/extraction\ (40\ mins)$$

$$= 8723.66\ MJ/hour$$

Assuming 43% efficiency:

$$P_{(kw)} = {}^{E_{(kJ)}}/_{t_s}$$

$$Assume\ 43\%\ efficiency:$$

$$P_{(kw)} = \frac{(8723.66)}{3.6} \times 0.43 = 1041.99\ Kwh$$

$$Cost\ of\ electricity\ (UK) = €0.112\ per\ Kwh$$

$$Value\ of\ electricity\ generated\ per\ extraction$$
$$= 1026.38\ Kwh\ \times 0.112\ €\ Kwh^{-1}\ =\ €116.70$$

$$Value\ of\ electricity\ generated\ per\ tonne\ of\ maize\ = €116.70 \times \frac{1000}{396}$$

$$=\ €294.71\ per\ tonne\ of\ maize$$

$$=\ €16.94\ per\ kg\ of\ wax$$

Therefore when subtracted from the cost of wax production:

$$Total\ COM\ cost = 21.50 - 16.94 =\ €4.56\ per\ kg\ of\ wax$$

2. Calculation assuming average efficiency of all technologies

When carrying out the same calculation above using the average efficiency of all technologies (27% efficiency), the total COM per kg of wax was found to be €10.87 per kg of wax.

Therefore if the biomass was to be utilised after the extraction for electricity production, the COM of the maize wax would decrease to **€10.87 per kg of wax** when taking the average energy efficiency of all available technologies (27%), while the cost is **€4.56** for every kg of wax when the most efficient technology is taken into consideration.

5.4 Conclusion

This is the first time a techno-economic assessment for the supercritical extraction of waxes from biomass has been carried out. The calculations were carried out using a methodology proposed by Turton *et al.*[237] The estimations were based on five main costs including fixed capital investment (FCI), labour costs (C_{OL}), raw material costs (C_{RM}), utility costs (C_{UT}) and cost of waste (C_{WT}). The COM for miscanthus wax extraction was found to be **€620 per tonne of miscanthus biomass** or **€148 per kg of wax**, with the FCI and C_{UT} contributing significantly to the COM. These costs are estimated on a number of assumptions the main ones being that the miscanthus biomass is milled not pelletised, all parts of the miscanthus plant were used and the miscanthus biomass is utilised solely for the extraction of waxes. In the case of the latter supercritical extraction of waxes from biomass should be the first step of a holistic biorefinery and therefore the biomass would be passed along for further processing to produce fuels and chemicals leading to reduction of C_{RM}. If the biomass was pelletised, miscanthus leaves were solely taken into consideration (4 times the wax yield) and the

miscanthus biomass was combusted following the extraction, then the COM of the wax could be reduced to as much as **€10.43 per kg of wax** (based on 27% combustion efficiency for electricity generation) or **€4.77 per kg of wax** (based on 43% efficiency).

The COM for maize stover was found to be significantly lower (**€88.89/ kg of wax**) when compared to that of miscanthus **(€148/ kg of wax**) due to the lower C_{RM} costs of maize stover combined with a much higher % yield of wax extracted from the biomass. The cost of extracting wax from maize can be lowered if pelletised leaves are solely utilised and the maize biomass is combusted following extractions, leading to an overall cost of **€10.87 per kg of wax (**based on 27% combustion efficiency for electricity generation) and **€4.56 per kg of wax** (based on 43% efficiency). It must be stated that these costs are estimated for an industrial supercritical plant with a yearly capacity of around 1600 tonnes of biomass.

Finally, it must be said that the biorefinery scenario seen here is quite basic (biomass is solely utilised for electricity generation). In this thesis, it has been shown that hydrolysis and fermentation of the biomass could lead to the generation of surfactants and biofuels. Furthermore, studies have shown that further processing of the biomass, such as microwave pyrolysis of the biomass, could lead to additional added-value products as well as increased calorific value of the remaining char.[192] This study has shown that, if certain parameters are taken into account, a high value product (wax) can be obtained for a low price, when thinking holistically.

Chapter 6

Hemp fibre waste residues as a source of added-value products.

6 Chapter 6

6.1 Introduction

In the previous chapters, the waxy constituents obtained from C_4 waste residues were investigated. This chapter will investigate the wax obtained from C_3 biomass waste stream. As such, hemp was selected as a possible C_3 waste residue and, in this chapter, the lipophilic extractives from hemp waste were investigated.

Hemp (*Cannabis sativa L.)* is a C_3 plant that has its origins in Central North-East Asia.[270, 271] It is one of the oldest cultivated non-food crops known dating back to around 5000 years ago.[270] From the 1500's to the 1700's hemp and flax were the two main fibre crops grown in Europe.[272] The quality and durability of the fibres (accumulated in the stem) made them popular for use in sail-making, paper-making and clothing.[271] However, there was a decline in hemp cultivation in Europe throughout the 1800's.[272] Post-World War II, a major decline occurred due to a number of reasons, including the discovery and synthesis of synthetic fibres, high-labour costs associated with hemp fibres, the large-scale manufacturing of cotton and the association of hemp with illegal substances (Δ^9-tetrahydrocannabinol (THC)). In 1971, a ban on hemp cultivation was imposed under the Misuse of Drug Act.[273]

Industrial hemp and marijuana (known for its psychotropic properties) originate from the same species (*Cannabis sativa L.*), however, while the latter is bred for its Δ^9-tetrahydrocannabinol (Δ^9-THC) content in the female flowers, industrial hemp has been bred for high fibre content in the stem.[270] The industrial hemp fibre has minimal amounts of Δ^9-THC (0.2% *w/v*), which displays the psychotropic properties – approximately 50 times less than that found in marijuana.[270] In the last 2 decades, as a result of a number of developments, there has been renewed interest in hemp cultivation in several European countries (Italy, Spain, Germany, the Netherlands, United Kingdom and France) as well as other parts of the world.[271, 272] Certain agricultural commodities have been overproduced within the EU which has led to the search for alternative uses for agricultural land.[272] Hemp offers a number of agricultural benefits, namely, pest and disease resistance, weed control and improvement of soil properties due to crop rotation.[271] Hemp generates very high yields under very low input, with around 15 – 25 tonnes of dry matter per hectare. Its large plasticity allows it to be cultivated under a wide range of agro-ecological conditions.[271, 272, 274]

There was an increase in cultivated areas in Europe, from 2762 hectares in 1989 to 41,682 hectares in 1998.[271] However, hemp cultivation was still restricted, with strict legislation until 2007 when certain cultivars were approved for cultivating in the entire European Union.[274] The first large-scale industrial plant in the UK was Hemcore, which has experienced large growth over the past few years.[274]

The main purpose for growing hemp is the extraction of its fibres from the stem.[271] Hemp stems consist of two main parts – the bast fibres (35%) having a high cellulose content (57 – 77%) and low lignin content (5 – 9%) and the woody core (65%) (or shiv), which has lower amounts of cellulose (40 – 48%) and a higher lignin content (21 – 24%).[275] Hemp fibres have always been known to be of very high quality and can be used in a number of industries such as paper-manufacturing, textiles industries, automotive industries and bio-building industries.[270, 274] It has also been used as a biofuel in certain countries such as Sweden.[274]

During the processing of hemp for fibre extraction, large amounts of hemp dust are generated, which could be a potential source of valuable chemicals. The exploitation of hemp dust for natural product extraction could potentially add value to this otherwise waste residue. In this chapter, wax extraction was carried out on a variety of different hemp dust samples, obtained from different parts of an industrial hemp processing plant (Harrison Spinks). Heptane Soxhlet extraction as well as $scCO_2$ extraction were carried out. The work carried out here forms part of work package 5 (WP5) of the MultiHemp project, a European FP7 consortium, and was done in collaboration with Camille Bainier (National College of Chemical Engineering of Mulhouse, France) and Marine Reinaud (École nationale supérieure de chimie de Lille, France).

6.2 Harrison Spinks

Harrison Spinks is a company that manufactures luxury mattresses and other minor components. They incorporate natural wool (for softness) as well as hemp fibres for structural support in their mattresses. They grow their own hemp fibre plants on 80 acres of arable land, on a farm in North Yorkshire, UK. The hemp is processed on site in a processing facility in order to extract the fibres from the plant and separate it out from the woody core or shiv. The fibres are incorporated into the mattresses while the shiv is used for animal bedding. Figure 6-1 is a schematic that illustrates how the processing facility is set-up.

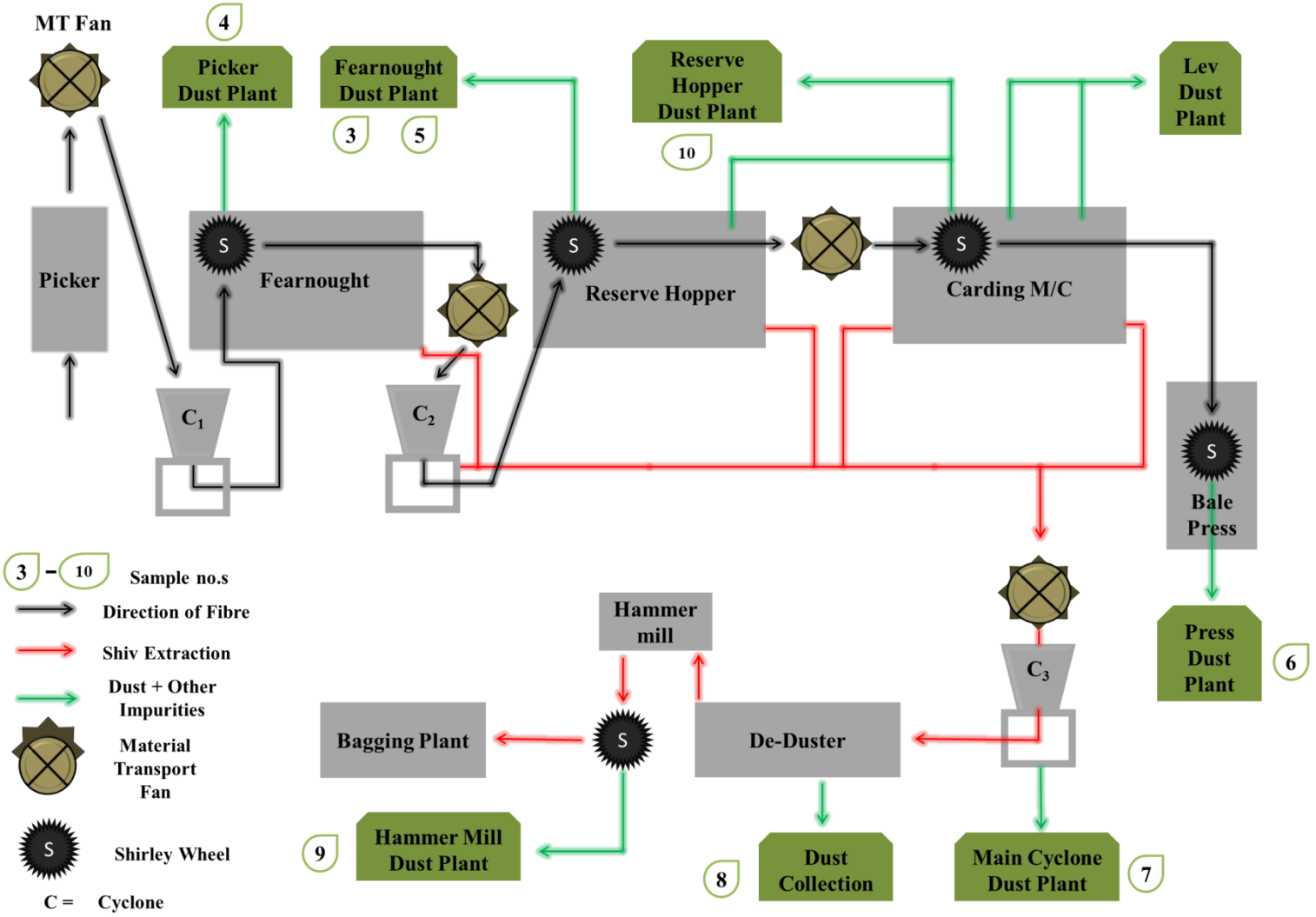


Figure 6-1 Schematic of the hemp processing plant (Harrison Spinks) and the paths taken by the fibre, shiv and dust.

The paths taken by the fibre and shiv are shown in Figure 6-1. The process involves the use of various types of machinery which aim to separate the fibres from the rest of the stem and a considerable amount of dust is generated at each step. These are collected in dust plants that are found across the facility. Approximately five tonnes of hemp is currently processed every week by the processing facility and around 18 – 33% of the total is hemp dust. This means that every week, approximately 900 – 1650 kg of hemp dust is generated and left unutilised.

Table 6-1 Dust samples and their origins from the processing facility.

Dust sample no.	Dust plant
3	Fearnought dust plant (1st sample)
4	Picker dust plant
5	Fearnought dust plant (2nd sample)
6	Press dust plant
7	Main cyclone dust plant
8	Dust collection (from rotary screen of de duster)
9	Hammer mill dust plant
10	Reserve hopper dust plant

Eight hemp dust samples (shown in Table 6-1) were obtained from different dust plants found in the processing facility (labelled in Figure 6-1) and Soxhlet extractions with heptane were carried out on each sample. The extractives obtained were characterised and compared in order to see whether different parts of the processing facility generated dust samples having different extractive compositions (and hence see whether different stages of the mechanical processing of hemp results in dust samples of varying composition). $ScCO_2$ extraction was subsequently carried out on one sample (selected depending on its characteristics) and an optimisation of the extraction process was carried out.

6.3 Soxhlet extractions of various hemp dust samples.

6.3.1 Composition of heptane extractives from different industrial samples from hemp fibre processing

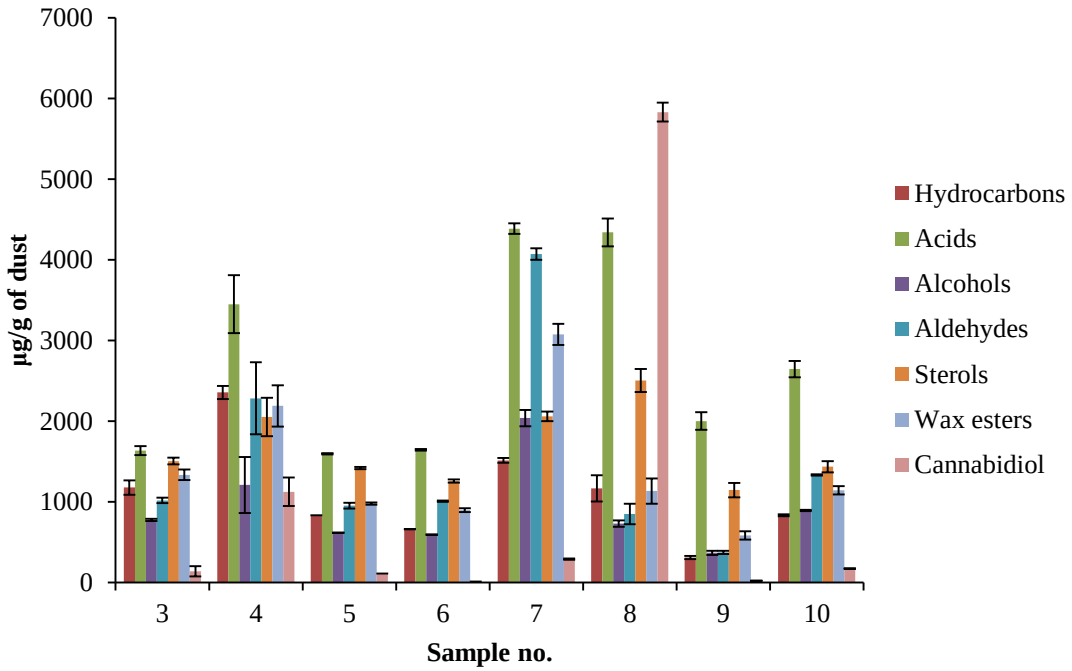


Figure 6-2 Concentration of compounds found in the heptane extractives from the various dust samples.

Lipophilic extractives from the various hemp dust samples were extracted by means of Soxhlet extractions using heptane. Figure 6-2 shows the distribution of the major families of lipophilic compounds found in the extracts from the hemp fibre processing plant in µg/g of dust. It can be seen that there is a similarity in lipophilic composition between sample 3, sample 5 and sample 6, the latter two of which are almost identical. Interestingly, sample 3 and sample 5 are waste residues collected from different parts of the same dust plant (referred to as Fearnought dust plant) and this therefore indicates that the type of machinery leads to the generation of waste residues having specific types and quantities of compounds.

Long-chain fatty acids were the most abundant compounds found in each sample with the exception of sample 8 (which is dominated by cannabinoids). The highest amount of fatty acids were found in sample 7 (4386.6 ±65.7 µg/g of dust) and sample 8 (4341.1 ±173.7 µg/g of dust). Saturated fatty acids having chain lengths of C_6 – C_{25} were identified in all heptane extracts with C_{16} predominating. Marques *et al.* investigated the composition of lipophilic extractives in hemp raw material and hemp cellulose pulps to investigate the effects that pulping has on the lipophilic constituents.[276] Lipophilic

extracts were obtained by Soxhlet extraction with acetone on the raw material and various cellulose pulps for 8 hours. Chain lengths of C_{16} – C_{26} were identified (with trace amounts of C_{28} acid detected in certain pulp samples).[276] Gutiérrez *et al.* also looked at acetone-extracted lipophilic extracts from hemp raw material in order to compare them with pitch deposits.[277] They found chain lengths of C_{16} – C_{30} and stated that C_{16}, C_{18} and C_{20} acids predominated (though no quantification data was given).[277]

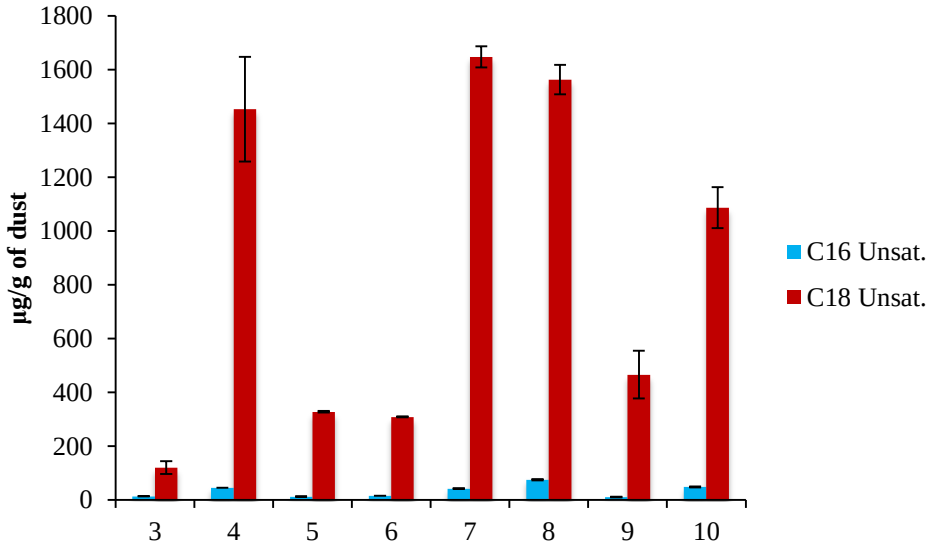


Figure 6-3 Distribution of unsaturated fatty acids in the various hemp lipophilic extracts in ug/g of dust.

Four unsaturated fatty acids were identified in the lipophilic extracts: palmitoleic acid, oleic acid, linoleic acid and linolenic acid. The unsaturated fatty acids constituted a large portion of the total fatty acid composition in sample 4, sample 7, sample 8 and sample 10 (approximately 40% of the total fatty acid composition) while relatively small amounts were found in sample 3 (approximately 7% of the total fatty acid composition). The largest amounts of unsaturated fatty acids were found in sample 7 (1698 ±39.9 µg/g of dust), sample 8 (1637.7 ±57.5 µg/g of dust) and sample 4 (1498.3 ±194.9 µg/g of dust). However, when looking at the composition of unsaturated fatty acids in the lipophilic extracts (mg/g of wax), sample 10 has a higher amount of unsaturated fatty acids in the lipophilic extracts (72 ±5 mg/g of wax) as shown in Figure 6-4 .

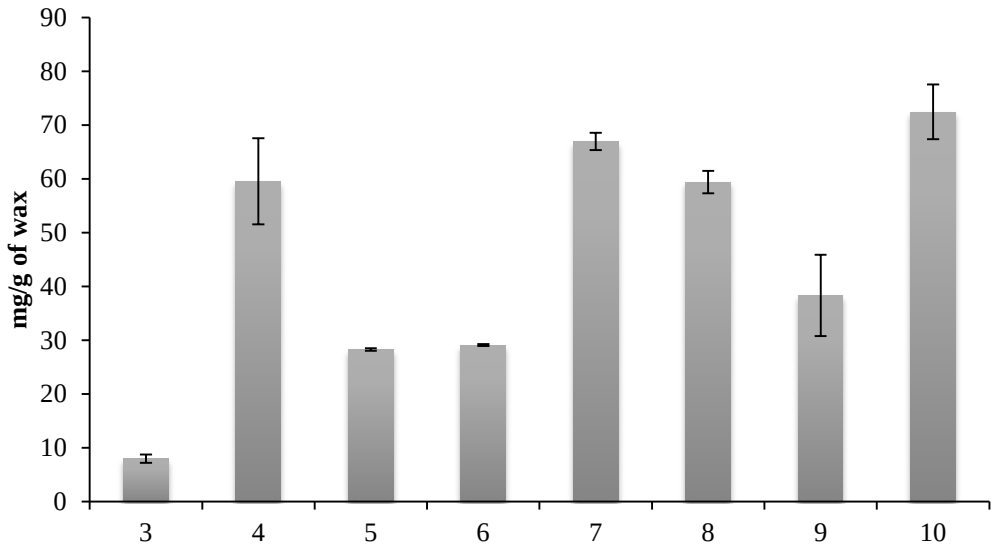


Figure 6-4 Amount of unsaturated fatty acids in the hemp lipophilic extracts in mg/g of wax.

Only linoleic acid and oleic acid were found in the lipophilic extractives from hemp and cellulose pulps in the study carried out by Marques *et al.* and there was a decrease in the total composition of unsaturated fatty acids during the pulping process.[276] Gutiérrez *et al.* also found oleic acid and linoleic acid in hemp biomass.[277]

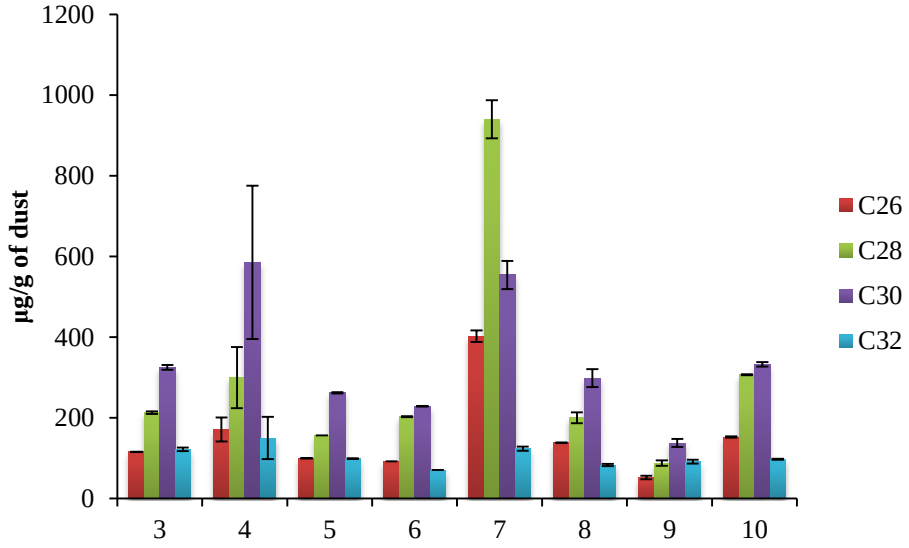


Figure 6-5 Most abundant alcohol chain lengths in the various hemp lipophilic extractives in µg/g of dust.

n-Policosanols having chain lengths from C_{22} to C_{32} were detected in all samples. Sample 7 had the largest amount of *n*-policosanols (2040.2 ±101.7 µg/g of dust) followed by sample 4 (1209.8 µg/g of dust) and sample 10 (893 ±9 µg/g of dust). Interestingly, there was a difference in the dominant alcohol chain length between sample 7 and the other samples. 1-octacosanol was the dominant alcohol chain length in

sample 7 (comprising 46% of the total fatty alcohol composition) while 1-triacontanol was found to predominate in the remaining samples. This suggests that there is a drastic change in the *n*-policosanol composition of the hemp waste residues generated when the hemp fibres passes through the main cyclone. It is only in this sample that 1-octacosanol is obtained in such significant quantities (939.9 ±46.9 µg/g of dust or 38.2 ±1.9 mg/g of wax). Similar chain lengths have been reported in the literature of alcohol composition in hemp and cellulose pulps, with 1-octacosanol predominating.[276] 1-docosanol, 1-octacosanol and 1-triacontanol were said to be the major alcohol chain lengths in another study on alcohol composition in hemp.[277]

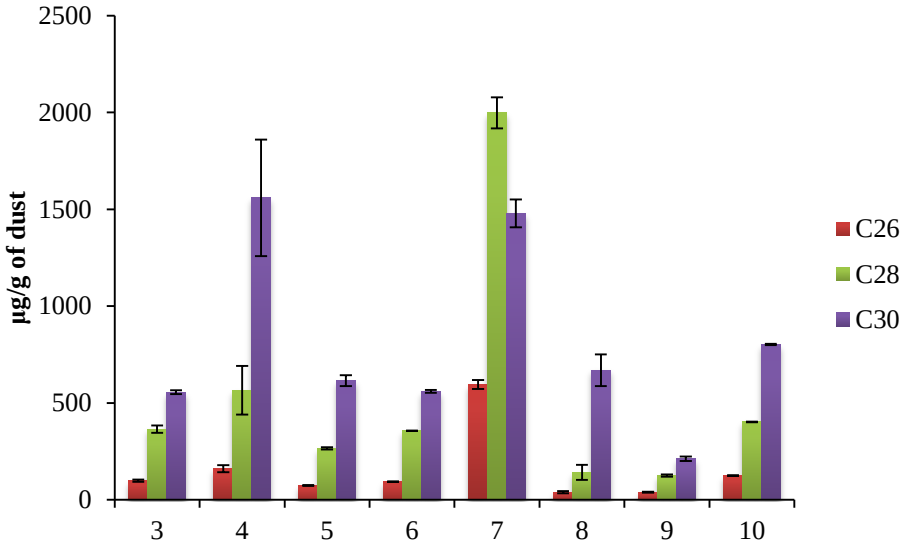


Figure 6-6 Distribution of aldehydes in the various hemp lipophilic extractives in µg/g of dust.

Figure 6-6 shows that there is a large abundance of fatty aldehydes in sample 7 (4071.9 ±71.6 µg/g of dust). Significantly lower amounts of aldehydes were found in the other samples, with sample 4 having the second highest amount of fatty aldehydes (2284 ±445.1 µg/g of dust). Chain lengths of C_{26} to C_{30} were identified. Once again, there was a difference in the dominant aldehyde chain length between sample 7 (octacosanal predominating) and the other samples (triacontanal predominating). The same chain lengths were detected in previous studies on hemp and hemp cellulose pulps, with triacontanal predominating.[276, 277] There was a significant reduction in aldehyde content after the pulping process.[276]

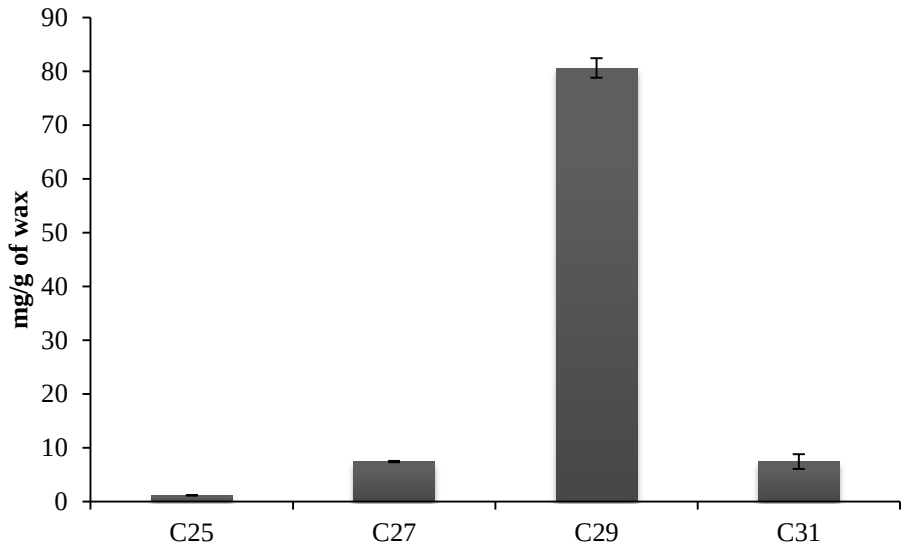


Figure 6-7 Distribution of alkanes in hemp lipophilic extract sample 4 in mg/g of wax.

It is interesting to study the long-chain alkane composition in the hemp lipophilic extractives in that it is different to other plant waxes due to the uneven distribution of alkane compounds. Chain lengths ranging from C_{25} – C_{31} were detected, with nonacosane (C_{29}) predominating. Similar amounts of C_{27} and C_{31} were found in all the extracts. Sample 4 demonstrates the largest amount of alkanes (2357.3 ±81.5 μg/g of dust) followed by sample 7 (1514.8 ±30.5 μg/g of dust). The alkane composition of hemp wax varies considerably from the alkane composition observed in the C_4 waxes, in that the latter demonstrated no predominance of one alkane. Figure 6-7 compares the % composition of different alkane chain lengths in hemp sample 4 (representative hemp sample) with the stem waxes of the various C_4 plants studied in this thesis.

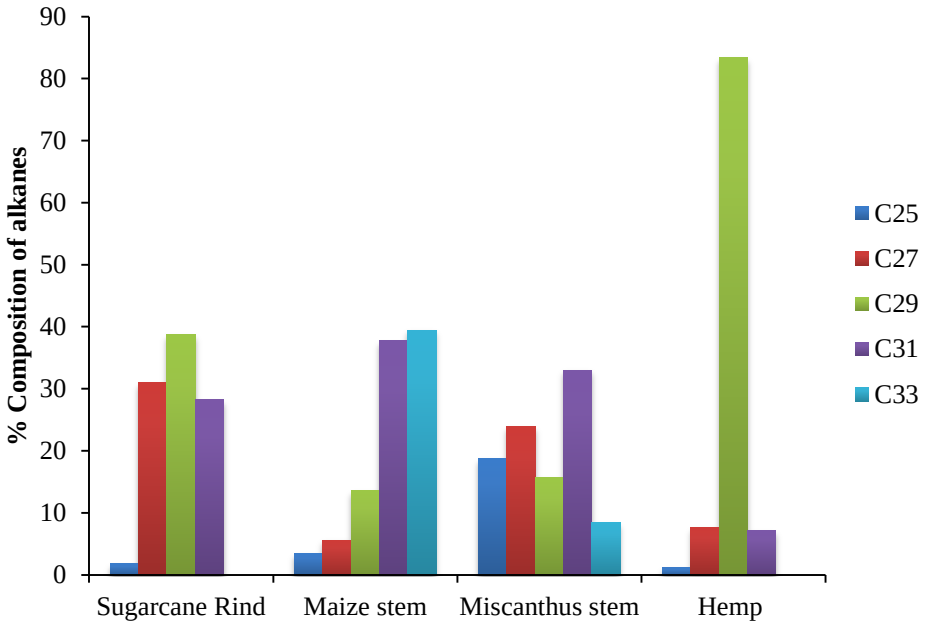


Figure 6-8 Comparison of the % composition of different alkane chain lengths in hemp, sugarcane rind, miscanthus stem and hemp.

Figure 6-8 shows that, while there is a dominant alkane chain length in the waxes extracted from sugarcane, maize and miscanthus, there is a more uniform distribution of chain lengths. In contrast, in the hemp wax, C_{29} comprises around 83% of the total alkane composition. The alkane composition is consistent with that reported in literature, where a predominance of C_{29} alkane was found in acetone-extracted waxes from hemp.[276, 277] Furthermore, relatively equal amounts of C_{27} and C_{31} were detected, which is consistent with the results of this study.[276]

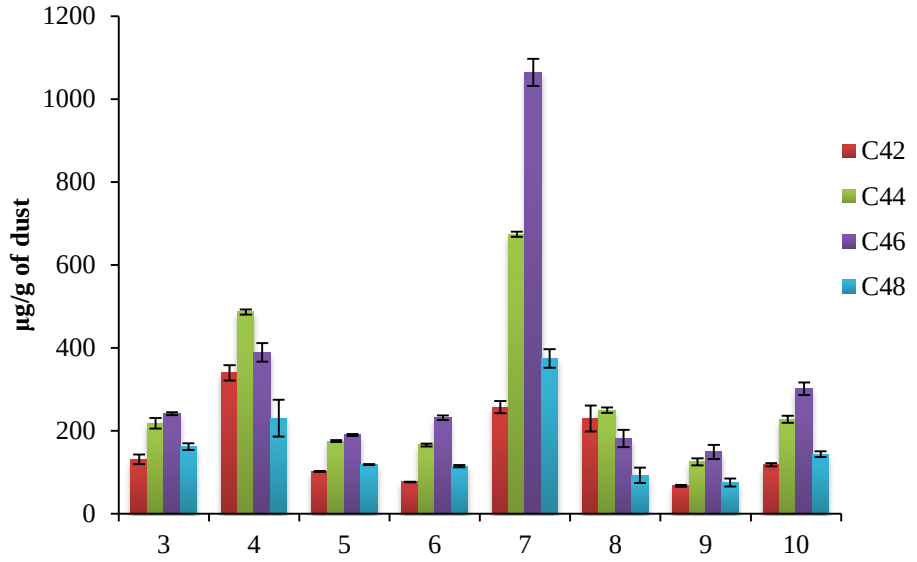


Figure 6-9 Distribution of major wax esters in hemp extracts in ug/g of dust.

Wax esters having chain lengths of C_{38} to C_{58} were present in all samples but, were most abundant in sample 7 (3076.6 ±129.9 μg/g of dust) followed by sample 4 (2189.7 μg/g of dust). There was a variation in the dominant wax ester chain length amongst the different samples, with C_{44} wax ester predominating in sample 4 and sample 8 (22.2% and 22% of total wax ester composition respectively), while C_{46} wax ester was the most abundant chain length in the remaining samples (34.6% of total wax ester composition in sample 7). A smaller distribution of wax esters were highlighted in previous studies on wax ester composition in hemp during the pulping process for paper production (C_{40} to $C_{50,}$ Marques *et al.*; C_{40} to C_{54}, Gutiérrez *et al.*) and it is interesting to note that all the wax esters were lost during the pulping process.[276, 277]

All seven hemp lipophilic samples investigated show three sterols (β-sitosterol, stigmasterol and campesterol), one steroid ketone (stigma-4-*en*-3-one) and the triterpenoid friedelin was detected in trace amounts. Sample 8 demonstrated the largest amount of sterols (2503.7 ±142.5 μg/g of dust). A wider range of steroid ketones (stigma-3,5-dien-7-one and 5α-stigmastan-3,6-dione) and triterpenoids (α-amyrin and β-amyrin) were found in previous studies (albeit in small quantities) in the hemp used for the production of cellulose pulps in paper manufacturing.[276, 277] However, there was a reduction in the steroid ketone during the pulping process while only a small amount of free sterols were present in certain cellulose pulps (no free sterols were present in the cellulose pulps that had undergone elemental chlorine free bleaching).[276]

Cannabinoids were also detected in the lipophilic extracts. Over 60 cannabinoid compounds have been determined in *Cannabis sativa L.*. However, only a small amount of these have been investigated in detail.[278] The major cannabinoid determined in lipophilic extracts was found to be cannabidiol (CBD). The mass fragmentation pattern of CBD from the lipophilic extract (sample 8) may be found in Figure 6-10 below.

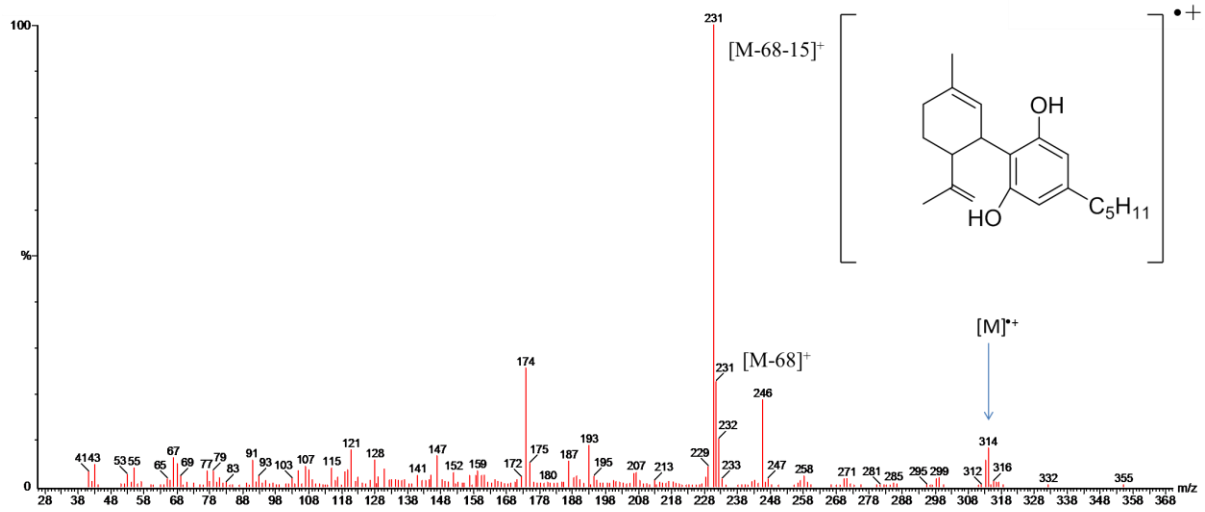
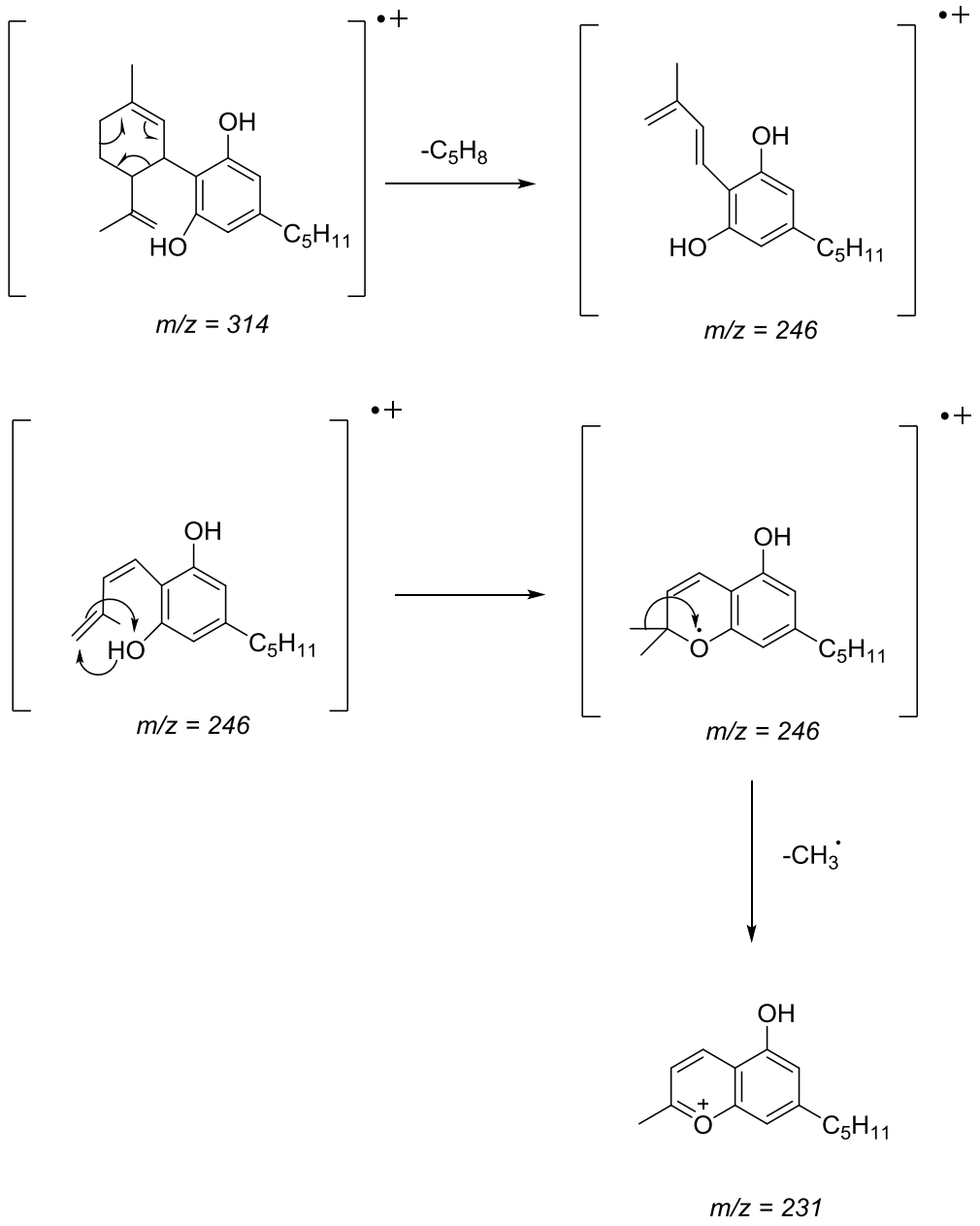


Figure 6-10 Mass spectrum of CBD found in hemp sample 8.

The mass fragmentation pattern of CBD is very similar to two other cannabinoids; cannabichromene (CBC) and cannabicyclol. However, there is a diagnostic peak at *m/z* 246 present in the CBD mass spectrum that is absent in the fragmentation patterns of the other cannabinoids.[278] This ion arises as a result of a retro-Diels Alder reaction of the cyclohexene ring as shown in scheme 6-1.[279-282]



Scheme 6-1: retro-Diels Alder reaction of cyclohexene ring of CBD.

Studies have shown that the mass fragment is a closed structure rather than an open structure.[279, 280] One of the two aromatic hydroxyl groups is available for ring closure as shown in scheme 6-1. This ring closure is required as the base peak in the mass spectrum (*m/z* = 231) results from the loss of a germinal methyl group.

Figure 6-11 shows that substantial quantities of CBD were found in sample no. 8 (5832.5 ±118.9 µg/g of dust). Considerably less amounts of CBD were found in the other dust samples (sample 4 had the second highest quantities, with 1125.4 ±117.8 µg/g of dust) which indicates that during the mechanical extraction and separation of the fibre from the woody core, there is accumulation of CBD at one stage of the process. Trace amounts of cannabinol and Δ^9-THC were also detected in the extract.

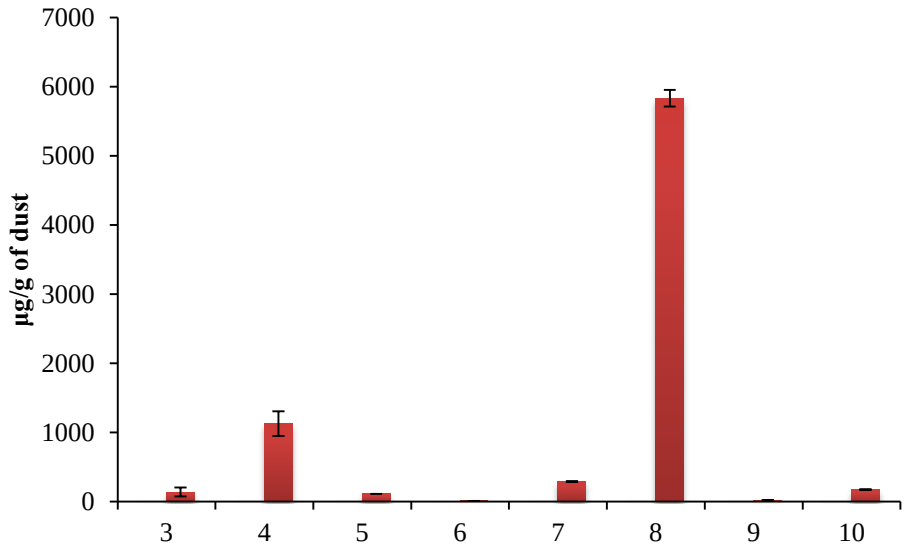


Figure 6-11 CBD content in the heptane extracts from the various dust samples.

Cannabinoids are present in all parts of the plant, but there is a variation in the cannabinoid content in different parts (different plant organs). Studies on cannabinoid content in fibre-type plant varieties have shown high levels of CBD and low-traces of tetrahydrocannabinol (Δ^9-THC) in the bracts (modified or specialised leaves associated with the flowers of plants), which is consistent with what was found in the dust samples in the current study.[283]

CBD has been given considerable attention over the past few years due to its plethora of therapeutic properties and pharmacological activities.[284-286] Unlike Δ^9-THC, it is a non-psychotropic compound and is well-tolerated, enabling it to be used to treat numerous disorders. CBD has low toxicity which has allowed for clinical-level studies on its therapeutic efficacy (alone or combined with various cannabinoids) in the treatment of a variety of central nervous system (CNS) and peripheral disorders.[285]

CBD exhibits high anti-inflammatory properties which could be used to treat neuro-inflammatory disorders. In fact, in 2011, the first phytocannabinoid drug Savitex® was approved in the UK (CBD combined with Δ^9-THC) for the treatment of multiple sclerosis muscle spasms.[287] The therapeutic effects of Δ^9-THC were found to be enhanced by CBD while a clear reduction in its negative effects was observed. Like most phytocannabinoids, CBD is an anti-emetic and it has also been found to induce sleep which has led to proposals for its use in the treatment of numerous sleep disorders.[288, 289]

Extracts containing CBD were also tested to treat various types of cancer due to its anti-tumoral properties.[290] One of the major discoveries of CBD has been its anti-psychotic

properties, making it a promising candidate for the treatment of schizophrenia.[291-293] Clinical trials to treat schizophrenia with purified CBD extracts have begun to take place by GW pharmaceuticals.[294] Studies have also shown positive results in the treatment of numerous degenerative diseases such as Parkinson's disease, Huntington's disease and Alzheimer's disease (neuroprotective properties arise as a result of the combination of CBD's anti-inflammatory and anti-oxidant properties).[295-297]

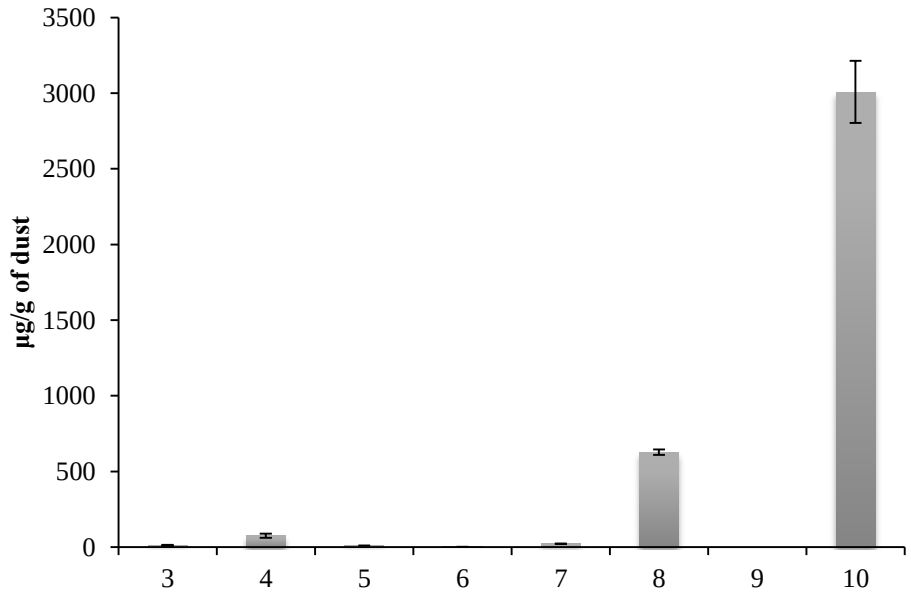


Figure 6-12 Distribution of phytol in the various hemp samples in ug/g of dust.

Interestingly, phytol was detected in significant quantities in sample 10 (3008.3 ±205.7 µg/g of dust), while it was only found in minute quantities in all the other samples. Furthermore, phytol was found to be the most abundant compound in the lipophilic extractives in sample 10 as shown in Figure 6-12. The substantial quantities of phytol in the hemp dust wax have not been observed in the C_4 waxes. Phytol has not previously been found in the lipophilic extractives of hemp.[276, 277]

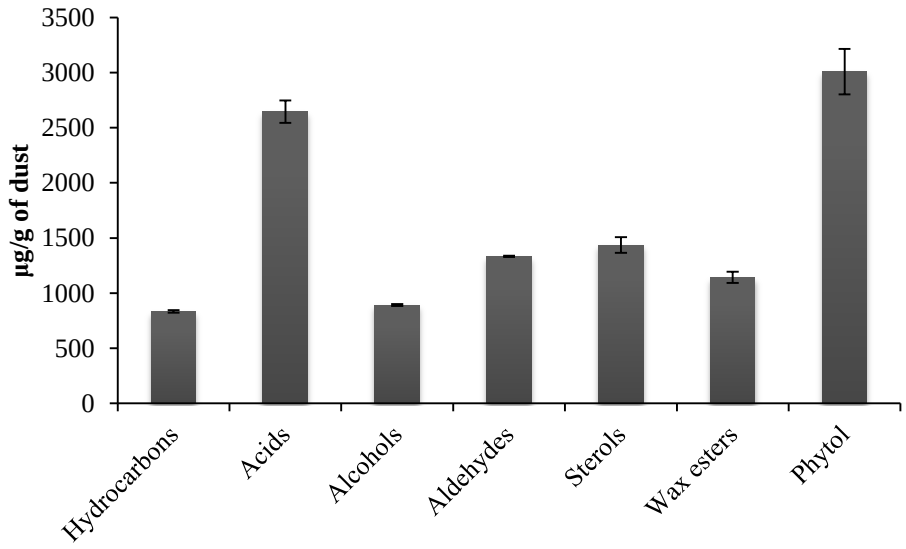


Figure 6-13 Distribution of compounds in sample 10 in µg/g of dust.

These results are promising as for the first time it has been demonstrated that hemp fibre waste residues from different parts of an industrial processing facility generate lipophilic extractives having different constituents (even though they originate from the sample hemp fibre plant). Hemp dust residues obtained from the main cyclone dust plant provide large amounts of long-chain saturated and unsaturated fatty acids, fatty aldehydes and wax esters. In this part of the process, the main cyclone separates off the lighter dust which ends up in the dust plant and lets the heavier shiv (woody core) pass on to the rotary screen de-duster which in turn further separates the dust out (sample 8) leaving only the shiv to be processed through the hammer mill. Waste residues from the picker dust plant (sample 4) are rich in hydrocarbons while lipophilic extracts from the rotary screen duster (sample 8) provide a steady source of high-value cannabinoids as well as phytosterols which are of medicinal and nutraceutical interest.[154, 163, 284, 286] The residues generated from the reserve hopper dust plant (sample 10) provide lipophilic extractives having considerable quantities of phytol which is of commercial interest for the fragrance industry.[176]

Previously in literature, it was found that the manufacturing of cellulose pulps from hemp resulted in a significant decrease in certain groups of lipophilic compounds that are present in the raw material hemp lipophilic extractives namely aldehydes, wax esters, steroid ketones, sterol esters and alkylferulates.[276] Free sterols have been shown to decrease in certain pulping processes.[276] In the hemp dust residues collected from the extraction of hemp fibre (for incorporation into bedding), this should not occur as the hemp biomass did not undergo any chemical pre-treatment. Furthermore, an advantage of extracting from hemp dust as opposed to the entire plant is that the woody core rich

in lignin and the fibre rich in cellulose have been separated enriching the sample with the epidermal part of the stem (which is where the waxy content is situated). Further work is needed to investigate the effects that mechanical pre-treatment have on the % composition of lipophilic constituents in the hemp fibres. It has already been demonstrated in this study that mechanical pre-treatment fractionates the lipophilic constituents, with certain groups of compounds more abundant in certain waste residues than in others (such as the cannabinoids in sample 8, phytol in sample 10, long-chain fatty aldehydes and wax esters in sample 7). It is also speculated that the mechanical pre-treatment might improve the biomass for solvent extraction as it could make it more easily accessible for solvent molecules to access solute particles. However, further work is required to confirm this.

6.4 Supercritical extraction and optimisation of lipophilic extractives from hemp sample no.8 (dust collected from the rotary de-duster plant)

Sample 8 was selected in order to carry out $scCO_2$ extraction and optimisation of lipophilic extractives as a result of the high concentrations of CBD and phytosterols present in the heptane extractives (when compared to the other samples). A 2x2 factorial experimental design was carried out; whereby the pressure and temperature were varied as follows: 80 bar 35 ^{o}C, 80 bar 65 ^{o}C, 240 bar 50 ^{o}C, 350 bar 50 ^{o}C, 400 bar 35 ^{o}C and 400 bar 65 ^{o}C. The results are summarised in Figure 6-14.

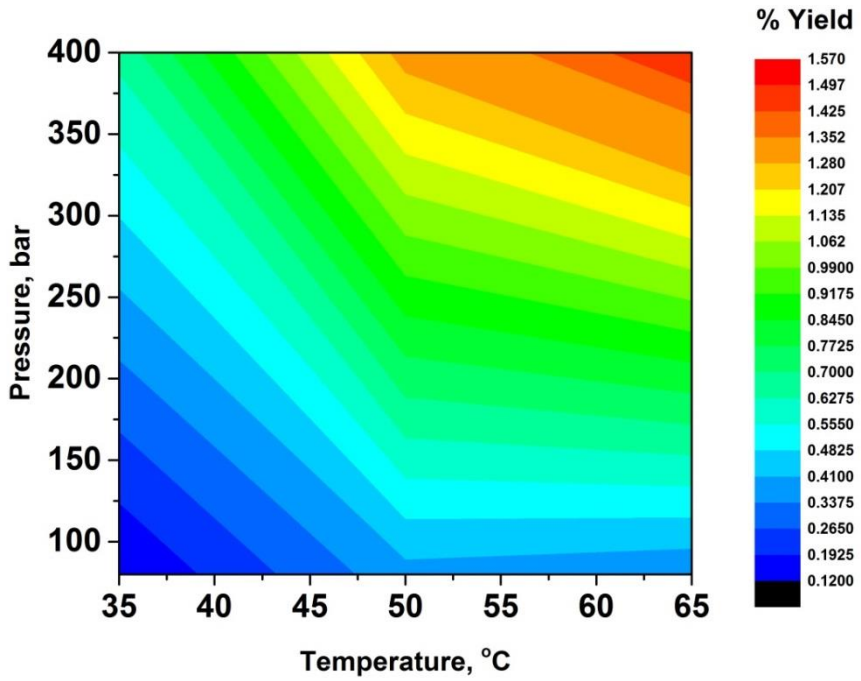


Figure 6-14 Contour plot showing % yield as a function of pressure and temperature.

Figure 6-14 shows that the highest extraction crude yields were obtained when the highest pressure and temperature were implemented (400 bar and 65 ^{o}C). However, it is interesting to see whether these conditions also give rise to the highest amounts of the compounds of interest (namely CBD and phytosterols). Therefore, different families of compounds obtained for each $scCO_2$ extraction were quantified and compared in order to see what effect varying the conditions (temperature and pressure) had on the composition of lipophilic extractives.

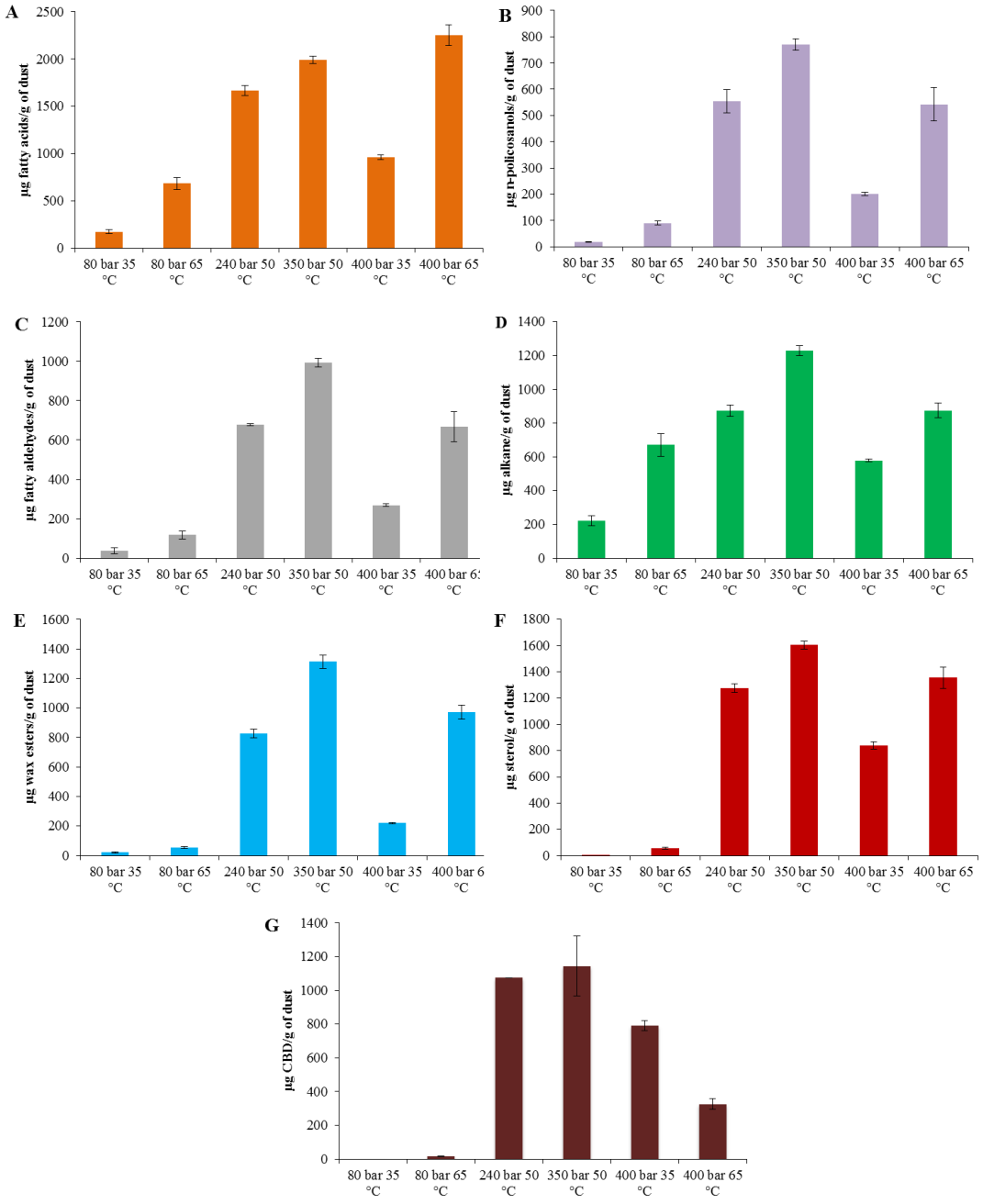


Figure 6-15 Quantities of A) Fatty acids B) *n*-Policosanols C) Fatty aldehydes D) *n*-Alkanes E) Wax esters F) Sterols and G) CBD in $scCO_2$ extracts with various conditions (temperature and pressure), in µg/g of dust.

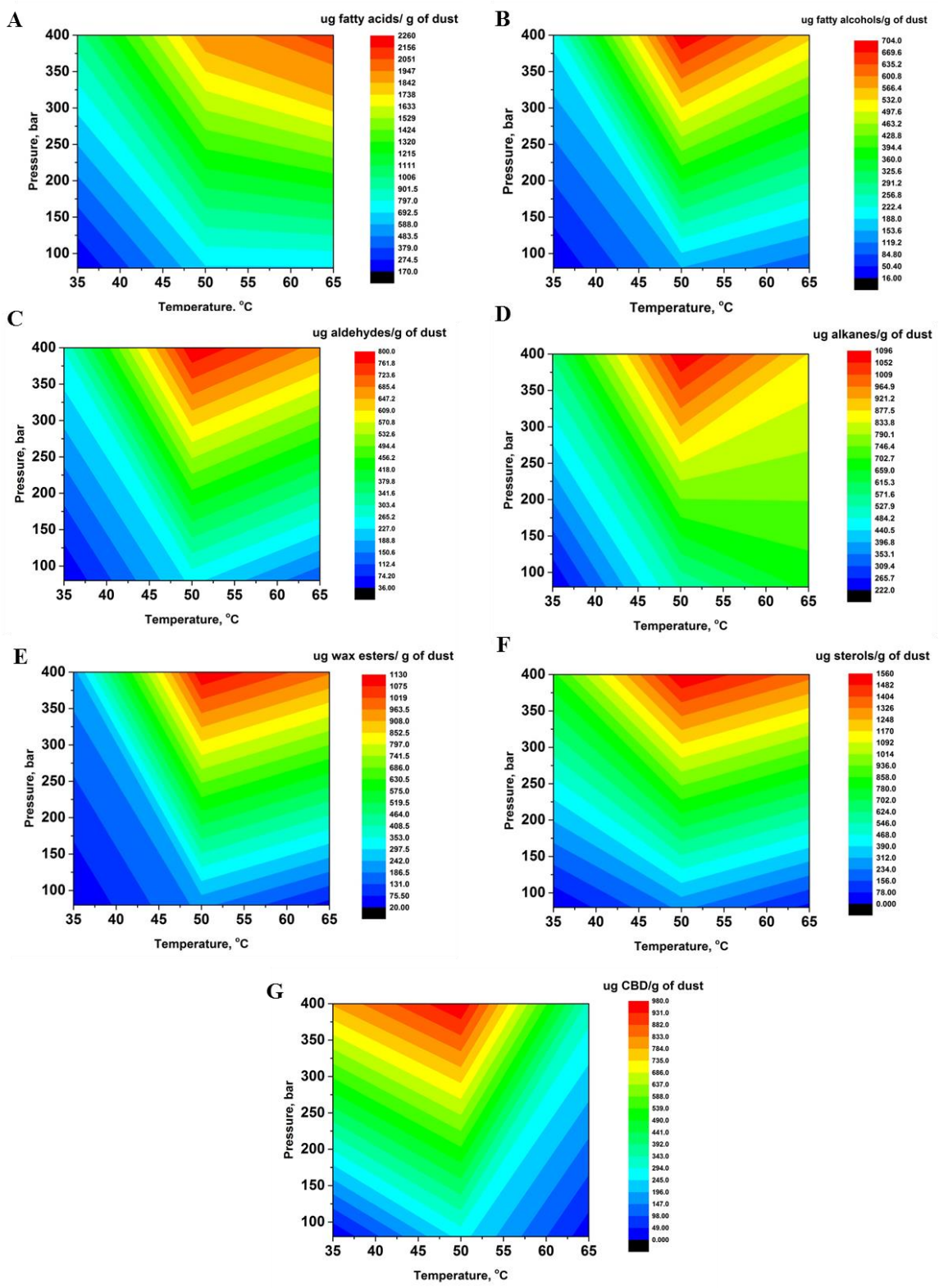


Figure 6-16 2D-contour plots showing the variation of A) Fatty acid concentration B) n-Policosanol concentration C) Fatty aldehyde concentration D) n-Alkane concentration E) Wax ester concentration F) Sterol concentration and G) CBD concentration with temperature and pressure.

Results (Figure 6-15) indicate that the highest yields of fatty acids were obtained with 400 bar and 65 ^{o}C (2252.8 ±108.5 µg/g of dust). Maheswari *et al.* modelled the solubility of fatty acids with $scCO_2$ at various pressures and temperatures using the

Chrastil equation.[216] They found that with fatty acids having chain lengths of C_{12}, C_{14} and C_{16}, increasing the pressure while keeping the temperature constant led to an increase in the solubility of fatty acids in $scCO_2$. Furthermore, they found that the solubility of C_{14} fatty acid greatly increased with an increase in temperature from 35 ^oC to 40 ^oC. They speculated that the increase in solubility occurred as a result of a phase change from solid tetradecanoic acid to liquid tetradecanoic acid. Similar results were obtained for C_{16} and C_{18} fatty acids.[216] This data correlates with what was observed here. Higher yields of saturated fatty acids were obtained at higher pressures and temperatures and it is speculated that the higher temperatures (65 ^oC) increase the solubility of fatty acids due to phase transitions (melting of the fatty acids) from solid to liquid (the melting point of hexadecanoic acid and octadecanoic acid are 62.9 ^oC and 69.3 ^oC respectively).

However, in the case of the other families of compounds, i.e. the long-chain fatty alcohols, aldehydes, *n*-alkanes, wax esters sterols and cannabinoids, the highest yields were achieved when conditions of 350 bar and 50 ^oC were utilised (771.2 ±21.7 μg/g of dust, 992.4 ±22.7 μg/g of dust, 1229.9 ±29.5 μg/g of dust, 1313 ±43.2 μg/g of dust, 1606 ±30.8 μg/g of dust and 1143.4 ±117.8 μg/g of dust respectively). It is not clear as to why these conditions yielded the highest concentrations of these groups of compounds. Besides the solubilities of the individual components in $scCO_2$, other factors have to be taken into consideration such as the interactions between the different components making up the epicuticular waxes as well as entrainer effects by the solute molecules in $scCO_2$.[72]

Figure 6-16 shows a number of 2-D contour plots, which try to model the yields of compounds extracted (in μg/g of dust) with varying pressure and temperature. Interestingly, for all groups of compounds, there seems to be a specific temperature (≈50 ^oC) at which the influence of pressure on yield changes. Once this temperature is reached the yield of compounds extracted drastically increases with increasing pressure.

It is interesting to note that, in the case of CBD (Figure 6-16 G), there seems to be a negative effect with temperature on the yield of CBD extracted. This contrasts to what was observed with the other groups of compounds, where temperature seems to aid in the extraction. However, higher yields were obtained at 400 bar and 35 ^oC than 400 bar and 65 ^oC and 50 ^oC seems to be the ideal temperature for the extraction of CBD. Solubility studies of CBD in $scCO_2$ have been carried out and seem to be in agreement with the data obtained in this current study.[298] In previous studies, the solubility of CBD

at varying pressures (113 – 206 bar) and temperatures (315, 326 and 334 K) was investigated. It was found that there was a higher CBD solubility at 327 K than at 334 K. It was concluded that liquid cannabinoids (such as CBD and cannabigerol (CBG)) display a decreased solubility in $scCO_2$ compared to solid cannabinoids. It was also concluded that the highest CBD solubilities were obtained at moderate temperatures.[298]

The melting point of CBD is 66 ^{o}C and the highest temperature utilised in the current optimisation study was 65 ^{o}C. Therefore, at this temperature the majority of CBD would be found in the liquid phase which would explain the decreased concentrations of CBD obtained when this extraction temperature was utilised (decrease in solubility of CBD). The solubility behaviour of CBD in $scCO_2$ is different to the psychoactive cannabinoids (Δ^9-THC and cannabinol), which show higher solubilities with higher temperatures. Therefore, the separation of CBD from the psychoactive cannabinoids by $scCO_2$ fractionation should be relatively straightforward.[298]

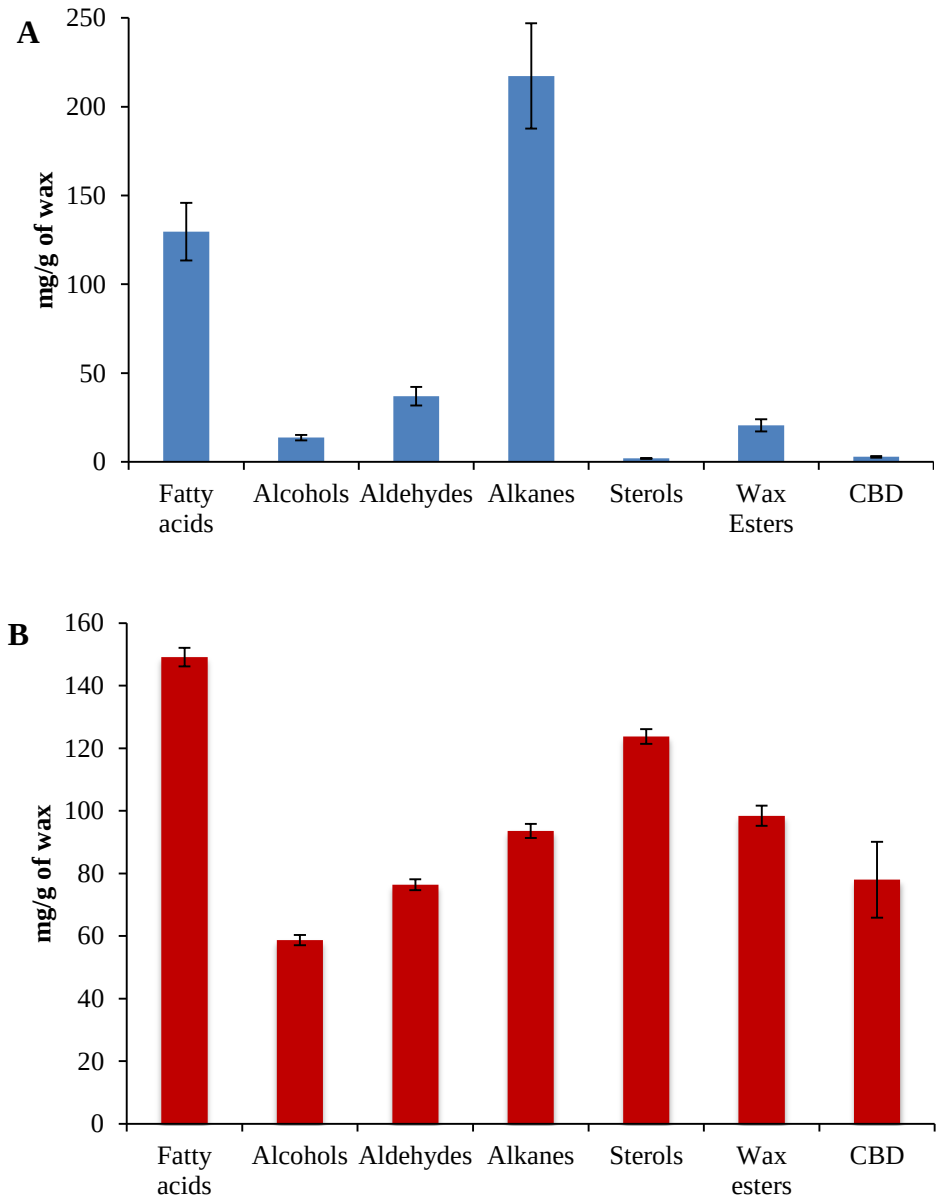


Figure 6-17 Quantities of groups of compounds obtained at A) 80 bar and 35 ^{o}C B) 350 bar and 50 ^{o}C in mg/g of wax.

Figure 6-17 illustrates the lipophilic composition of the $scCO_2$ extracts at 80 bar and 35 ^{o}C (lowest conditions) and 350 bar and 50 ^{o}C in mg/g of wax. It can be seen that in the extraction conducted at low pressure and temperature, the extract primarily constitutes *n*-alkanes (217.3 ±29.7 mg/g of wax) followed by long-chain fatty acids (129.7 ±16.2 mg/g of wax). The remaining groups of compounds are only found in minute quantities. It is known that certain groups of compounds such as sterols and cannabinoids show limited solubility at the supercritical point which explains the low abundance of these groups of compounds.[203, 215, 298] Therefore, at low extraction conditions, there is selectivity towards specific groups of lipophilic compounds. However, in the $scCO_2$ extracts obtained at 350 bar and 50 ^{o}C, there is a much wider variation in the groups of

compounds obtained with long-chain fatty acids the most abundant compounds. There is a significant increase in the concentrations of sterols, wax esters and CBD.

Therefore, it can be concluded that even though conditions of 400 and 65 ^{o}C gave rise to the highest % yield of crude wax, the factorial experimental design shows that, with the exception of the long-chain fatty acids, conditions of 350 bar and 50 ^{o}C gave rise to the highest quantities of *n*-policosanols, fatty aldehydes, *n*-alkanes, wax esters, sterols and cannabidiol.

Further work is needed to investigate why the specific temperature of 50 ^{o}C causes a drastic increase in the yield of compounds extracted with increasing pressure for the majority of compounds (fatty acids, alcohols, aldehydes, alkanes, wax esters and sterols).

6.5 Conclusion

In this chapter it has been demonstrated that the processing of hemp for fibre extraction and shiv separation generates significant amount of dust residues which contain large groups of valuable chemicals that could potentially be exploited. Interestingly, dust collected from different stages of the mechanical process gave rise to lipophilic extractives that have significantly different amounts of hydrophobic components. Dust sample 7 (collected from the main cyclone dust plant) gave rise to considerable quantities of long-chain saturated and unsaturated fatty acids, fatty aldehydes and wax esters, while dust from the picker dust plant (sample 4) was found to contain significant amounts of hydrocarbons. Dust sample 8 (obtained from the de-duster) was the only sample to contain significant quantities of cannabinoids (mainly CBD), while significant amounts of phytol were only found in the dust collected from the reserve hopper dust plant. This is interesting as it shows that the mechanical processing of hemp leads to the fractionation of lipophilic constituents, which should make the isolation and purification of these compounds easier.

$ScCO_2$ extraction was conducted on dust sample 8 (dust from the de-duster) and optimisation of the process was carried out using the factorial experimental design. Interestingly, it was found that, although conditions of 400 bar and 65 ^{o}C gave rise to the highest % yield of crude wax, in-depth analysis on the concentrations of each family of compounds showed that 350 bar and 50 ^{o}C gave rise to the highest yields of the majority of compounds (with the exception of fatty acids).

Chapter 7

Experimental

7 Experimental

7.1 Chemicals

7.1.1 Solvents

Organic solvents *n*-hexane, *n*-heptane and toluene were purchased from Fisher Scientific UK Limited. Acetone, dichloromethane (DCM), ethanol and ethyl acetate were obtained from VWR (International) chemicals. Deuterated (*d*-) chloroform was purchased from Aldrich.

7.1.2 Gases

Carbon dioxide (supercritical grade – 99.99%), helium, hydrogen and nitrogen were sourced from BOC Ltd. and all were used without any further purification.

7.1.3 Standards

Linoleic acid (99%), 1-octacosanol (90+%) and stigmasterol (95%) were obtained from Acros Organics. Azelaic acid (98%), dodecanal (≥95%), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, ≥99%), tetradecane (≥99%) and Vitamin K_1 (≥99%) were purchased from Aldrich. Stearic acid (98%) was obtained from Alfa Aesar. Hentriacontane (≥98%) and Δ^9-tetrahydrocannabinol solution (1 mg/ml ±5% in methanol, analytical standard for drug analysis) were purchased from Fluka. Friedelin (Technical grade) was obtained from Santa Cruz biotech. Cannabidiol solution (1 mg/ml in methanol, certified reference material), phytol (≥97%), β-sitosterol (≥70%) and stearyl palmitate (≥99%) were purchased from Sigma. ASTM® D5442 C16 – C44 Qualitative Retention Time Mix (containing docosane, dotriacontane, eicosane, hexacosane, hexadecane, hexatriacontane, octacosane, octadecane, tetracontane, tetracosane, tetratetracontane and triacontane; 8.3% (*w/w*) each component) was sourced from Supelco.

7.1.4 Other chemicals and materials

Carnauba wax was purchased from Acros Organics. Candelila wax was obtained from Aldrich. Beeswax was purchased from Fisher Scientific Limited. The cellulose thimbles for the soxhlet extractions were obtained from Fisher Scientific Limited. Phosphomolybdic acid hydrate was obtained from Aldrich. TLC K60 silica gel aluminium sheets were purchased from Merck. 100 g Biotage SNAP HP-Sil cartridge for flash chromatography were utilised for flash chromatography.

Zeolite, sodium disilicate (SKS 6), sodium percarbonate, Ufarol TCT 90A, sodium bicarbonate, sodium citrate, polypeptide Donlar, fatty acid methyl ester ethoxylates (FAMEE) and tetraacetylethylenediamine (TAED) were kindly provided by Ecover.

7.2 Biomass

7.2.1 Sugarcane

One-year old sugarcane plants were provided by Fazenda Cercadinho (Casa Branca, SP, Brazil). The leaves and rind were separated from the stalk. The rind, formed by a thin, darker and harder external layer of the plant stalk, was removed to leave the internal part of the stalk. This internal part was milled using a knife mill SL-31 (Solab Científica) to remove the majority of the sugar juice; the solid waste produced is the bagasse. All samples were rinsed to remove surface dust (leaf and rind), or residual sugar (bagasse). They were then dried in a convection oven at 60 °C for 24 hours. The biomass was removed, weighed and placed in the convection oven once more. At specific intervals the biomass was weighed until a constant weight was achieved. All the samples (rind, leaf and bagasse) were ground prior to supercritical extraction and passed through a 2 mm sieve.

7.2.2 Miscanthus

The leaf and stem samples used in this study originated from one *M. x. giganteus* genotype (H0118) and one of *M. sinensis* (H0121, also known as MS-90-2) from the WUR-PB collection of miscanthus. All shoots of a few fully-grown multi-tillered plants of both genotypes were collected by 5-11-2010 from a field nursery established in 2003 at Wageningen. At that time the shoots still had in part green leaves. The shoot material of each genotype was subsequently divided in a leaf and stem fraction and were than dried separately in a forces-air oven at 70 ^{o}C to constant weight. The dry matter content of stems from genotype H0118 (MG) and H0121 (MS) at harvest was 39.2% and 40.2%, respectively. The respective figures for the leaves from both genotypes were 35.2% and 38.9%. The stems and leaves of both genotypes were milled using a Glen Creston Ltd. cutting mill with a 1 mm grate.

7.2.3 Maize

Maize biomass was provided by the French National Institute for Agricultural Research (INRA). The maize was harvested after R6 stage (silage) and the cobs were removed. The husks, leaves and stems were separated and dried in a vacuum oven at 70 ^{o}C to

constant weight. These were then milled using a Glen Creston Ltd. cutting mill with a 1 mm grate.

7.2.4 Generic Miscanthus

Miscanthus giganteus was grown under field conditions in York, North Yorkshire, UK. The biomass was harvested and dried in a vacuum oven at 60 ^{o}C. The biomass collected represented the sixth year of harvest. A hammer mill was used to mill the sample giving particle sizes of 1 mm. The composition of miscanthus was found to be cellulose (34% ±2.5%), hemicellulose (42 ±2.5%), lignin (28 ±2%) and ash (0.83 ±0.03%). The initial weight prior to milling, the final weight, the milling time required as well as the electricity and heat requirements (calculated based on equipment specifications) were recorded.

7.2.5 Generic Maize stover

Maize stover was obtained from plants grown under field conditions near York (UK). The maize was harvested after R6 stage (silage) and the cobs removed. Stover samples were milled to 50 mm particles prior to pre-treatment using a hammer mill. The initial weight prior to milling, the final weight, the milling time required as well as the electricity and heat requirements (calculated based on equipment specifications) were recorded.

7.2.6 Hemp farm (*Santhica sp.)* samples

The hemp dust residues were obtained from different dust plants situated in various points along the Harrison Spinks processing facility (diagram of the processing facility may be found in Chapter 6) situated in Tadcaster, North Yorkshire, UK. The hemp dust samples that were obtained are labelled in Table 7-1. Originally ten samples were collected from the processing facility, however the first two samples (sample 1 and sample 2) were found to contain too many impurities and these were therefore discarded. The original hemp fibre plants are grown on 80-acres of arable land, on a farm in North Yorkshire.

Table 7-1 Dust samples and their origins from the hemp processing facility.

Dust sample no.	Dust plant
3	Fearnought dust plant (1st sample)
4	Picker dust plant
5	Fearnought dust plant (2nd sample)
6	Press dust plant
7	Main cyclone dust plant
8	Dust collection (from rotary screen of de duster)
9	Hammer mill dust plant
10	Reserve hopper dust plant

Dust samples were dried in a vacuum oven at 30 ^{o}C for several days to constant weight. Figure 7-1 is illustrates the various dust samples collected.

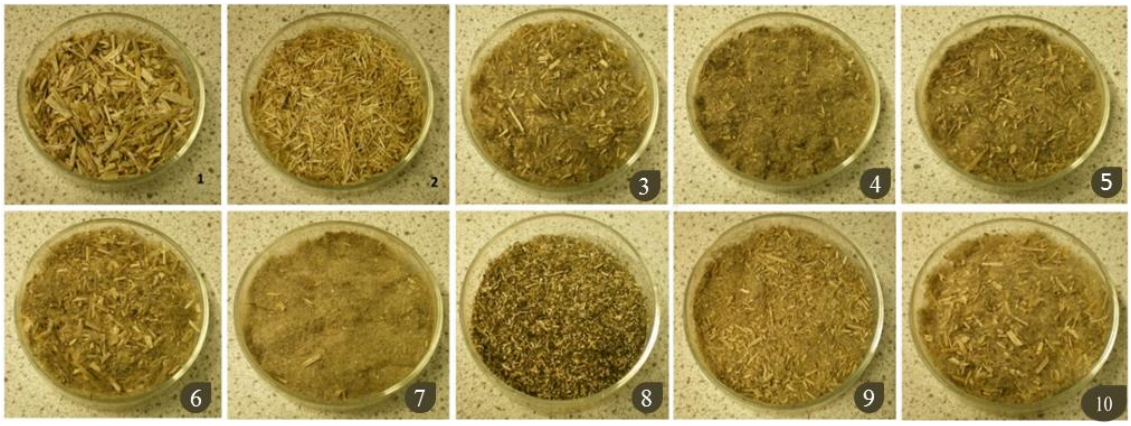


Figure 7-1 Dust samples collected from the hemp processing facility (Harrison Spinks). The first two samples contained too many impurities and were discarded.

7.3 Soxhlet extraction of biomass

11g of milled biomass (miscanthus, maize, sugarcane or hemp biomass) was placed in a Soxhlet thimble which was inserted into the Soxhlet apparatus. This was fitted to a 250

ml round bottom flask containing hexane (200 ml) or heptane (200 ml). A Radleys Discovery Technologies 2006T thermocouple was used to monitor the temperature during the extraction. The solution was allowed to reflux for 4 hours. The resulting solution was filtered (to remove any biomass present in the product) and the solvent was removed *in vacuo*. The samples were further dried at room temperature for 24 hours before weighing to ensure the removal of traces of residual solvent. The crude wax product was weighed and the % yield calculated. For each biomass (miscanthus, maize or sugarcane), three extractions were carried out and an average % yield calculated.

7.4 Lab-scale supercritical fluid extraction of miscanthus, sugarcane, maize and hemp biomass

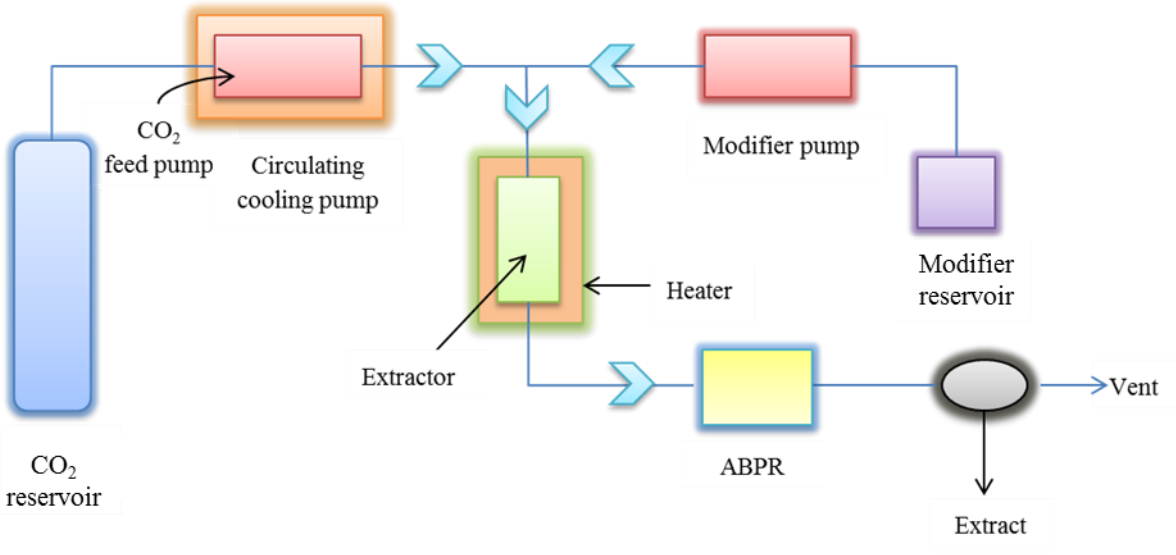


Figure 7-2 Simplified schematic illustrating the SFE-500 set-up

The $scCO_2$ extractions were carried out using a SFE-500 provided by Thar technologies. Supercritical fluid grade carbon dioxide (99.99%) was used to conduct the extractions. ≈100 g of milled miscanthus biomass (miscanthus, maize, sugarcane or hemp biomass) was placed into the 500 cm^3 extraction vessel and connected to the extraction system. Liquid CO_2 passed through a pre-heater set at the required temperature. The extractor was heated to the required temperature (range of extractions from 35 ^oC – 65 ^oC) and 5 minutes were allowed for it to equilibrate. An internal pump was used in order to obtain the required pressure (range of extractions from 80 bar – 400 bar). An automated back pressure regulator (ABPR) maintained the pressure throughout the system. The system was run in dynamic mode, in which the carbon dioxide which contained the epicuticular lipids, was allowed to flow into the collection vessel. A flow rate of 40 g min^{-1} of liquid

CO_2 was applied for miscanthus, maize and sugarcane, while 35 g min^{-1} was applied for hemp. The extraction was carried out for 4 hours.

When the extraction was terminated, depressurisation of the system was carried out over a period of 15 mins – 75 mins (depending on the pressure applied). The wax was collected by rinsing the collection vessel twice with approximately 100 ml of DCM. Anhydrous magnesium sulphate was added to any product samples that contained water (small amounts of water could be extracted by $scCO_2$ during the extraction process). The solution was subsequently filtered and DCM was passed through the filter paper to re-dissolve any wax found on the filter paper. The solvent was removed *in vacuo*. The crude wax product was weighed and the % yield was calculated. The plant material was removed and a brush was used to clean the extraction vessel. The system was washed in dynamic mode using a combination of $scCO_2$ and ethanol (10%) for 45 minutes at the extraction pressure. The pump supplying the modifier was then turned off and carbon dioxide was allowed to pass through the system for an additional 20 minutes.

7.5 Lab-scale supercritical extraction of generic miscanthus and maize stover (kinetics).

The supercritical carbon dioxide extractions were carried out using a SFE-500 provided by Thar technologies. Supercritical fluid grade carbon dioxide (99.99%) was used to conduct the extractions. ≈107 g of milled biomass (miscanthus or maize stover) was placed into the 500 ml extraction vessel and connected to the extraction system. The required temperature and pressure were applied. The reaction vessel was heated to the required temperature (50 ^{o}C for miscanthus, 65 ^{o}C for maize stover) and 5 minutes were allowed for it to equilibrate. An internal pump was used in order to obtain the required pressure (350 bar for miscanthus, 400 bar for maize stover). The pressure of the system was maintained by means of an ABPR. The system was run in dynamic mode, in which the carbon dioxide which contained the epicuticular lipids, was allowed to flow into the collection vessel. A flow rate of 40 g min^{-1} of liquid CO_2 was applied and the extraction was carried out for 4 hours. The system was stopped at specific time intervals; whereby samples were collected after 5, 10, 20, 30 and 40 mins followed by every 20 minutes for the remaining 3 hours and 20 mins. The % yield of wax extracted was recorded for each sample collected. When the extraction was terminated, depressurisation of the system was carried out over a period of 60 mins for the miscanthus biomass and 75 mins for the maize stover biomass.

7.6 Semi-pilot scale supercritical extraction and fractionation of maize stover biomass

Figure 7-3 A)Thar SFE extraction system used for the semi-pilot extraction and fraction of wax from maize stover. B) Fractional vessels set at the different pressures and temperatures.

The semi-pilot supercritical carbon dioxide extractions were carried out using a Thar technologies SFE-500. Supercritical fluid grade carbon dioxide (99.99%) was used to conduct the extractions. ≈1.8 kg of maize stover were loaded into 2 X 5 L extraction vessels and connected to the extraction system. The required temperature and pressure were applied (400 bar and 65 ^{o}C). The reaction vessel was heated to 65 ^{o}C and 5 minutes were allowed for it to equilibrate. Two internal pumps were used in order to obtain the required pressure (400 bar). The pressure of the system was maintained by an ABPR. The system was run in dynamic mode, in which the carbon dioxide which contained the lipids was allowed to flow into three fractionating vessels, each having a different pressure and temperature (150 bar/50 ^{o}C, 85 bar/35 ^{o}C and ATM/50 ^{o}C). The pressures of the three fractionating vessels were set manually (manual back pressure regulators). A flow rate of 300 g min^{-1} of liquid CO_2 was applied and the extraction was carried out for 4 hours. The lipids were collected by depressurising the fractionators. ≈20 kg of maize stover was extracted to ensure that sufficient biomass was available for downstream processing as well as enough wax was collected for applications testing.

7.7 Typical thin layer chromatography analysis (TLC) of lipids

The crude wax (30 mg) was dissolved in DCM (1 ml). The extract was applied approximately 1 cm from the bottom of the 20 cm x 20 cm silica gel 60 F_{254} TLC plate (Merck) using a micropipette. The separation on the silica gel plate was obtained in 15 minutes using 5 ml of developing solvent *n*-hexane: ethyl acetate (3:1) in a horizontal developing chamber. The plates were immersed in a staining solution (10% w/w phosphomolybic acid in ethanol). The plate was then immediately dried at 100 ^{o}C for around 2 minutes in order to reveal the position of the components. The positions were recorded and compared to the movement of standards conducted under identical conditions.

For the eluent that was obtained after flash chromatography, the different fractions were spotted onto the TLC plate without concentrating or diluting the samples. The fractions were combined based on their spots revealed by TLC and GC/GC-MS were used to analyse the combined fractions.

7.8 Flash chromatography to isolate triterpenoids in sugarcane leaf wax.

Flash chromatography was carried out using a Biotage® Isolera™ Four. The crude extract (1 g) was dissolved in 10 ml of DCM in a round bottom flask which contained a small amount of silica gel (high-purity grade for flash chromatography). The solvent was removed *in vacuo* in order to coat the wax onto the silica gel. The mixture was then transferred on top of a 100 g Biotage SNAP HP-Sil cartridge. The flow rate and the equilibration flow rate were set to 50 ml/min. The solvent system employed was hexane (75%) and ethyl acetate (25%). A step gradient system was selected in which the various solvent concentrations were typically changed in large increments and summarised in Table 7-1. The eluent was collected in 15 ml fractions.

Table 7-2 Step Gradient system (CV = Column volumes).

Gradient	Solvents	Mix.	Length (CV)
1	Hexane/ethyl acetate	6%	1.0
2	Hexane/ethyl acetate	6 – 42%	8.1

3	Hexane/ethyl acetate	42 – 50%	1.8
4	Hexane/ethyl acetate	50%	2.0
5	Hexane/ethyl acetate	50 – 57%	1.4
6	Hexane/ethyl acetate	57 – 60%	0.5
7	Hexane/ethyl acetate	60%	1.0
8	Hexane/ethyl acetate	60 – 64%	0.5
9	Hexane/ethyl acetate	64 – 95%	1.4
10	Hexane/ethyl acetate	95 – 100%	1.0

7.9 Analysis of wax extracts.

7.9.1 Derivatisation prior to HT-GC (High temperature-gas chromatography) analysis

30 mg of crude wax extract was silylated by adding 200 µL N,O-*bis*-(trimethylsilyl)-trifluoro-acetamide and 100 µL toluene. The solution was heated in an oven for 45 minutes at 75 ^{o}C.

7.9.2 High temperature- gas chromatography (HT-GC) method for analysis of waxes

HT-GC analysis was performed on an Agilent Technologies 6890N Network GC System. A ZB-5HT capillary column (30m x 250 µm x 0.25 µm nominal) was fitted at constant pressure of 22.35 psi. The carrier gas used was helium. The injector temperature and the flame ionisation detector temperature were maintained at 300 ^{o}C. The samples were injected by automated injection (1 µl injection volume) with a split ratio of 5:1. An initial oven temperature of 60 ^{o}C was maintained for 1 minute. The temperature was increased at a ramp rate of 8 ^{o}C min^{-1} until 360 ^{o}C and held at this temperature for 30 minutes.

Quantification of the lipid components was carried out by means of internal standard calibration and response factors (R_f). Six point linear calibration graphs were produced using external standards for the quantification of hydrophobic compounds (fatty acids,

alcohols, aldehydes, alkanes, wax esters, sterols and triterpenoids); whereby the mass ratios of the six samples were plotted against the area ratios. The R_f values for each external standard was obtained using the following equation:

$$\frac{Mass_{product}}{Mass_{standard}} = R_f \times \frac{Area_{Product}}{Area_{Standard}}$$

The standards that were used were octadecanoic acid, linoleic acid, azelaic acid, 1-octacosanol, dodecanal, hentriacontane, hexadecanoic acid octadecyl ester (Palmitic acid stearyl ester), stigmasterol and cannabidiol. Silylated calibration curves were also produced for the groups of compounds that were silylated (fatty acids, alcohols and cannabinoids).

7.9.3 HT-GC-MS (High temperature-gas chromatography mass spectrometry) procedure for analysis of wax

HT-GC-MS was performed on a Perkin Elmer Clarus 500 GC coupled with a Clarus 500 quadrupole mass spectrometer. This was fitted with a DB5HT capillary column (30 m x 250 μm x 0.25 μm nominal) at constant pressure of 22.35 psi. The carrier gas used was helium. The temperature of the injector was 300 ^{o}C and the flow rate was set to 1.2 ml/min. The initial oven temperature was maintained at 60 ^{o}C for 1 minute. The temperature was then ramped at a rate of 8 ^{o}C min^{-1} until 360 ^{o}C and held for 10 minutes. The Clarus 500 quadrupole mass spectra was operated in the electron ionisation mode (EI) at 70 eV, a source temperature of 300 ^{o}C, quadrupole at in the scan range of 30 - 1200 amu per second.

Another method was developed for the analysis of wax esters. The temperature of the injector was 380 ^{o}C and the flow rate was set to 1.2 ml/min. The initial oven temperature was maintained at 100 ^{o}C for 1 minute. The temperature was then ramped at a rate of 10 ^{o}C min^{-1} until 380 ^{o}C and held for 20 minutes. The Clarus 500 quadrupole mass spectra was operated in the electron ionisation mode (EI) at 70 eV, a source temperature of 300 ^{o}C, quadrupole at in the scan range of 30 - 1200 amu per second.

The data was collected with the PerkinElmer enhanced TurboMass (Ver5.4.2) chemical software and compounds were identified by analysing the mass fragmentation patterns, comparison of mass fragmentation patterns with spectra contained in the NIST library (v. 2.2) and by direct comparison with standard compounds.

7.9.4 Fourier Transform-infrared Spectroscopy (FT-IR analysis)

FT-IR analysis of the wax samples was carried out using a BRUKER Vertex 70 FT-IR spectrometer fitted with Specac Golden Gate ATR. The FT-IR was equipped with a DigiTect^{TM} DLATGS detector with integrated preamplifier scanning over a wavelength range of 4000 – 500 cm^{-1} at a resolution of 4 cm^{-1}. The spectra were collected using a rapid scan software running under OPUS 5.5 and the spectrum of each sample was calculated from an average of 16 scans.

7.9.5 Carbon-13 Nuclear Magnetic Resonance (^{13}C NMR)

^{13} C NMR spectra were obtained on a JNM-ECS 400 operating at 100 MHz, typically 2048 scans for ^{13}C NMR. The chemical shifts were recorded at 25 ^{o}C in deuterated chloroform ($CDCl_3$).

7.9.6 Differential scanning calorimetry (DSC) analysis

The thermal characteristics of the wax samples were measured on a MDSC Q2000 modulated differential scanning calorimeter. The wax extract (2.5 mg) was weighed into an open aluminium DSC pan and analysed under nitrogen gas using a three-stage heating profile in order to remove any prior thermal character. The DSC measurements were recorded against an empty aluminium reference pan in the final heating cycle of analysis. In determining the melting point, the cell was purged with a flow of nitrogen gas (150 mL/min) and was cooled by nitrogen (150 mL/min) in a refrigerated cooling system. The sample was heated from 20 to 105 ^{o}C at a rate of 10 ^{o}C/min. It was held at 105 ^{o}C for 1 minute and cooled to –10 ^{o}C at 10 ^{o}C/min. The sample was held at –10 ^{o}C for 1 min and then heated from –10 to 105 ^{o}C at 10 ^{o}C/min and held for 1 min at 105 ^{o}C. The melting point range was determined using the differential scanning calorimetry (DSC) curves of the last heating cycle.

Thermal characteristics of commercially available waxes (carnauba wax, candelila wax and beeswax) were also measured to compare with the waxes obtained in this study.

7.10 Applications testing of maize stover waxes

7.10.1 Foam measurements (Maize stover wax)

Foamability and foam stability measurements were analysed using a Krüss Dynamic Foam analyser DFA 100. A typical sample comprised 0.32 g of commercially available washing powder and 0.01 – 0.02 g of maize stover wax. A control sample was also prepared. 40 ml of tap water was added to each sample and heated to 50 ^{o}C. Air was

pumped through at a flow rate of 0.3 l/min for 18 seconds. Total height, foam height and liquid height as well as foam decay were measured.

7.10.2 Washing Machine tests

A standard formulation used in washing machine detergents was prepared by mixing together the following:

Table 7-3 Compounds that are found in the detergent formulation prepared.[218]

Compound	Mass (g)
Zeolite	21
SKS 6 (Sodium disilicate)	17
Sodium percarbonate	26
Ufarol TCT/90A	17.5
Sodium bicarbonate	9
Sodium Citrate	4
Polypeptide Donlar	4
FAMEE (Fatty acid methyl ester ethoxylates)	5
TAED (Tetraacetylethylenediamine)	3

The washing machine (Siemens SIWAMAT XLP 1330) was loaded with 10 towels and 10 dish towels along with the formulation, a soil ballast sheet (SBL 2004, 8 g soil piece, wfk-Testegewebe GmbH) and a known amount of the maize stover wax sample (1.5 g, 3 g). A 60 ^{o}C programme was set. A webcam was setup to monitor the washing machine run and measure the foam height. The experiment was run in triplicates. A number of blank runs (without the wax sample) were also carried out.

7.11 Biomass Thermochemical Pre-treatment of Maize stover

Biomass pre-treatment was carried out in a pressurised vessel with a 100 L total capacity. 2-3 kg of maize stover were loaded into the vessel together with 20-30 kg of water (pre-treatment media) in a 1/10 solid loading proportion. The pre-treatment was carried out at 160 ^{o}C ±5 ^{o}C for 40 minutes, after which the vessel was cooled down to the discharge temperature (80 ^{o}C). The solid and liquids were separated after pre-treatment and the solid was stored in a cold room while the liquor was frozen. A number of parameters were recorded including the initial biomass weight, final biomass weight (wet), water consumption, pre-treatment time, electricity and heat requirements (calculated based on equipment specifications).

7.12 Hydrolysis of maize stover

10% dry matter content was used in a total volume of approximately 30 litres to perform the enzymatic hydrolysis of the pre-treated biomass. Prior to hydrolysis at pilot scale, shake flasks experiments were done in order to determine the enzyme dosage and hydrolysis time. The enzyme cocktail used was Cellic CTec2 (Novozymes). Hydrolysis was done over a 24 h period using an enzyme dosage of 10% (based on dry weight of raw material), at 55 °C and 5.2 pH. Analyses of monosaccharides, polysaccharides and common inhibitory substances were carried out after enzymatic hydrolysis.

7.13 Fermentation of maize stover

7.13.1 Fermentation with *Saccharomyces cerevisiae* Thermosacc.

The hydrolysate was subjected to fermentation without filtration. The fermentation organism was *Saccharomyces cerevisiae* Thermosacc. Temperature, pH and agitation were set to 30 °C, 5.0 and 150 rpm respectively. Fermentations were performed in shake flasks.

7.13.2 Fermentation with *Starmerella bombolica*

100 ml of hydrolysate was fermented in 500 ml shake flasks, with the addition of 0.25% yeast extract and 0.025% urea (modified from Rau *et al.*).[299] The pH of the medium was adjusted to 3.5 with citric acid. After autoclaving the hydrolysate

was inoculated with *Starmerella bombicola* and grown for 3 days at 25°C and 120 rpm and the optical density (OD) and the Brix value were measured in the supernatant. In total 4 maize hydrolysate samples were tested, as well as glucose as a reference, in duplicate.

The overall yield was calculated as followed:

$$Yield\ (X/S) = \frac{OD_{t=0,5h} - OD_{t=48,5h}}{Brix_{t=48,5h} - Brix_{t=0,5h}}$$

Chapter 8

Conclusion and Further work.

8 Chapter 8

8.1 Chapter 2

In this study, different types of sugarcane waste (rind, leaves and bagasse) were analysed for their lipid content, in order to potentially add value to these waste agricultural residues. Epicuticular waxes from the rind, leaves and bagasse have been successfully extracted using $scCO_2$ as well as hexane Soxhlet extractions. Different groups of lipophilic compounds were determined including fatty acids, alcohols, aldehydes, alkanes, wax esters, sterols and triterpenoids. It has been shown that different botanical parts of the plant contain waxes that have a variation in the type and quantity of lipophilic molecules.

The rind wax was dominated by long-chain fatty aldehydes (C_{24} – C_{36}) and *n*-policosanols (C_{24} – C_{34}), which together constituted approximately 83% of the total wax composition. The predominant chain length for each group was octacosanal (375.2 ±86.9 mg/g of wax) and 1-octacosanol (237.9 ±30.3 mg/g of wax). The relatively large quantities of these groups of compounds should make for simple isolation and purification which could be of commercial significance due to their nutraceutical properties.[141, 148] Minor quantities of long-chain alkanes, long-chain fatty acids and wax esters were also identified.

In contrast, a much wider variety of compounds were found in the wax extracted from the leaves. This current study reports the first full characterisation of sugarcane leaf wax. The major group of compounds identified were the triterpenoids (constituting 169 mg/g of wax), followed by unsaturated and saturated fatty acids and phytosterols. Triterpenoids are of commercial interest due to their significant pharmaceutical properties.[172-174] This is the first time that the exploitation of leaf thrash for epicuticular waxes has been considered. The wax extracted from the bagasse was found to contain the highest amount of wax esters.

These results, together with the DSC thermographic data, demonstrate that different sugarcane waxes can be utilised in different applications. A comparison between $scCO_2$ and Soxhlet hexane was also carried out. It was found that, for the majority of groups of compounds, $scCO_2$ extracted larger quantities of lipophilic molecules than hexane.

8.2 Chapter 3

In a biorefinery, it is important to utilise as much of the biomass as possible. In this chapter, the incorporation of $scCO_2$ extraction of waxes as a first stage in an integrated biorefinery prior to destructive technologies was investigated. The type and quantity of waxy components from maize was determined and the effect of $scCO_2$ extraction on the downstream processing of the biomass was examined.

The first part of the chapter looked into characterising and quantifying the $scCO_2$-extracted hydrophobic components making up different parts of the maize stover i.e. the stems, husks and leaves. The highest yields of waxes were extracted from the leaves followed by the husks and stems. It was found that, even though there was a similarity in the types of compounds extracted from the different parts of the stover, there was a variation in the abundance of each family of compounds. This difference was correlated to the melting point profiles of the lipophilic extractives. In terms of wax composition (mg/g of wax), the husk wax had the highest amount of wax esters which resulted in it having the highest melting profile. Interestingly, high concentrations of unsaturated fatty acids (linoleic, linolenic and oleic acids) were found in the stem and husk waxes while only minute quantities were detected in the leaf extracts. The leaf extracts had the highest amount of *n*-policosanols, steroid ketones and *n*-alkanes.

In the second part of the chapter, it was demonstrated for the first time that $scCO_2$ extraction would have numerous benefits if integrated in a holistic maize stover biorefinery. As far as the author is aware, this is the first time that $scCO_2$ fractionation of wax into fractions having different waxy constituents was attempted. Three fractions (Fraction A, Fraction B and Fraction C) containing different compositions and melting points were obtained. Fraction A had the highest melting profile, consisting of high-molecular weight compounds. A phytosterol-rich fraction was obtained in Fraction B, which is of significant interest as phytosterols have numerous nutraceutical properties and the concentration of phytosterols in one fraction should make for simpler isolation and purification.[153, 154, 157, 300] Fraction C was demonstrated as an effective de-foaming agent that can be implemented in detergents in place of non-renewable defoaming agents. Lab-foam and washing machine tests indicated that Fraction C successfully reduces foam while having no negative effects on the performance of the detergent.

Finally, $scCO_2$ was found to have a positive effect on downstream processing of the maize stover biomass. Fermentation data for surfactant production shows that there was a higher glucose consumption (19%) and greater growth (18%) for the $scCO_2$-extracted maize stover when compared to non-treated maize stover. Furthermore, fermentation to ethanol showed a 40% increase in overall ethanol production using $scCO_2$-extracted maize stover as compared to the non-treated stover. Therefore this shows that $scCO_2$ extraction is ideal for the generation of valuable waxy compounds in a holistic biorefinery, enhancing fermentation processes and the economic viability of the whole biorefinery.

8.3 Chapter 4

Miscanthus is a promising feedstock for a holistic biorefinery due to its rapid growth and excellent productivity. This chapter showed the benefits of incorporating $scCO_2$ extraction of waxes in a miscanthus biorefinery. $ScCO_2$ extraction of waxes from the leaves and stems of two different miscanthus species (MS and MG) was carried out. Larger wax yields were obtained for the leaves when compared to the stems. There were appreciable differences in the complex lipid mixtures of long-chain fatty acids, policosanols, fatty aldehydes, hydrocarbons, sterols, steroid ketones and wax esters among other compounds between the two species. Analysis showed that there were higher quantities of linoleic acid, linolenic acid and oleic acid in the MS waxes than the MG waxes, while significantly higher amounts of long-chain aldehydes were found in the MG lipophilic extracts. There was also a variation in composition (mg/g of wax) between the stem and leaf waxes; with the former having higher quantities of phytosterols and wax esters.

Furthermore the pre-treatment of *miscanthus x giganteus* leaves with $scCO_2$ had beneficial effects in its downstream processing. Saccharification was found to be enhanced upon $scCO_2$ treatment as compared to untreated samples. A 22% increase in sugar release was observed with the $scCO_2$-extracted miscanthus leaves compared to no pre-teatment, while a 26% increase in sugar release was observed when compared to the ethanol-treated leaves.

8.4 Chapter 5

Although previous chapters have demonstrated that $scCO_2$ extraction is an effective pre-treatment step in a C_4 biorefinery, this work cannot be developed further unless an economic evaluation of the manufacturing process is taken into account. This is the first instance where an economical assessment of the $scCO_2$ extraction of waxes from biomass has been investigated, based on a methodology proposed Turton *et al.*[237] The extraction costs (COM) for the extraction of waxes from miscanthus and maize stover were estimated; whereby five main costs were used for the estimation: Fixed capital investment (FCI), labour costs (C_{OL}), raw material costs (C_{RM}), utility costs (C_{UT}) and waste treatment costs (C_{WT}). C_{UT} costs include costs associated with electric power used for the CO_2 pumps, refrigeration costs and costs associated with the CO_2 heaters. The costs estimated in this study are for an industrial supercritical plant with a yearly capacity of around 1600 tonnes of biomass.

The COM for miscanthus wax extraction was found to be €148 per kg of wax (or €620 per tonne of miscanthus biomass), with the FCI and C_{UT} contributing significantly to the COM. A number of assumptions were used to calculate the COM, the main ones being that the miscanthus biomass is milled not pelletised, all parts of the miscanthus plant were used and the miscanthus biomass is utilised solely for the extraction of waxes. In the case of the latter, $scCO_2$ extraction of waxes from biomass would be the first step of a holistic biorefinery and therefore the biomass would be passed along for further processing to produce fuels and chemicals leading to reduction of the C_{RM} costs. If the biomass was pelletised, miscanthus leaves were solely taken into consideration (4 times the wax yield) and the miscanthus biomass was combusted following the extraction for electricity generation, then the COM of the wax could be reduced to as much as €10.43 per kg of wax (based on 27% efficiency in the combustion of miscanthus biomass for electricity generation) or €4.77 per kg of wax (based on 43% combustion efficiency for electricity generation).

The COM was found to be significantly lower for maize stover (based on average C_{RM} for maize stover €88.89/ kg of wax) when compared to that of miscanthus (€148/ kg of wax). This is as a result of the lower C_{RM} costs of maize stover combined with a much higher % yield wax extract. Once again, the cost of extracting wax from maize stover can be reduced if pelletised leaves are solely used and the maize biomass is combusted following extractions to generate electricity. This would lead to an overall cost of

€10.87 per kg of wax (based on 27% combustion efficiency for electricity generation) and €4.57 per kg of wax (based on 43% combustion efficiency for electricity generation).

8.5 Chapter 6

This chapter looked at utilising hemp waste as a source of chemicals. During the processing of hemp for fibre production, large amounts of hemp dust are generated which could be a potential source of valuable chemicals and add value to this otherwise waste residue. Up to 33% of the hemp by mass is effectively lost during processing in the form of dust, which is left unutilised or burnt for energy. Hemp dust samples were collected from a hemp processing facility in North Yorkshire (Harrison Spinks) and heptane Soxhlet extractions and $scCO_2$ extractions were carried out.

Herein, it was demonstrated that dust residues from fibre processing contain significant quantities of high value lipophilic molecules including fatty acids, policosanols (fatty alcohols), fatty aldehydes, hydrocarbons, sterols, triterpenoids and cannabinoids (Cannabidiol (CBD)). Interestingly, dust collected from different stages of the mechanical process gave rise to lipophilic extractives that have significantly different amounts of these hydrophobic components. This is interesting as it shows that the mechanical processing of hemp leads to the fractionation of lipophilic constituents, which should make the isolation and purification of these compounds easier. Of particular interest is CBD, which has low toxicity and is non-psychotropic. CBD has attracted much attention for clinical-level studies on its therapeutic efficacy (alone or combined with various cannabinoids) in the treatment of a variety of central nervous system (CNS) and peripheral disorders.

$ScCO_2$ extraction was conducted on dust samples and optimisation of the process was carried out using the factorial experimental design. It was found that conditions of 400 bar and 65 ^{o}C gave rise to the highest % yield of crude wax; however in-depth analysis on the concentrations of each family of compounds showed that 350 bar and 50 ^{o}C gave rise to selective extraction of higher value compounds (with the exception of fatty acids). The results obtained in this study may open new doors in hemp waste utilisation.

8.6 Further work

8.6.1 Characterisation and quantification of high-molecular weight compounds

It was found that it was not possible to characterise and quantify all of the molecules constituting the epicuticular waxes. A considerable portion of the miscanthus wax (leaves and stem wax samples for both miscanthus species) was unidentified while approximately 21% of maize stover wax Fraction A by composition was identified and quantified. This suggests that the remaining composition consists of high-molecular weight compounds such as phospholipids and triglycerides. These molecules cannot be determined by GC and GC-MS and therefore further work could involve investigating the use of other analytical techniques (gas-liquid chromatography, high performance-liquid chromatography, supercritical fluid chromatography, size-exclusion chromatography etc.) to identify and quantify these high-molecular weight compounds so that a complete wax profile can be obtained.

8.6.2 Further investigations into the defoaming properties of maize stover wax Fraction C

A full-detailed mechanism for the defoaming properties of the maize stover wax Fraction C should be carried out. A mechanism has been proposed, whereby the large quantities of saturated and unsaturated fatty acids become saponified in the aqueous washing powder solution, forming salts (due to the presence of calcium and magnesium in the hard water) which act as defoamers. However, it would be interesting to determine in greater detail what brings about the defoaming effect by looking at the individual compounds constituting the wax (using standards). Comparing the defoaming results of the standards with that of the wax as a whole would make it possible to conclude whether the defoaming effect is brought about by individual groups of compounds or through a synergistic effect by the different groups of compounds constituting the wax.

In the current study, the quantity of maize stover wax used in the washing machine runs was 3 g and 1.5 g, making up ≈2.7% and ≈1.4% of the total detergent formulation respectively. Further work could involve using smaller amounts of wax in the washing machine runs (since antifoam could make up 0.8 – 4 % of the total detergent formulation) to see whether the same defoaming effect is observed with smaller quantities of wax. Reducing the amount of wax utilised for each washing machine run would have significant positive effects from an economical viewpoint.

8.6.3 Further work on maize stover wax Fractions A and B.

It would also be interesting to conduct application testing on the other maize stover fractions (Fraction A and Fraction B). This could involve isolating and purifying the phytosterols found in wax Fraction B (which had large quantities of phytosterols) followed by subsequent enzymatic esterification reactions to produce phytosteryl esters, which have a much higher solubility in the human digestive system, and would therefore greatly improve the nutraceutical effects found in the phytosterols.

8.6.4 Effect of $scCO_2$ extraction on the downstream processing of sugarcane waste.

In the current study, the effects of $scCO_2$ extraction on the downstream processing of miscanthus and maize stover were investigated. Unfortunately, it was not possible to carry out this study on sugarcane due to insufficient quantities of biomass. Therefore future work could involve carrying out the same investigation on sugarcane bagasse, to see the effects that $scCO_2$ extraction has on the hydrolysis of bagasse and subsequent fermentation to produce biofuels.

8.6.5 Semiochemical properties of hemp wax?

It has been demonstrated that long-chain hydrocarbons can acts as semiochemicals and an alkane fraction isolated from wheat straw wax was successfully used as a natural aphid insecticide.[126] The lipophilic extractives in the hemp dust were found to contain considerably large amounts of long-chain hydrocarbons compared to the C_4 waxes. It would therefore be interesting to isolate the hydrocarbon fraction found in the hemp lipophilic extractives and test this as a natural insecticide.

8.6.6 Fractionation of CBD from the hemp lipophilic extractives.

CBD has attracted much attention for clinical-level studies on its therapeutic efficacy (alone or combined with various cannabinoids) in the treatment of a variety of central nervous system (CNS) and peripheral disorders. One of the hemp dust samples was found to contain substantial amounts of CBD. Optimisation studies have shown that the solubility of CBD decreases in $scCO_2$ with increasing temperature, which was contrary to the other groups of molecules found in the lipophilic extractives. It would therefore be interesting to carry out a $scCO_2$ fractionation of the crude hemp dust wax in order to try and isolate the CBD from the remainder of the lipophilic constituents in order to get a CBD fraction of relatively high purity which would be of considerable value.

8.6.7 Extraction of lipophilic molecules from the whole plant or parts of the plant

The work carried out on hemp involved extracting lipophilic extracts from hemp dust residues generated during the mechanical processing of hemp for fibre extraction. It would be interesting to carry out extraction of waxes from the actual plant (raw material) or different parts of the plant (leaves, stems etc.) in order to compare the waxy content in the plant with the dust residues generated during hemp processing in order to see whether there is loss of lipophilic molecules during fibre extraction.

Appendix A

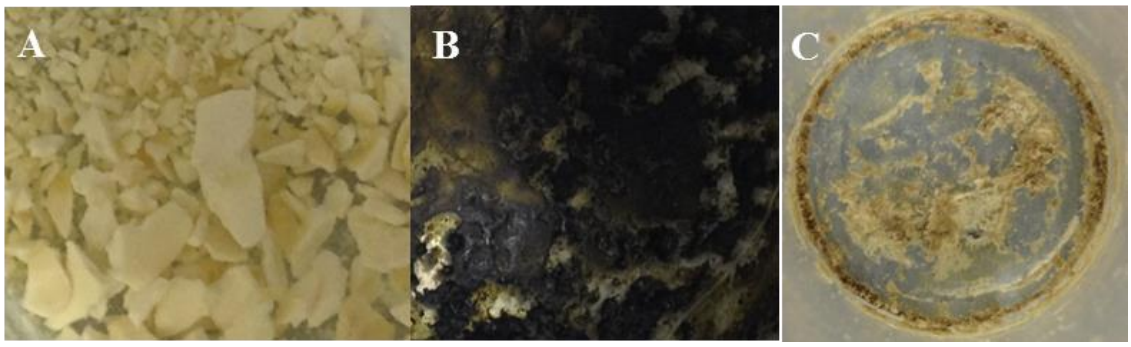


Figure D-1 Textures of A) Sugarcane rind wax B) Sugarcane leaf wax C) Sugarcane bagasse wax.

List of abbreviations

$	Dollars
$\boldsymbol{E_T^N}$	Normalised polarity
€	Euro
Δ	Delta
≈	Approximately
1^o	Primary
2-D	Two dimensional
2^o	Secondary
ASTM	American Society for Testing and Materials
ATM	Atmospheric pressure
BSTFA	Bis(trimethylsilyl)trifluoroacetamide
C	Carbon
CBD	Cannabidiol
CER	Constant extraction rate
Cm	Centimetre
CO	Carbon monoxide
CO_2	Carbon dioxide
C_{OL}	Cost of operational labour
COM	Cost of Manufacturing
COP	Coefficient of performance

C_{RM}	Cost of raw materials
C_{UT}	Cost of utilities
C_{WT}	Cost of waste treatment
d-	Deuterated
DC	Direct costs
DCM	Dichloromethane
DSC	Differential scanning calorimetry
EI	Electron ionisation
EPA	Environmental Protection Agency
EU	European Union
FAE	Fatty acid elongase complex
FAMEE	Fatty acid methyl ester ethoxylates
FAO	Food and Agricultural Organisation of the United Nations
FAS	Fatty acid synthase complex
FC	Fixed costs
FCI	Fixed capital investment
FER	Falling extraction rate
FI	Field ionisation
FT-IR	Fourier transform-Infrared spectroscopy
G	Gram
GC	Gas chromatography
GE	General Expenses

H	Hour
H	Hydrogen
Ha	Hectare
HDL	High density lipoprotein
HT-GC	High temperature-gas chromatography
HT-GCMS	High temperature-gas chromatography mass spectrometry
i.e.	That is
IR	Infrared spectroscopy
K	Kelvin
Kcal	Kilocalorie
kg	Kilogram
KI	Kovat's Index
kJ	Kilojoule
KOH	Potassium hydroxide
Kw	Kilowatt
L	Litre
LDL	Low density lipoprotein
LED	Light emitting diode
m	Metres
M. giganteus	*Miscanthus x. giganteus*
M. sinensis	*Miscanthus sinensis*
m/z	Mass-to-charge-ratio

M^+	Molecular ion
MA	Non-treated maize stover
MA $scCO_2$	Supercritical extracted maize stover
mg	Milligram
MG	*Miscanthus x. giganteus*
min	Minute
misc.	Miscanthus
MJ	Megajoule
ml	Millilitre
mm	Millimetre
mol	Molar
MS	Mass spectrometry
MS	*Miscanthus sinensis*
MT	Metric tonne
N	Newton
NaOH	Sodium hydroxide
NIST	National Institute of Standards and Technology
nm	Nanometre
Nmols	Nanomoles
NMR	Nuclear Magnetic Resonance
no.	Number
^oC	Degrees Celsius

OD	Growth
odt	Oven dried tonne
P	Pressure
p-	Para
P_c	Critical pressure
R_f	Response factor
rpm	Revolutions per minute
s	Second
S. bombolica	*Saccharomyces bombolica*
scCO_2	Supercritical carbon dioxide
SCF	Supercritical fluid
SFE	Supercritical extraction
SKS-6	Sodium disilicate
T	Temperature
TAED	Tetraacetylethylenediamine
T_c	Critical Temperature
THC	Δ^9-Tetrahydrocannbinol
TLC	Thin layer chromatorgraphy
TMS	Trimethylsilane
TR	Trace amount
TS	Total sugars
UK	United Kingdom

US	United States
v.	Version
v/v	Volume/volume
VLCFA	Very long chain fatty acid
w/w	Weight/weight
y	Year
α	Alpha
β	Beta
γ	Gamma
µ	Microgram
µl	Microlitre
π	Pi

Chapter 9

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9 References

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