Characterisation and supercritical extraction of lipophilic molecules from C4 biomasses

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Abstract

Herein, we describe the characterisation of a range of hydrophobic molecules from supercritical CO_2 extracts of C_4 biomass, namely miscanthus, maize and sugarcane bagasse. In addition to the characterisation of these molecules, this report highlights relevant high-value applications for the extracted products, including use in pharmaceuticals, personal care products and cosmetic applications. A range of interesting molecules have been identified in the wax extracts including, long-chain *n*-alkanes, fatty acids, fatty alcohols, wax esters, sterols, triterpinoids, beta-diketones amongst other compounds.

1. Introduction

The preliminary scope of this work was to study the lipid metabolite profile of three different types of C4 biomass, miscanthus, maize and sugar cane bagasse; in order to identify and extract high-quality compounds which would potentially add value to these feedstocks. The present work forms part of work package 6 (WP6) which deals with generating added value from biomass. The work highlighted in this report focussed on identifying added value products from the hydrophobic component of the C4 grasses and therefore dealt with extracting waxes and wax-like products from maize, miscanthus and sugar cane bagasse and also identifying possible commercial applications for these products. 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.[1] However, often the term ‘plant wax’ is used collectively to describe the complex mixture of surface lipids covering the aerial tissues of herbaceous plants.[2]

Two types of extractions were carried out: (i) Soxhlet extractions with hexane (ii) supercritical carbon dioxide ($scCO_2$) extraction. To the author’s knowledge, there is no published work dealing with the extraction from miscanthus, maize or sugarcane bagasse using $scCO_2$. Furthermore, no work has been reported on analysing the separated botanical components. Therefore, different parts of the plant, such as the leaves and stems were separated and looked at individually.

Three types of miscanthus were investigated: *M. giganteus* (H0118) and two genotypes of *M. sinensis* (H0121 and H0156). The leaves and stems of each were extracted using two different solvents: (i) Hexane and (ii) $scCO_2$. The different components of the maize waste, i.e. the leaves, stems and husks were separated and analysed individually. Due to restrictions

on the transport of biomass from Brazil, it was a significant challenge to obtain samples of sugarcane bagasse, leaves and bark for extraction and analysis within the EU. Sugarcane bagasse samples were obtained from Processum (Sweden) and results of extraction are demonstrated within this report. The extractions of sugarcane leaves and bark were conducted at the University of Sao Paulo (USP) during a student exchange visit between March to May 2012. The detailed analysis of the sugarcane leaves and bark will not be the subject of discussion in this report but, will be presented at the annual sunlibb meeting in Paris September 2012.

2. Experimental

2.1 Soxhlet extraction:

11g of milled biomass (miscanthus, maize or sugarcane bagasse) 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). The solution was allowed to reflux for 4 hours. The resulting solution was filtered and the hexane was removed *in vacuo*. The crude wax product was weighed and the % yield calculated. The results are summarised in the Results section. For each biomass (miscanthus, maize or sugarcane bagasse), three extractions were carried out and an average % yield calculated.

2.2 Supercritical Fluid Extraction

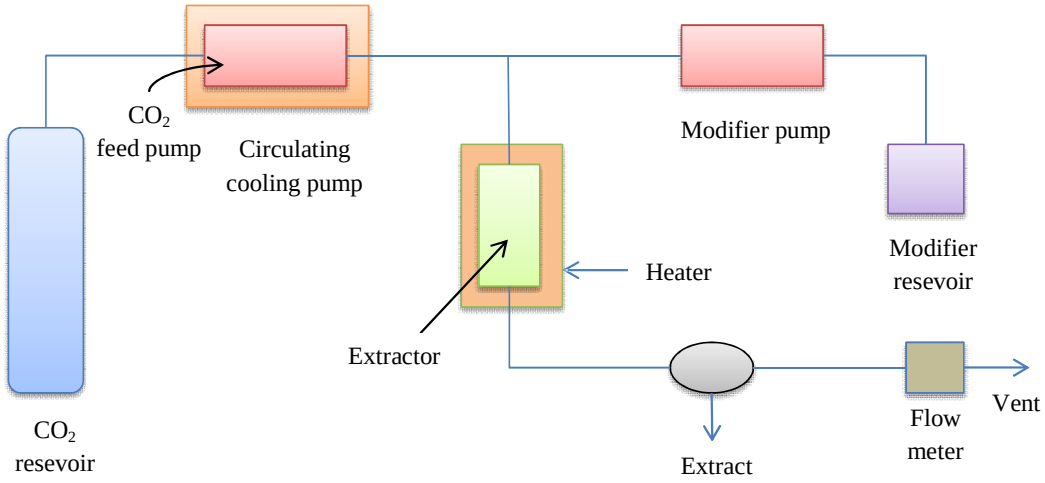


Figure 2.01 Schematic of the supercritical extraction system.

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. 100 g of milled biomass (miscanthus, maize or sugarcane bagasse) 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 50 ^{o}C and 5 minutes were allowed for it to equilibrate. An internal pump was used in order to obtain the required pressure (350 bar). The system was run in dynamic mode, in which the carbon dioxide which contained the epicuticular lipids, were 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. When the extraction was terminated, depressurisation of the system was carried out over a period of 4 hours. The wax was collected by rinsing the collection vessel twice with approximately 100 ml of DCM. The solvent was removed *in vacuo*. The crude wax product was weighed and the % yield was calculated. The results are summarised in the Results section. 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 supercritical carbon dioxide 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.

2.3 HT-GC (High temperature-gas chromatography procedure for analysis of wax

HT-GC analysis was performed on an Agilent Technologies 6890N Network GC System. In order to obtain adequate separation of the key reaction components, a general method was created which involved the following:

A ZB-5HT capillary column (30m x 250 μm x 0.25 μm nominal) was fitted at constant pressure of 22.35 psi. 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 40:1. 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.

2.4 HT-GC (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 EI-mass spectrometer. This was fitted with a DB5HT capillary column (30m x 250 μm x 0.25 μm nominal) was fitted at constant pressure of 22.35 psi. 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.

3. Results and Discussion

The primary analytical tools that were used for analysing the lipid components were gas chromatography and mass spectrometry. Identification of the various hydrophobic compounds was carried out principally by field-ionisation mass spectrometry (FI-MS) and electron-ionisation mass spectrometry (EI-MS). The EI-mass spectra and FI-mass spectra were obtained by utilizing a high temperature gas chromatography coupled to either a field impact mass spectrometer or an electron-ionisation mass spectrometer.

The compounds were identified mainly from 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 being investigated. Other methods implemented for identifying the molecules include calculating the Kovat's indices (KI's) of the various peaks present in the GC chromatograms and comparing them with known KI's of various lipid compounds in published literature, 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 and standards.

3.1 Extract yields of Miscanthus

As stated previously, two species of Miscanthus were investigated: *Miscanthus giganteus* (H0118) and 2 genotypes of *Miscanthus sinensis* (H0121 and H0156). The leaves and stems of each were separated and analysed individually. Figure 1 illustrates the total % yield of crude extract obtained by soxhlet extraction and supercritical extraction for the stems and leaves for each genotype.

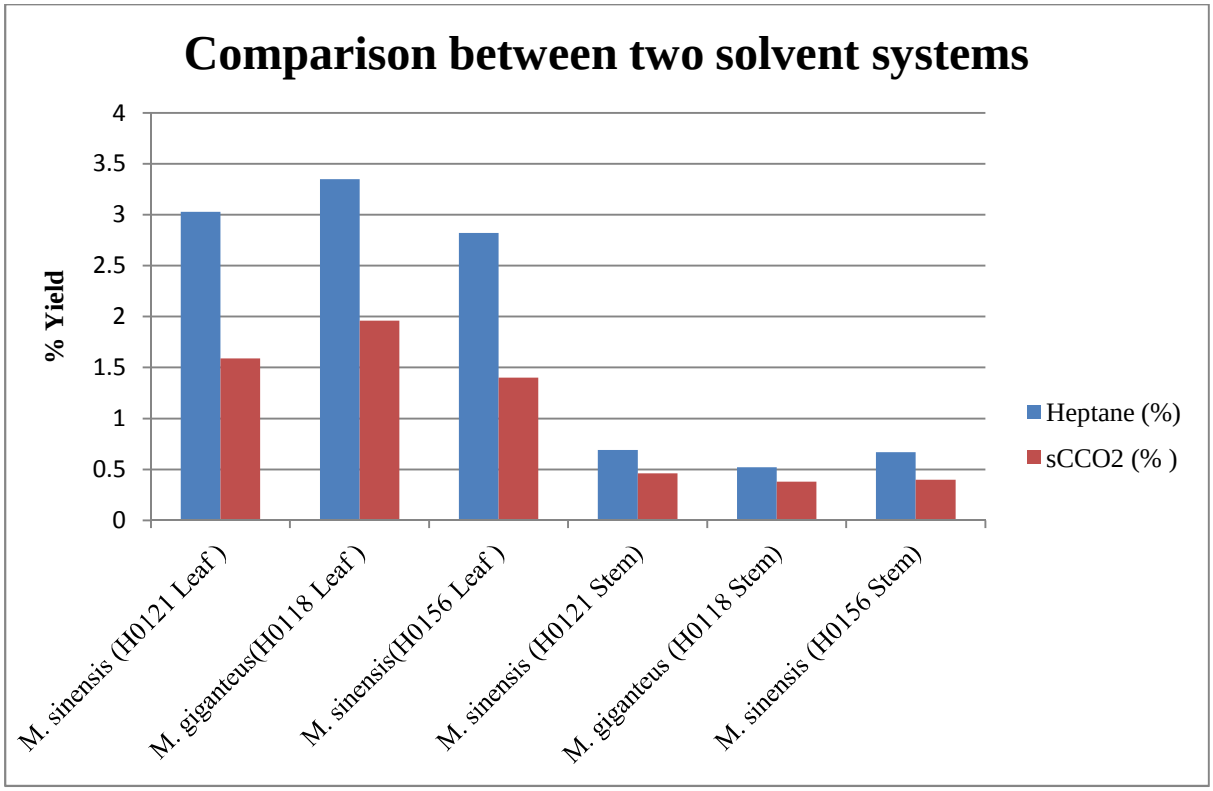


Figure 3.01 % yield of epicuticular wax obtained from the leaves and stems from each genotype of miscanthus.

The results obtained indicate that soxhlet extractions of both the leaves and stems of all genotypes have a much higher % crude yield compared to supercritical extractions (Figure 3.01). Soxhlet extractions with heptane are known to co-extract a number of polar compounds such as pigments (namely chlorophyll), sugars and polar lipids.

In both types of extractions and for all genotypes, a much higher % yield was obtained from the leaves than the stems. The leaves have a much higher surface area to volume ratio and as such are more prone to loss of water and are more susceptible to mechanical damage from various environmental factors. Therefore larger quantities of wax are required in order to prevent loss of water via transpiration, minimise leaching losses and prevent mechanical injury from environmental conditions. The leaf surface wax is particularly important for minimizing the amount of water lost through evaporation, especially when the stomatal pores of the plant are closed in response to reduced turgor.

The results also show a trend for both types of extractions, in which the highest % yield of wax extracted from the leaves occurred with leaves of *Miscanthus giganteus* (H0118) (3.35% for soxhlet extraction, 1.96% for supercritical extraction) while the lowest % yield was

obtained from leaves of *Miscanthus sinensis* genotype 2 (H0156) (2.82% for soxhlet extraction, 1.4% for supercritical extraction). In contrast, for both types of extractions the highest % yield of wax extracted from the stems of *Miscanthus sinensis* (H0121) (0.69% for soxhlet extraction, 0.46% for supercritical extraction) while the lowest % yield was obtained from stems of *Miscanthus giganteus* (H0118) (0.52% for soxhlet extraction, 0.38% for supercritical extraction).

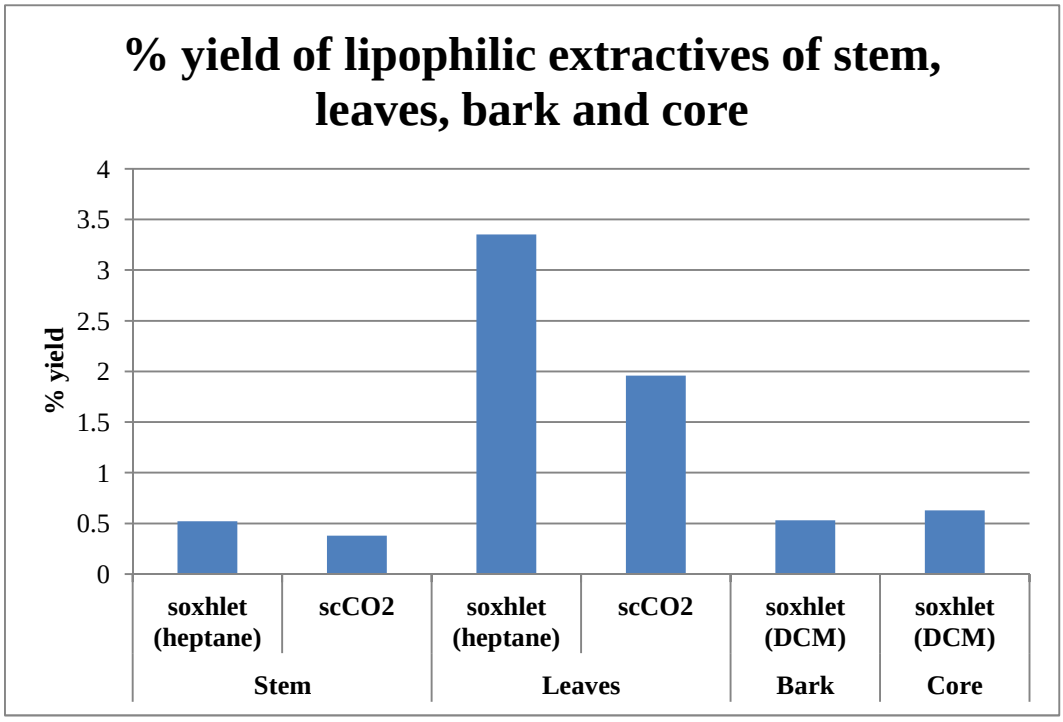


Figure 3.02 Comparison of % yield of lipophilic extractives of stem, leaves, bark and core of *M. giganteus*. DCM extraction yields taken from *Villaverde et al.*.[3]

Villaverde *et al.* investigated the chemical composition of the lipophilic fraction of the bark and core of *Miscanthus giganteus*.[3] Figure 3.02 compares the % yields of crude lipophilic extractives of the stem, leaves (obtained in this investigation) and the core/bark data as reported by Villeverde *et al.*. The % yields obtained by Villeverde *et al.* for the bark (0.53%) and core (0.63%) are comparable with the % yield of crude lipophilic extractive obtained for the stem (0.52%) using soxhlet extraction with heptane.[3]

3.2 Extract yields of Maize

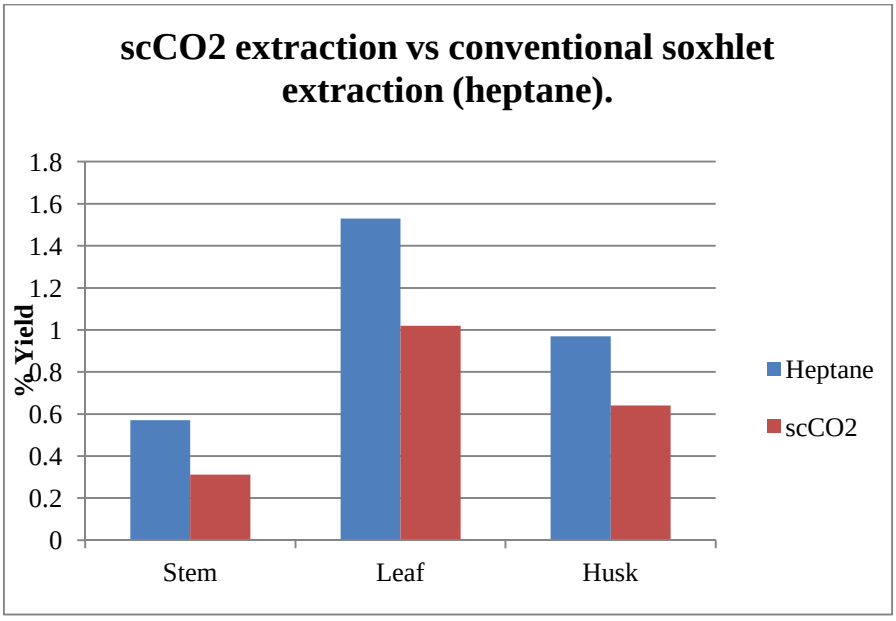


Figure 3.03 % yield of epicuticular wax extracted from the stems, leaves and husks of maize by conventional soxhlet extraction and $scCO_2$ extraction.

Figure 3.03 illustrates the % yield of epicuticular wax extracted from the stems, leaves and husks of maize by conventional soxhlet extraction and $scCO_2$ extraction. As was the case with miscanthus, the highest % yield of wax was obtained from the leaves, followed by the husk and stem.

3.3 Extract yields of sugarcane and sugarcane bagasse.

Wax was extracted from sugarcane bagasse by soxhlet extraction and supercritical carbon dioxide extraction. The percentage yield of wax extracted was found to be 0.68% for soxhlet extraction and 0.53% for supercritical extraction. In addition, soxhlet extractions were carried out on sugarcane leaves and bark using hexane. The percentage yield extracted from the bark was found to be 1.38% while that of the leaves is 2.45%.

3.4 Characterisation of extracts

3.4.1 Long chain Hydrocarbons (*n*-alkanes).

The long-chain *n*-alkanes were identified by means of gas chromatography coupled mass spectrometry and by calculating Kovats indices.

In the EI-mass spectrum of long-chain alkanes, the molecular ion peak was not identified as it was either absent or present in low intensities. However, the molecular ion peaks were identified from the FI-mass spectra and therefore the chain-length of the *n*-alkanes could be determined. 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_nH2_{n-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.

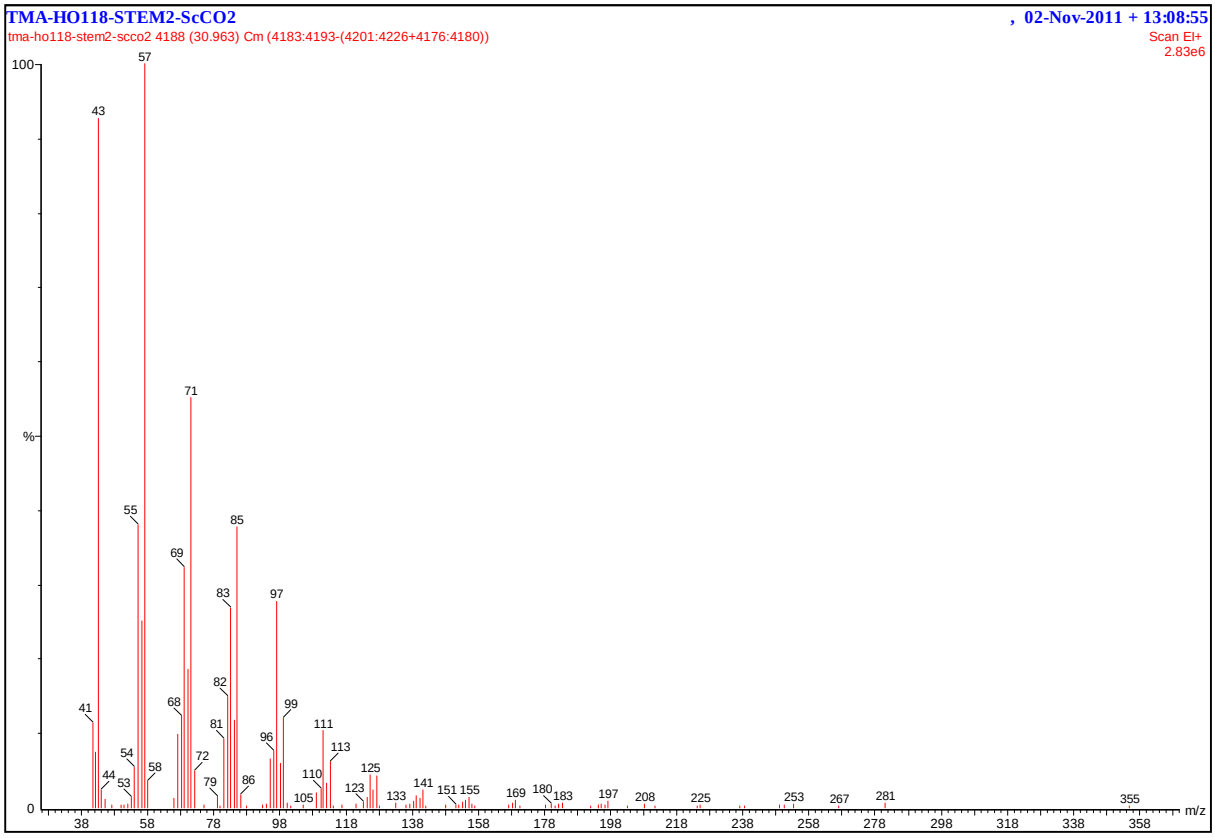


Figure 3.04 Mass spectrum of nonacosane (C29) from *Miscanthus giganteus*.

3.4.1.1 Miscanthus

Miscanthus sinensis (H0121) demonstrated *n*-alkanes with chain lengths ranging from C_{23} to C_{31}. The *n*-alkanes within *Miscanthus giganteus* (H0118) had chain lengths which varied

from C_{23} to C_{29}, while *Miscanthus sinensis* (H0156) exhibited *n*-alkanes having chain lengths in the range of C_{23} to C_{31}.

In the stem of *Miscanthus sinensis* (H0121), the alkane pattern consists of long-chain alkanes containing chain lengths of C_{23}, C_{27} and C_{29}. The dominant alkane was found to be nonacosane which constituted up to 51.9% of the total long-chain hydrocarbons in the stem. The leaves were characterised by long-chain hydrocarbons varying in chain length from C_{23} to C_{31}. The major alkane in the leaves was found to be hentriacontane, which constituted 41.1% of the total alkanes in the leaves.

A similar hydrocarbon pattern was found in the stems and leaves of *Miscanthus sinensis* (H0156). In the stem, the alkanes that were found in the extract were C_{23}, C_{27} and C_{29}, with the latter being the major alkane in the stem (62.2%). In the leaves, C_{25} and C_{31} alkanes were also present, with the latter constituting up to 46.9% of the total alkanes.

On the other hand, in the stem of *Miscanthus giganteus* (H0118), heptacosane and nonacosane were found in similar amounts, comprising approximately 28.7% and 29.3% of the total hydrocarbons respectively. In the leaves of *Miscanthus giganteus*, the major alkane was found to be nonacosane (44.9% of the total alkanes).

Not much is known about the long-chain hydrocarbon pattern in Miscanthus. In the study carried out by Villeverde *et al.* only one long-chain alkane was found in the bark and core of *Miscanthus giganteus* when extracting with DCM and in very small amounts. Heptacosane was found in concentrations of 11 mg/kg in the bark and 2 mg/kg in the core.[3] Therefore, a wider range of long-chain hydrocarbons were extracted from the leaves and stems using supercritical carbon dioxide.

3.4.1.2 Maize

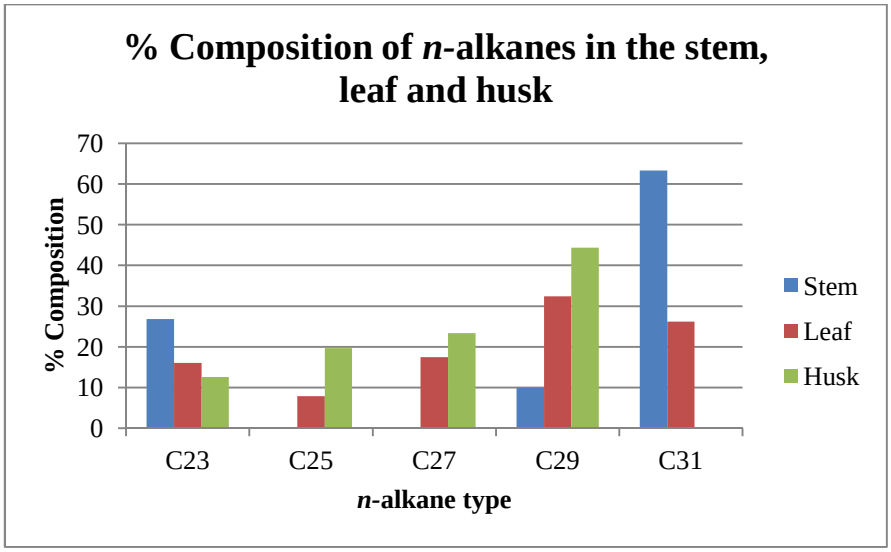


Figure 3.05 % Composition of *n*-alkanes in the stem, leaf and husk of maize.

Figure 3.05 indicates the type of hydrocarbons found in the stems, leaves and husks of maize. Similarly to miscanthus, supercritical extraction of the various maize components gave rise to long-chain hydrocarbons varying in chain length from C_{23} to C_{31}. As was seen in the results for both genotypes of *Miscanthus sinensis* (H0121 and H056), pentacosane was not present in the stem. However, heptacosane was also absent, while hentriacontane was detected. The leaves contained the greatest variety of alkanes, ranging from C_{23} to C_{31} with C_{29} being the dominant alkane. The husk contained long-chain *n*-alkanes varying in length from C_{23} to C_{29}, with the latter being the most dominant alkane (44.3% of the total alkanes).

3.4.1.3 Sugarcane

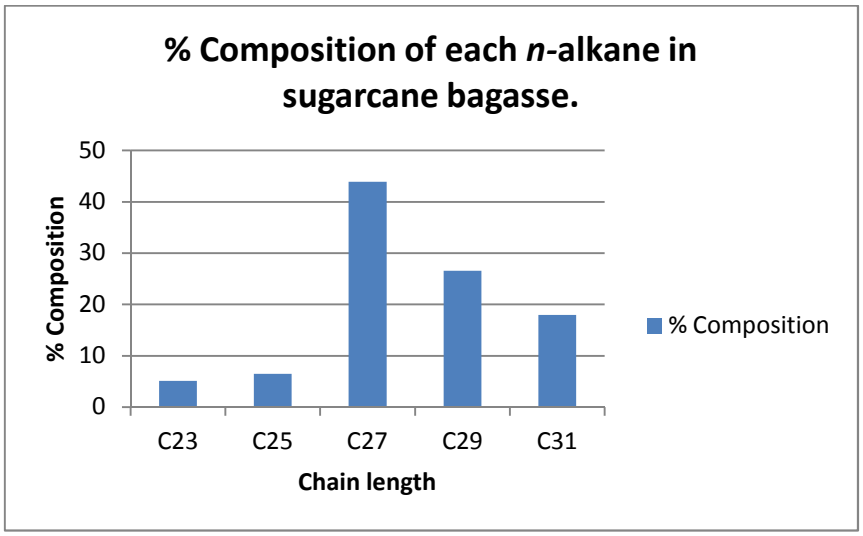


Figure 3.06 % Composition of each *n*-alkane in sugarcane bagasse.

Similarly to miscanthus and maize, in sugarcane bagasse the hydrocarbon pattern ranges from C_{23} to C_{31}. The distribution of alkanes may be seen in figure. In this case however heptacosane (C_{27}) was found to be the dominant alkane followed by nonacosane (C_{29}) and hentriacontane (C_{30}) (Figure 3.06).

3.4.1.4 Applications of long chain alkanes

Long-chain hydrocarbons have been shown to display semiochemical properties, where they play a role in plant-insect interactions.[4] Work has been carried out in which the 'pseudocopulatory' behaviour of male bees, *Andrena nigroaena*, towards the flowers of *Ophrys sphegodes*. 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 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}.[4]

3.4.2 Long-chain fatty acids

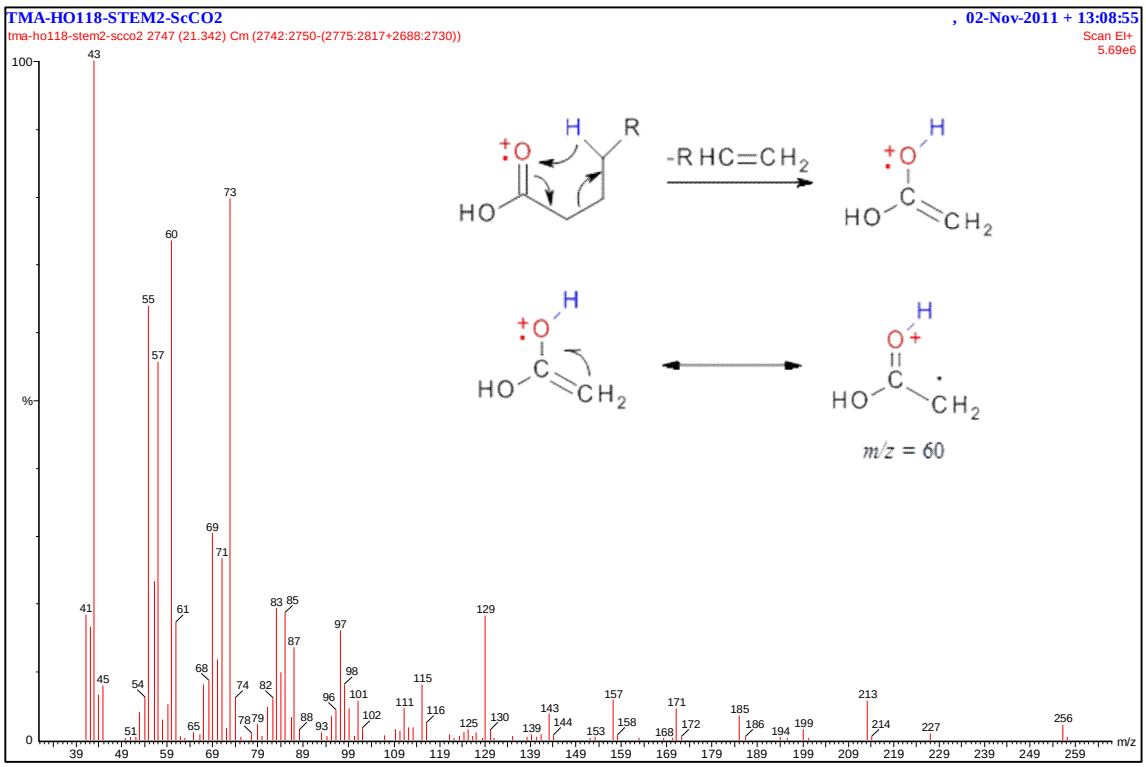


Figure 3.07 Mass spectrum of hexadecanoic acid (C16) from *Miscanthus giganteus*.

The long-chain fatty acids were identified using gas chromatography coupled with mass spectrometry. As was the case with the long-chain hydrocarbons, the molecular ion peak was either present in low abundances or absent in the EI-mass spectra (Figure 3.07). However the molecular ion peaks were determined from the FI-mass spectra, and therefore the chain-length of the acid could be determined. In the EI-mass spectra of long-chain acids, 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.).

However, 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 general definition for the McLafferty rearrangement is the "transfer of a γ-hydrogen to a double-bonded atom, through a six-membered transition state, with β-bond cleavage". 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, (ii) 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.

3.4.2.1 Miscanthus

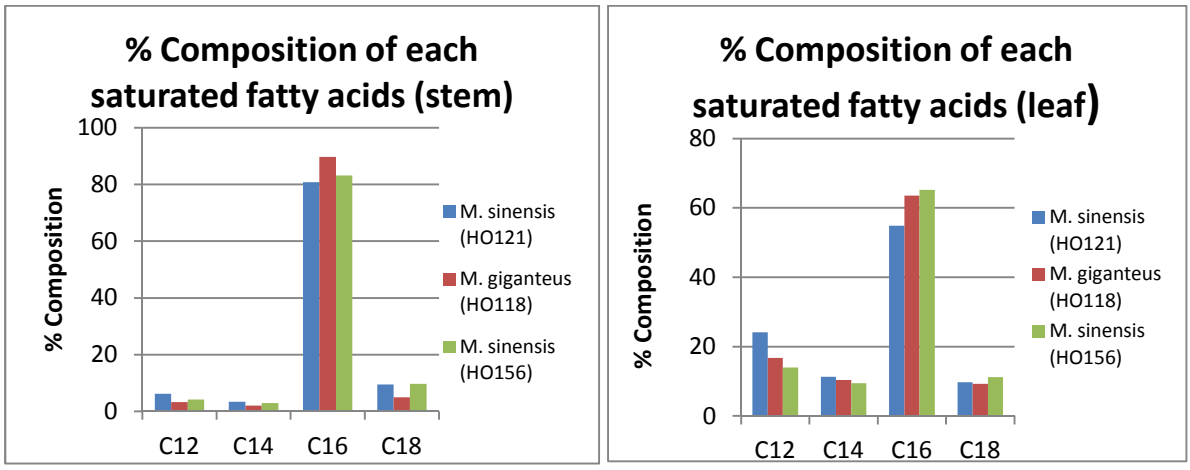


Figure 3.08% Composition of each saturated fatty acid in the a) stem and b) leaf of the various miscanthus genotypes.

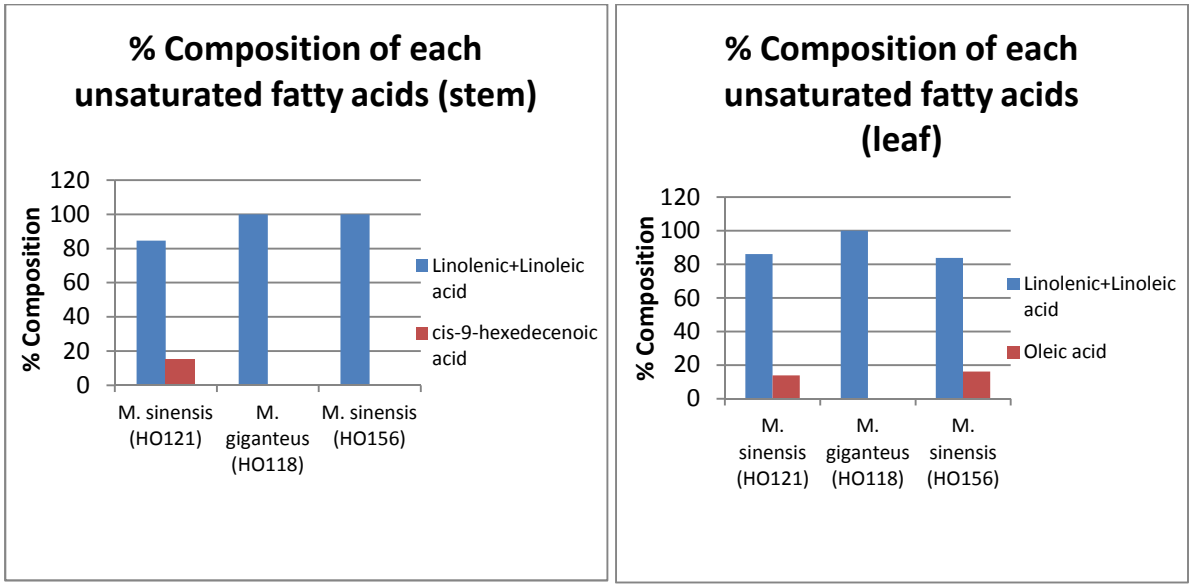


Figure 3.09% Composition of each unsaturated fatty acid in the a) stem and b) leaf of the various miscanthus genotypes.

The lipophilic extractive of the leaves and stems of all genotypes of Miscanthus were found to contain fatty acids having an even number of carbon atoms, which complies with what is normally found in the plant kingdom. In the leaves and stems of all genotypes, the greatest concentration of fatty acid was hexadecanoic acid (C16:0). This constituted up to 54.9% of the total saturated fatty acid composition in *Miscanthus sinensis* (H0121), 63.5% of the total saturated fatty acid composition in *Miscanthus giganteus* (H0118) and 65.3% of the total composition in *Miscanthus sinensis* (H0156). The percentage composition of hexadecanoic acid in the stems and leaves of each sample are summarised in Figure 3.09. The chain length of saturated fatty acids in the stem and leaves, ranged from C_{12} to C_{18}. In the study carried out by Villeverde *et al.* a number of saturated fatty acids were found in the bark and core of *Miscanthus giganteus*, when extracting with DCM, ranging from C_6 to C_{30}. This was not the case in this investigation, where a smaller number of fatty acids were found in the stem and the leaves when extracting with supercritical CO_2. This is probably due to the increased solubility of the acids in the more polar DCM. In their study, hexadecanoic acid was the most abundant saturated fatty acid in the core of *Miscanthus giganteus* (which is consistent with this study) and is the second most abundant saturated fatty acid in the bark following octacosanoic acid.[3]

In addition to saturated fatty acids, two unsaturated fatty acids were present in the stems and leaves of all Miscanthus species: linoleic acid (18:2) and linolenic acid (18:3). These contain

two and three unsaturated bonds respectively. These were identified by means of standards and from FI-MS and EI-MS data. Their composition varied in the leaves and stems of the different species. Since the two compounds were found to co-elute, a combined % composition of unsaturated C_{18} acids was determined. In *Miscanthus sinensis* (H0121), the combined % composition of linoleic and linolenic acid was found to be of the total fatty acids in the stems and in the leaves. In both genotypes of *Miscanthus sinensis* (H0121 and H0156), another C_{18} unsaturated acid was identified, oleic acid, which has one double bond in its structure (C18:1). In *Miscanthus sinensis* (H0156), in addition to the C_{18} unsaturated acids, a small amount of *cis*-9-hexedecenoic acid was found, which formed around 15.3% of the total unsaturated fatty acids. Villeverde *et al.* reported the presence of linoleic acid in the bark and core of *Miscanthus giganteus* samples but, no linolenic acid was found.[3] In addition to linoleic acid however, they reported the presence of three other unsaturated acids, *trans*-9-hexadecenoic acid, *cis*-9-octadecenoic acid and *trans*-9-octadecenoic acid.[3]

3.4.2.2 Maize

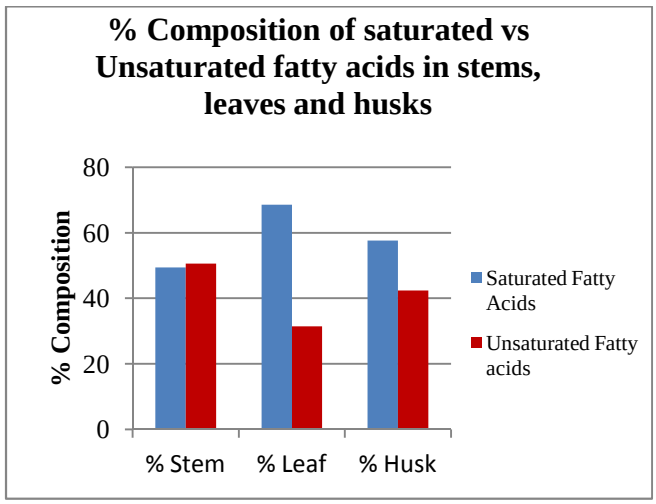


Figure 3.10 % Composition of saturated and unsaturated fatty acids in the extracts from the stems, leaves and husks.

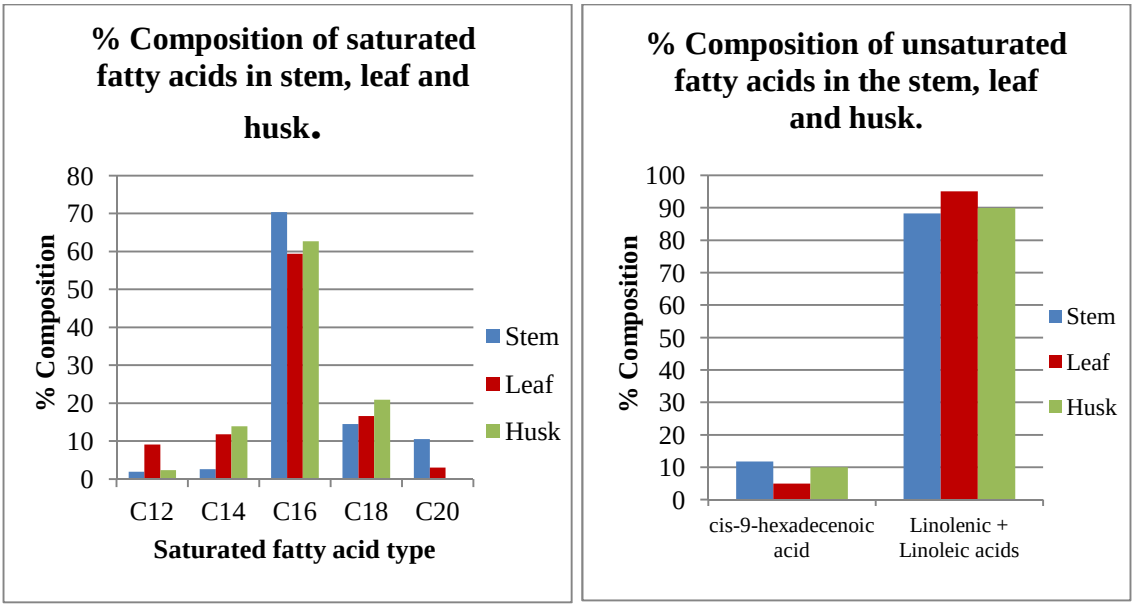


Figure 3.11 Composition of each saturated fatty acid in the a) saturated fatty acids and b) unsaturated fatty acids found in the stems, leaves and husks of maize.

Long-chain fatty acids with an even number of carbon atoms were found in the lipophilic extractives of the stems, husks and leaves. Both saturated and unsaturated fatty acids were found in maize. In the extract from the husks and leaves, the % composition of saturated fatty acids is higher, while in the stem there is a slightly higher % composition of unsaturated fatty acids, as can be seen in Figure 3.10.

Similar to miscanthus, the dominant long-chain saturated fatty acid was found to be hexadecanoic acid (C_{16}) in the stems, leaves and husks of maize, followed by octadecanoic acid (C_{18}) and tetradecanoic acid (C_{14}) (as shown in Figure 3.11). In the lipophilic extract from the husk, saturated fatty acids ranging from C_{12} to C_{18} were identified. However in the stems and leaves, a small amount of eicosanoic acid (C_{20}) acid was also present.

Two chain lengths were identified for the unsaturated fatty acids: C_{16} and C_{18}, with the latter being the dominant chain length. Linoleic and linolenic acids were the unsaturated C_{18} acids identified while *cis*-9-hexadecenoic acid was the C_{16} acid identified.

3.4.2.3 Sugarcane Bagasse

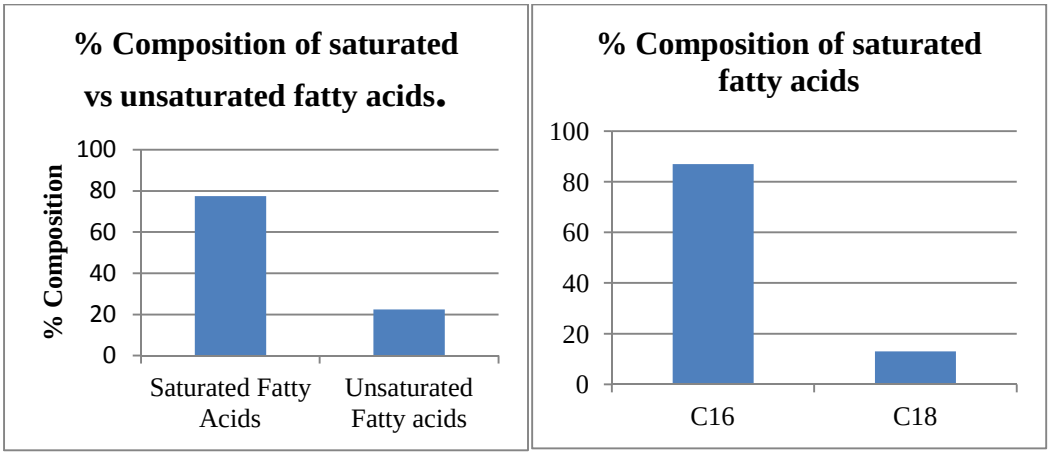


Figure 3.12 % composition of a) saturated vs unsaturated fatty acids b) % distribution of saturated fatty acids.

In sugarcane bagasse there is a higher proportion of saturated fatty acids compared to unsaturated fatty acids (Figure 3.12). In contrast to maize and miscanthus, only two fatty acids were identified in the wax extract; hexadecanoic acid (C16) and octadecanoic acid (C18), with the former being the dominant alkane. Two unsaturated fatty acids were identified in the extract: linolenic and linoleic acid.

3.4.2.4 Applications of long-chain fatty acids

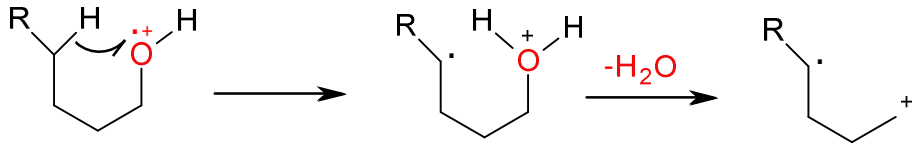
Polyunsaturated fatty acids are known to have an effect on serum cholesterol in humans.[5] The hypocholesterolemic effect of linoleic acid has been well established.[6-8] Horrobin *et al.* has shown that increasing the intake of linoleic in one's diet leads to a reduction in plasma cholesterol, though large amounts need to be consumed.[8] 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.[8] Studies have shown that in normolipidemic men, α-linolenic acid is just as effective in lowering blood cholesterol as linoleic acid.[9] Work has shown that different types of diets, which varied in the composition of unsaturated fatty acids, had similar cholesterol-lowering results.[9]

Furthermore, Zhao *et al.* looked into the effects of α-linolenic acid on cardiovascular risk factors and vascular inflammation in hypercholesterolemic men and women.[10] The results indicate that a diet which is high in α-linolenic acid leads to a significant decrease in cardiovascular risk by reducing lipoprotein levels and lipids in the blood and by exhibiting vascular anti-inflammatory effects, in which vascular inflammation is reduced as well as

endothelial dysfunction.[10] Other studies have also shown that α-linolenic acid-rich diet has significant cardioprotective effects.[11]

3.4.3 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 often a distinct and prominent peak that is 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 1. The thermal decomposition of higher alcohols on hot inlet surfaces often exaggerates the M – 18 peak.



Scheme 1.

3.4.3.1 Miscanthus

In the stem of *Miscanthus giganteus* the only fatty alcohol that was present was found to be octacosan-1-ol (C28:0). Villeverde *et al.* found four different types of fatty alcohols in the bark and core however, octacosan-1-ol was the only fatty alcohol that was found in reasonable quantities (81 mg/kg in the bark and 25 mg/kg in the core). The other fatty alcohols (hexancosan-1-ol, heptancosan-1-ol and pentacosan-1,2-diol) were found in quantities less than 7 mg/kg of dry weight.[3]

No long-chain alcohols were detected in the wax extracts from maize.

3.4.3.2 Sugarcane bagasse

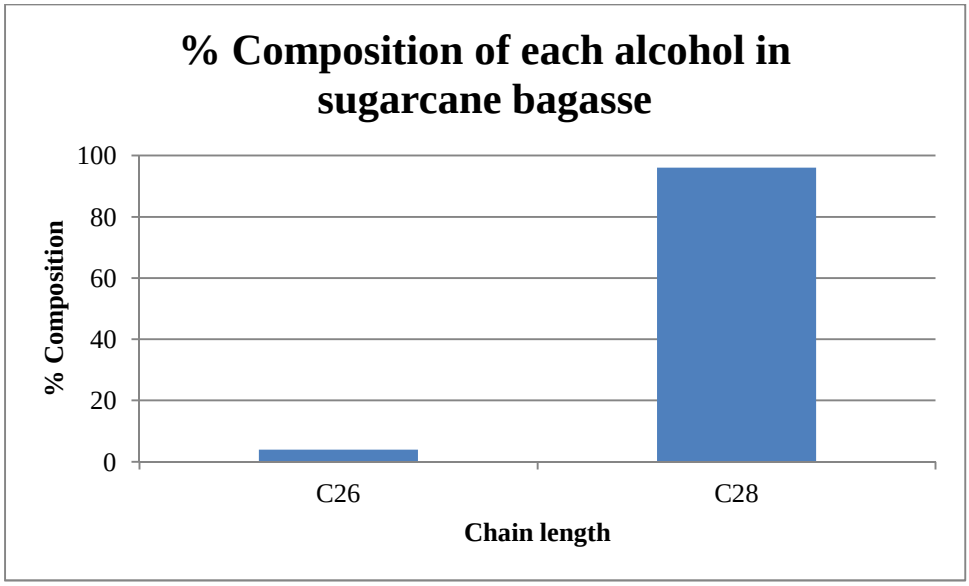


Figure 3.13 % Composition of each alcohol in sugarcane bagasse.

The two alcohols found in sugarcane bagasse were hexacosanol (C_{26}) and octacosanol (C_{28}), the latter accounting for around 96.1% of the total long-chain alcohols. (Figure 3.13)

3.4.3.3 Applications of long-chain alcohols

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.[12]

Policosanol therapy has been found to improve risk factors that are linked with arteriosclerosis which leads to significant improvement in cardiovascular health. First of all, the functionality of endothelial cells which line the arterial walls has been found to improve on intake of policosanol supplements.[12] This is important as when endothelial cells are damaged or malfunctioned, the resulting irregular arterial walls could release clotting factors and encourage aggregation of platelets and inflammation, leading to the formation of atherosclerotic plaques and blood clots.[13]

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.[12] It has also been proposed that the protective functions of policosanols can be used to treat patients suffering from intermittent claudication.[12] This refers to the muscle pain that is experienced during physical activities in the lower extremities. It is linked with vascular

disease as muscle pain is experienced with narrowing of arteries and decrease in blood flow.[13] Patients suffering from intermittent claudication showed improvement when 20 mg/day of policosanols was administered.[14]

3.4.4 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 was also identified, which is characteristic of aldehydes and alcohols. To determine which of the two functional groups was present the sample was silylated.

3.4.4.1 Miscanthus

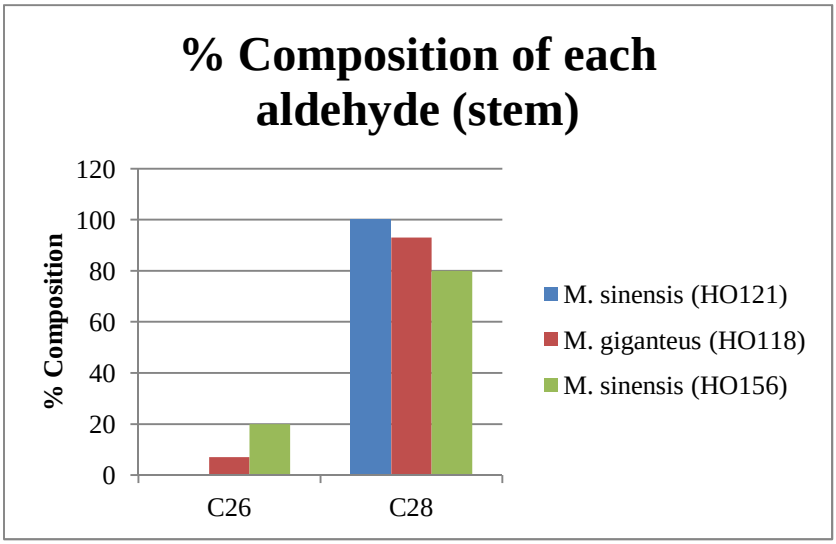


Figure 3.14% composition of each aldehyde in the stem of the three miscanthus samples.

Two long-chain aldehydes were found in the stem extracts of miscanthus: hexacosanal (C_{26}) and octacosanal (C_{28}). The dominant aldehyde was octacosanal which was found in considerable amounts (>80% of the total aldehyde composition) in all miscanthus samples (Figure 3.14). In the leaf extracts, octacosanal was the only aldehyde identified, no hexacosanal was detected.

No long-chain aldehydes were detected in the wax extracts from maize.

3.4.4.2 Sugarcane bagasse

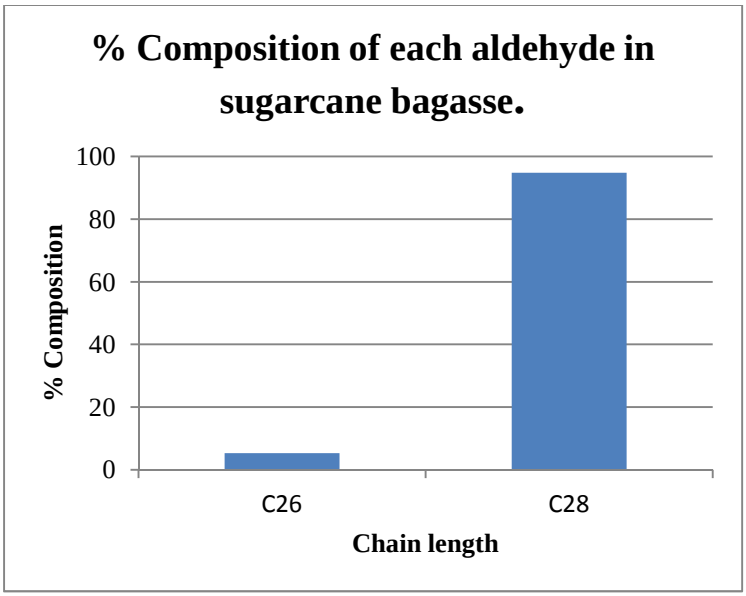


Figure 3.15 % Composition of each aldehyde in sugarcane bagasse.

Similarly to miscanthus two long-chain aldehydes were identified in the wax extract: hexacosanal (C26) and octacosanal (C28), with the latter once again being the dominant aldehyde, accounting for 94.8% of the total long-chain aldehydes (Figure 3.15).

3.4.5 Sterols

3.4.5.1 Miscanthus

In the leaves and stems of all three Miscanthus genotypes, three types of phytosterols were found: β-sitosterol, stigmasterol and campesterol. Stigmasterol is the dehydrogenation product of β-sitosterol. The structure of β-sitosterol can be seen in Figure 3.16.

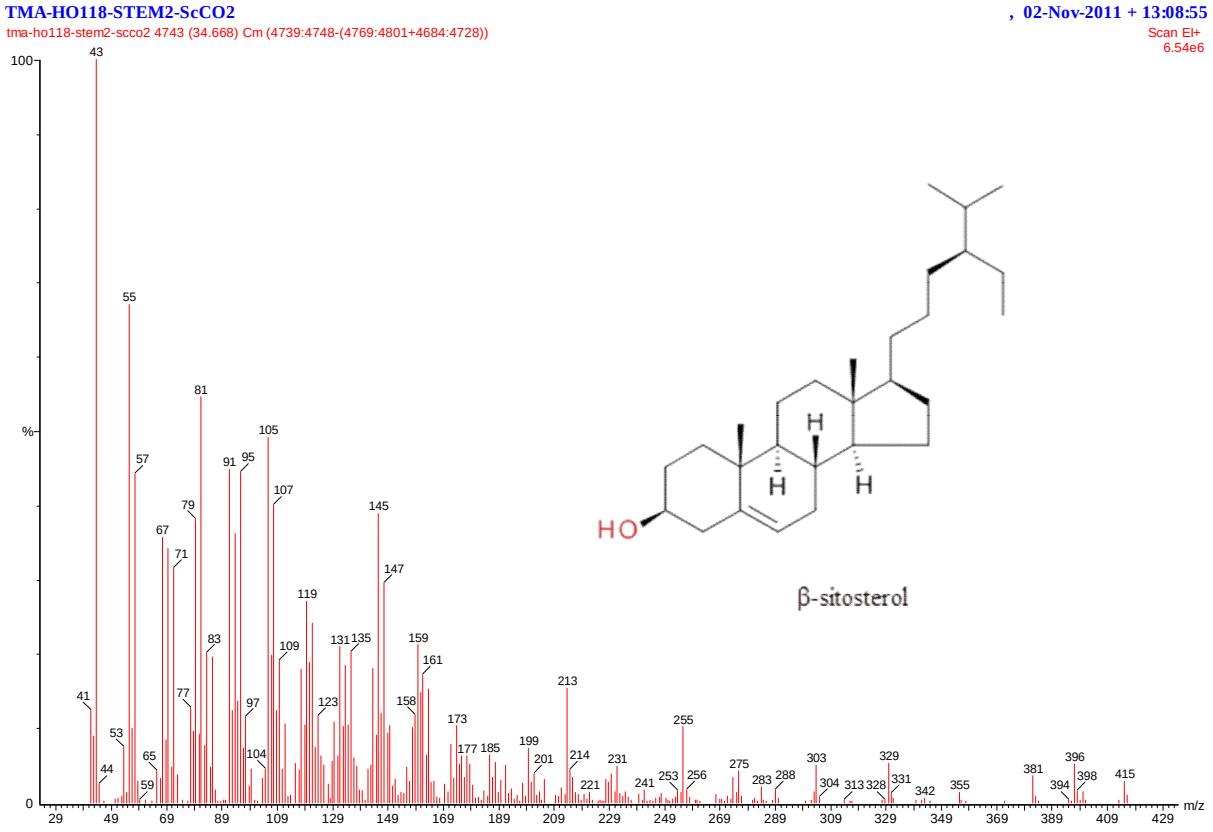


Figure 3.16 Mass spectrum and structure of β-sitosterol from *Miscanthus giganteus*.

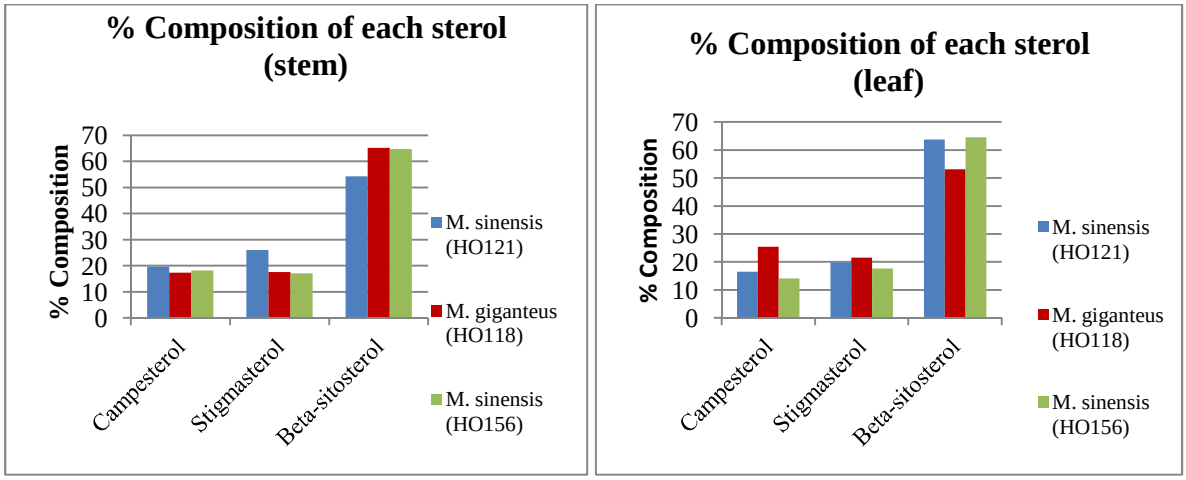


Figure 3.17% Composition of each sterol in the a) stem and b) leaves of the various miscanthus samples.

In all stem and leaf lipophilic extractives, the most abundant sterol which was present was β-sitosterol, followed by stigmasterol and campesterol (Figure 3.17). This is consistent with what is reported in literature, as β-sitosterol and campesterol are the two most abundant

sterols found in the plant kingdom. The results are in good agreement with those obtained by Villeverde *et al.*, who found β-sitosterol in the largest quantities in the bark and core.

3.4.5.2 Maize

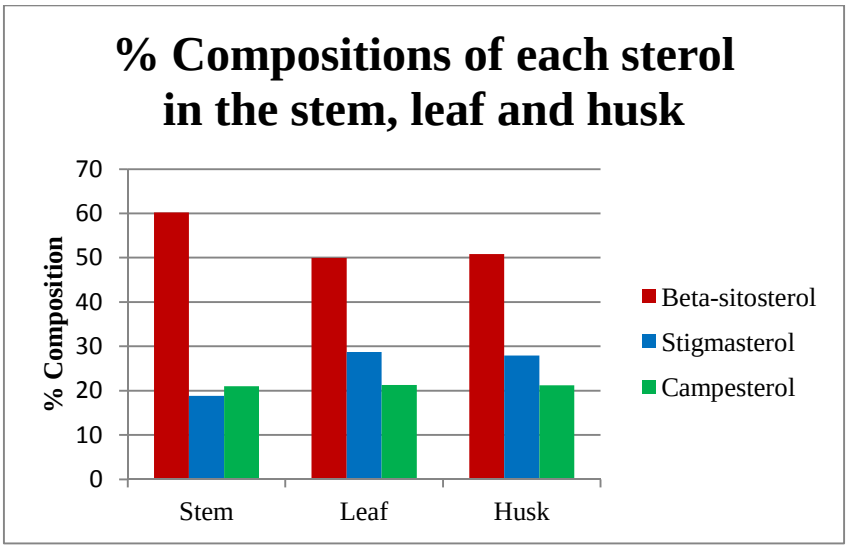


Figure 3.18 Composition of each phytosterol in the stems leaves and husks of maize.

Similar results were found in the maize extracts: β-sitosterol, stigmasterol and campesterol were the three sterols identified, with β-sitosterol being the most abundant sterol, constituting around 60.2%, 49.9% and 50.8% of the total sterols in the stems leaves and husks respectively (Figure 3.18). As far as the authors are aware, no reports have been found which indicate the presence of phytosterols in maize.

3.4.5.3 Sugarcane Bagasse

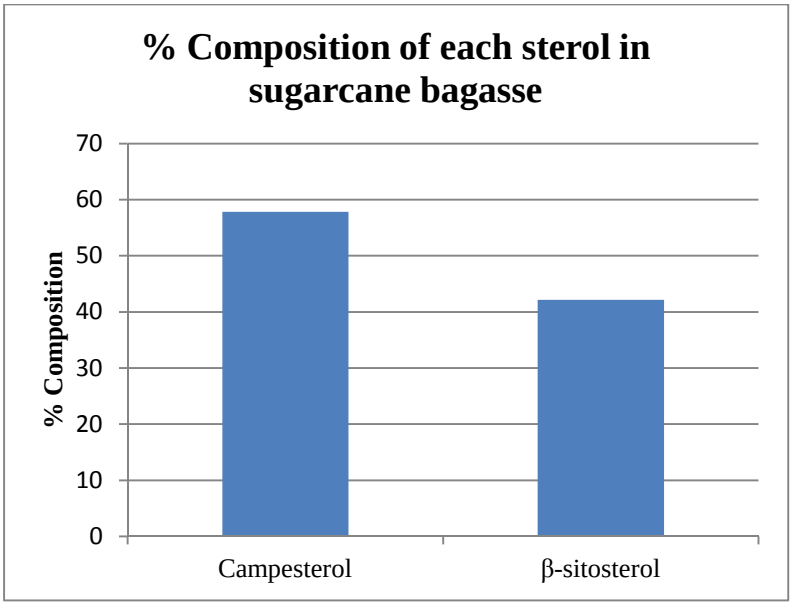


Figure 3.19 %Composition of each sterol in sugarcane bagasse.

In contrast to maize and miscanthus, only two sterols were detected in the wax extract from sugarcane bagasse: Campesterol and β-sitosterol (Figure 3.19). In addition, campesterol was found to be the major sterol and not β-sitosterol (as was the case with maize and miscanthus).

3.4.5.4 Applications of phytosterols

Phytosterols are of particular interest as they have a variety of potential biological and physiological applications. They are widely known to act as efficient anticancer compounds.[15] It has been estimated that the risk of cancer can significantly decrease, by as much as 20% with a phytosterol-enriched diet.[16] The three main phytosterols that are present in the human diet are β-sitosterol, campesterol and stigmasterol. The absorption of phytosterols by the body is limited; with less than 20% of dietary campesterol and 7% of dietary β-sitosterol.[15] Controlled intestinal perfusion studies carried out on healthy normal men indicated that the bioavailability of campesterol, stigmasterol and β-sitosterol were found to be 10%, 5% and 4% respectively.[17]

However, even though there is limited absorption, the amount of phytosterols absorbed is significant nonetheless. A number of controlled epidemiological studies and observational analysis suggest that consumption of phytochemical-enriched diets lead to a significant decrease in common cancers namely colon, breast and prostate cancers.[18-24] Population studies carried out by Shimizu *et al.* have shown that there is a relatively low incidence of colon, breast and prostate cancers in Asian countries due to high consumption of phytosterol-

enriched diets.[4] However, there is a significant increase in the risk of these cancers when Asians relocated to western countries and switched to diets that are more animal-based.[18]

In Uruguay, a number of case control studies took place, where the role of phytosterols in the risk of developing certain cancers was investigated.[19-22] They looked into whether or not a relationship existed between phytosterols intake and the incidence of developing lung, stomach, breast or oesophageal cancer. Results from the studies indicated that, in all four types of cancer, there is a potential protective role of phytosterols.[19-22] Mc Cann *et al.* carried out controlled studies to study the effect that phytosterol intake had on the risk of ovarian cancer.[23] Their results showed that there were significant reductions in the risk of ovarian cancer "for the highest quintile intakes of stigmasterol (OR = 0.42, $p<0.05$, 95% CI = 0.20 – 0.87).[23]

Another important application of phytosterols is their involvement in cholesterol metabolism and atherosclerosis. In order for whole-body cholesterol levels to be maintained, three processes need to be precisely controlled and regulated: (i) *De novo* cholesterogenesis (ii) cholesterol absorption and (iii) cholesterol excretion. If these processes become imbalanced, this could result in a significant increase in plasma cholesterol concentrations, accumulation of cholesterol in a variety of tissues and an increased risk of cardiovascular disease.[25] There has been extensive clinical as well as epidemiological evidence which shows that there is a strong relationship between atherosclerotic cardiovascular disease and high plasma cholesterol, especially low-density (LDL) cholesterol.[26] LDL-cholesterol concentrations have to be kept as close to an optimal level as possible in order to reduce the risk and burden of coronary atherosclerosis.[26]

It has been known for quite some time that phytosterols are effective in reducing plasma LDL-cholesterol levels with minimal side-effects and without significantly changing high-density (HDL)-cholesterol and triglycerides.[27] Phytosterols thus have a hypocholesterolemic role in humans and animals.[28,29] A number of studies have looked into the effects of phytosterols on disorders of lipid metabolism and atherosclerosis in humans since Peterson showed how β-sitosterol lowered cholesterol levels in chickens in 1951.[30-34] Data indicates that dosages of 2 – 2.5 g/day of phytosterols lowers LDL-cholesterol levels by up to 14% on average.[35]

3.4.6 Wax Esters

3.4.6.1 Miscanthus

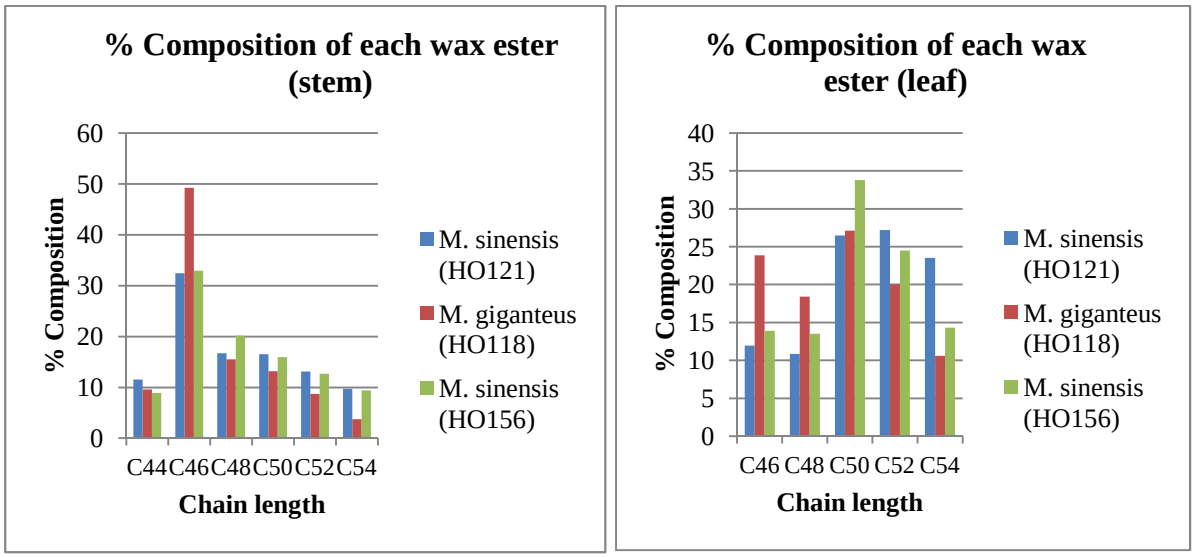


Figure 3.20 %Composition of each wax ester in a) stem b) leaf.

A number of wax esters were detected in the wax extracts from miscanthus ranging in chain length from C_{44} to C_{54} (Figure 3.20). However a limitation of GC-FID and GC-MS is that it is unlikely to see any compounds over a molecular weight of one thousand and therefore there is a great chance that not all of the wax esters were identified. In the stem of all miscanthus samples, the dominant wax ester was found to be octacosanyl octadecanoate (C_{46}) followed by octacosanyl eicosanoate (C_{48}) and octacosanyl docosonoate (C_{50}). On the other hand, in the leaves the major wax ester was found to be octacosanyl docosonate (C_{50}). Furthermore, in the leaves of all miscanthus samples, no C_{44} (octacosanyl hexadecanoate) was detected.

3.4.6.2 Maize

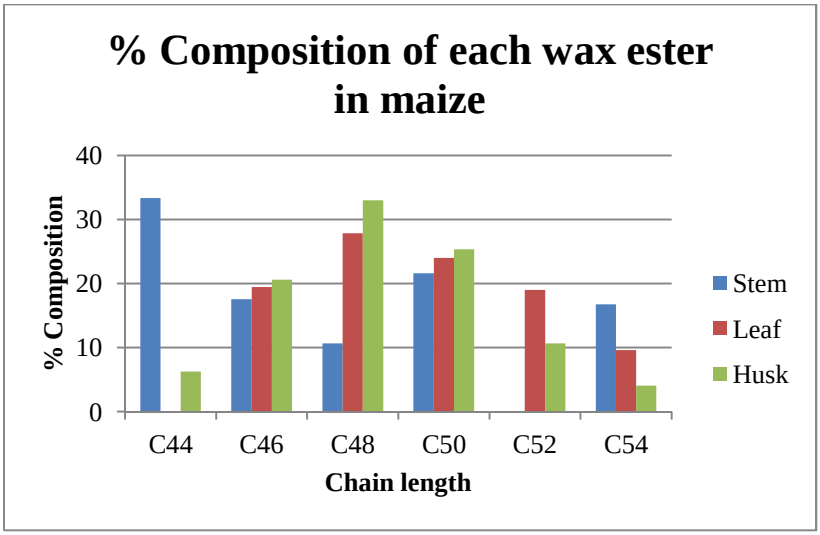


Figure 3.21 % Composition of each wax ester in maize.

Once again, in maize the chain length of wax esters detected was in the range of C_{44} to C_{54}. In the leaves and husk the dominant wax ester was found to be octacosanyl eicosanoate (C_{48}) followed by octacosanyl docosanoate (C_{50}) while in the stem octacosanyl hexadecanoate (C_{44}) was found to be the dominant wax ester, as can be seen in Figure 3.21. Similarly to miscanthus, no octacosanyl hexadecanoate was detected in the wax extract from the leaves, while no octacosanyl tetracosanoate (C_{52}) was found in the stem.

3.4.6.3 Sugarcane bagasse

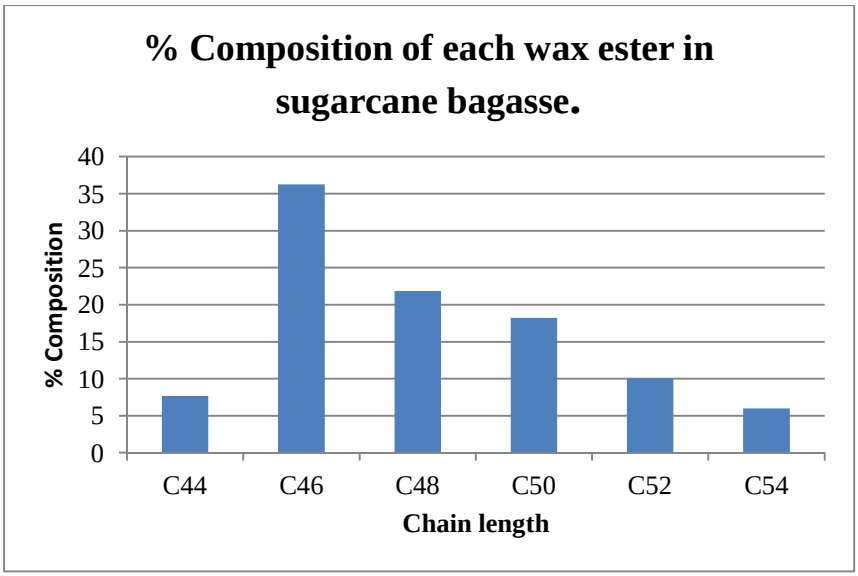


Figure 3.22 %Composition of each wax ester in sugarcane bagasse.

The wax ester pattern in sugarcane bagasse is similar to that of the miscanthus stem, with the major wax ester being octacosanyl octadecanoate (C_{46}) followed by octacosanyl eicosanoate (C_{48}) and octacosanyl docosonoate (C50). Wax esters have significant value in cosmetic application.

3.4.7 Additional compounds identified within extracts

In the leaves of all miscanthus species, a considerable amount of phytol was detected. Phytol is a fragrance ingredient that is widely utilised in a variety of products including toilet soaps, shampoos and other toiletries, fine fragrances, detergents and household cleaners.[36]

In the leaves of all miscanthus species, a considerable amount of the triterpinoid lupeol was detected. Triterpenes exhibit a wide range of biological activities which has led to an ever-increasing interest worldwide.[37] The greatest amount of interest has been on their cholesterol-lowering properties. Lupeol is of particular interest and has gained widespread focus by medical professionals, researchers and pharmaceutical makers alike.[37] It is considered to be an adequate anti-inflammatory agent, inhibiting inflammatory effects both *in vitro* as well as in animal models. Lupeol has also been shown to act as an anti-cancer agent.[38] It was shown to instigate apoptosis in pancreatic cancer cells. A variety of reports have illustrated the *in vivo* and *in vitro* efficacy of lupeol in preventing the growth of prostate cancer cells. Studies have shown that a multipronged strategy is adopted by lupeol for displaying anti-cancer behaviour which involves: (i) inhibiting the growth of human cancer cells (ii) causing apoptotic death of cancer cells.[38]

Beta-diketones: Beta-diketones were detected in waxes extracted from sugarcane. Sugarcane bagasse was found to have a lower proportion of beta-diketones compared to sugarcane. A reason for this is that beta-diketones have interesting chelating properties and they therefore have the potential to chelate with metals within the vanasse and be removed from the bagasse into aqueous solution during sugar extraction. Therefore an interesting application of beta-diketones would be their use as metal chelators which would be ideal for the recovery of precious metals or use in personal care products. They also form super hydrophobic coatings.

Conclusions

The lipophilic profile of the different C4 biomasses has been characterised using supercritical extraction systems. The supercritical system extracts free lipids only and does not liberate those lipids that are bound to the plant. Successful extractions of lipids were carried out using

supercritical carbon dioxide at 350 bar and 50 ^{o}C at a flow rate of 40g min^{-1} for four hours. Results have shown that from the three species of plant investigated, the highest extraction yields were obtained for miscanthus. The main classes of lipophilic components that were identified in the wax samples from C4 biomasses included long-chain *n*-alkanes, fatty acids, fatty alcohols, wax esters, sterols, triterpinoids, beta-diketones amongst other compounds.

A range of interesting molecules have been identified in the wax extracts of maize miscanthus and sugarcane bagasse which have potential for use in a variety of high value applications including pharmaceutical, personal care products and cosmetic applications. Fatty alcohols, fatty acids and phytosterols have proven to be effective cholesterol-lowering agents. Phytosterols and lupeol have shown to act as anti-cancer molecules while beta-diketones have applications as metal chelates as surface coating agents.

Sugarcane bagasse and miscanthus have similarities in the chemical composition whereas maize has a different chemical profile. The wax ester pattern of miscanthus and sugarcane bagasse is similar, as is also the fatty alcohol and fatty aldehyde fractions.

Although not investigated as part of this report, it would be of interest for the future commercial success of these products to develop efficient fractionation methods in order to isolate and purify families of compounds. Supercritical carbon dioxide is an ideal solvent for conducting fractionation experiments due to the ease of tunability of solvent properties (variation of temperature and pressure of the product separators). Further work is needed to both optimise the extraction yield and fractionation process. In addition, the modification of extracts including the esterification of plant sterols would be of significant interest and also of commercial importance.

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