Supercritical CO2 extraction as an effective pre-treatment step for wax extraction in a miscanthus biorefinery

*Thomas M. Attarda, C. Rob McElroya, Richard J. Gammonsa, John M. Slatterya, Nontipa Supanchaiyamatb, Claire Lessa Alvim Kameic, Oene Dolstrac, Luisa M. Trindadec, Neil C. Bruced, Simon. J. McQueen-Masond, Seishi Shimizua and Andrew J. Hunt\*a*

a Department of Chemistry, University of York, Heslington, York, UK. YO10 5DD, email: [andrew.hunt@york.ac.uk](mailto:andrew.hunt@york.ac.uk).

b Materials Chemistry Research Centre, Department of Chemistry, Khon Kaen University, Khon Kaen, Thailand

c Wageningen UR - Plant Breeding, Wageningen University and Research Centre, P.O. Box 386. 6700 AJ Wageningen. The Netherlands.

d Department of Biology, University of York, Wentworth Way, York, YO10 5DD, UK

**KEYWORDS**

Miscanthus, supercritical, carbon dioxide, extraction, waxes, saccharification

The Authors would like to dedicate this paper to Professor James H. Clark in celebration of his 65th birthday. Thank you for inspiring a generation of scientists to go green.

**ABSTRACT**

The use of supercritical carbon dioxide (scCO2) to extract valuable lipophilic compounds from miscanthus was investigated and subsequent enzymatic saccharification was carried out to determine the impact of scCO2 extraction on downstream processing of miscanthus. Two miscanthus genotypes (*Miscanthus x. giganteus and Miscanthus sinensis*) were investigated and characterised. A diverse range of molecules were detected including long-chain hydrocarbons, fatty acids, *n-*policosanols, aldehydes, wax esters, sterols and steroid ketones. Quantification data indicates that there is a considerable difference among each species in the quantities of specific compounds. The waxes also exhibited significant differences in melting temperature, thus illustrating the opportunity for utilisation in various applications. In addition to the isolation of valuable chemical compounds, the scCO2 pre-treatment also had a beneficial effect on the downstream processing of the biomass. The total sugars released after saccharification was found to increase by around 20% when coupled with scCO2 extraction, as compared to untreated samples.

**INTRODUCTION**

The biorefinery concept is analogous to current petroleum refineries, dealing with the conversion of biomass into a number of added-value products such as chemicals, energy and materials.1 However, the majority of first generation biorefineries utilise only single technologies and focus mainly on feedstocks that are in competition with food and feed.1-2 Recently the uses of wastes, agricultural residues and energy crops have become an important aspect of green chemistry that can be exploited in biorefinery applications.3-8 Miscanthus, a perennial grass, has been extensively grown for energy production. It is a C4 photosynthetic plant and therefore demonstrates high photosynthetic activity, high rates of CO2-fixation, high radiation and utilises water efficiently, thus leading to high rates of growth and productivity.9-12 Research has demonstrated the potential for incorporating miscanthus within a biorefinery framework, whereby chemicals, fuels and materials can be produced, when using this biomass as a feedstock.13-16Miscanthus side-streams, including waxes, can be exploited for a range of applications.

Over a century ago paraffin, a by-product residue from the petroleum refining industry, was introduced as an abundant and low cost alternative to natural waxes. It has since been the dominant source of wax.17 A decline in petroleum wax supply coupled with their ever-increasing costs and the switch to ‘greener’ alternative products by consumers has led to new opportunities for renewable natural waxes.18

Traditional organic solvents such as hexane, toluene, dichlor­omethane (DCM) and chloroform have been used to extract waxes from biomass, but the use of these is becoming restricted due to a number of human health (toxicological) and environmental issues.19 ScCO2 has shown to be a suitable alternative green solvent for extracting natural products from biomass.20-21 CO2 is a renewable solvent, has low levels of toxicity and is non-flammable. Furthermore, it is cheap and easily recycled.22 The scCO2 extraction of waxes from C3 plants has been well reported.19, 23-26 However, there is very limited work concerning supercritical extraction of waxes from C4 plants.27-28 In addition, only limited work has been conducted with respect to the extraction of molecules from *Miscanthus x. giganteus* and the extraction solvent utilised in the study was DCM.29 To the best of the authors’ knowledge there is no work investigating scCO2 extraction of waxes from miscanthus.

This study highlights the advantages of incorporating scCO2 extraction of waxes as an initial step in a miscanthus biorefinery. The scCO2 extraction of waxes was conducted on the leaves and stems of two miscanthus species; *Miscanthus sinensis and Miscanthus x. giganteus*. The lipophilic constituents of the waxes were identified and quantified in order to identify potential applications for this unutilised resource, which is frequently considered to be an agricultural waste. In addition, saccharification was carried out on the scCO2 extracted miscanthus and compared to untreated and ethanol-washed miscanthus in order to investigate potential additional benefits of scCO2 extraction on the biomass. This allowed for the assessment of any beneficial effect on the amount of sugars released during saccharification after using scCO2 to extract waxes. It was thought that the extraction of waxes in this way opens the biomass structure and facilitates enzymatic hydrolysis of the polysaccharide components.

**MATERIALS AND METHODS**

Materials and Sample preparation

Samples of leaves and stems from two miscanthus species (*Miscanthus x. giganteus* genotype HO118 and *Miscanthus sinensis* genotype H0121) were collected from the WUR-PB collection of miscanthus. Collection of the shoots occurred on the 5th November 2010 from a field nursery set up at Wagenin University in 2003. The shoots were taken from numerous fully-grown multi-tillered plants. The leaves and stems were separated and subsequently dried at 70 oC using a forced-air oven until constant weight was achieved. A Glen Creston Ltd. cutting mill with a 1 mm grate was used to mill the miscanthus biomass. The MG stem and leaf dry matter content was 39.2% and 35.2% respectively while those for the MS stem and leaf were 40.2% and 38.9% respectively.

Supercritical Fluid Extraction (SFE)

Supercritical Fluid Extraction of miscanthus samples was conducted according to the method as previously reported in the literature.30 The optimal conditions (pressure and temperature) were selected following an optimisation study (factorial experimental design) whereby different extractions were carried out at varying pressures (80, 240, 325, 350 and 400 bar) and temperatures (40, 50 and 60 oC). The optimal conditions were found to be 350 bar and 50 oC at 40 g min-1 CO2. DCM was utilised in this study to ensure that all the lipids were removed from the separator in order to get an accurate % yield. However, the use of non-green solvents is not required for collection of extracts on an industrial scale. A method for the recovery of waxes on a lab-scale that does not require the use of DCM will be presented in future work by the authors.

Preparation, pre-treatment and saccharification of *Miscanthus x. giganteus* samples

Preparation, pre-treatment and saccharification of *Miscanthus x. giganteus* samples were conducted according to the method previously reported in the literature.31

Wax analysis methods

Derivatization and HT-GC (and HT-GC-MS) procedures for wax analysis were conducted according to a previously published methodology. 28 DSC analysis was conducted according to a previously published method*.*27 The standard deviation (errors) in the quantification data have been calculated based on three replicates.

**RESULTS AND DISCUSSION**

Supercritical extraction of waxes from *M. x. giganteus and M. sinensis*

A 2x2 factorial experimental design was conducted on the leaves of MG to identify the optimal conditions (pressure and temperature) leading to the highest % yield of wax from miscanthus biomass (see supplementary information). Various different temperatures and pressures were investigated and it was found that the optimal conditions were 350 bar and 50 oC. The leaves and stems of two miscanthus species were investigated; *M. x. giganteus* (MG) and *M. sinensis* (MS). The % extraction yield of wax from the stems and leaves of MS and MG (by scCO2 extraction) may be viewed in Figure 1.

**Figure 1. % crude yields of waxes extracted from MS and MG stems and leaves. (MS – *miscanthus sinensis,* MG – *miscanthus giganteus*).**

Figure 1 highlights large wax contents in the leaves of both MG and MS, when compared to the stems. This is not surprising as the leaf surface area to volume ratio is much higher, meaning that the risk of water loss *via* transpiration and mechanical damage is similarly much greater than the stems. Therefore, higher wax content is found in the leaves to minimise these risks.

ScCO2 extractions of MG leaf exhibited the highest wax yields (1.96 ±0.03%), while leaves of MS were found to be 1.59 ±0.1%. The opposite was observed in the stems, whereby the largest quantities of wax were obtained from the MS stems (0.46 ±0.04%) when compared to the MG stems (0.38 ±0.03%). Soxhlet extractions were also undertaken using heptane. Although the % crude yields were higher (% yields of: MS stem 0.69%, MS leaves 3.03%, MG stem 0.52% and MG leaves 3.35%), the % composition of compounds identified was lower due to the large number of unwanted co-extractives obtained using heptane (due to the lower selectivity of conventional organic solvents).

Table 1. Quantification data of the lipophilic compounds found in the waxes from MS and MG (in μg/g-1 of biomass). MS – *miscanthus sinensis* MG – *miscanthus giganteus*.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Compounds | MS ScCO2 | | MG ScCO2 | |
|  | **Stems** | **Leaves** | **Stems** | **Leaves** |
| Pentanoic acid (C5H10O2) | 0.8 ±0.3 | 2.3 ±0.3 | 0.4 | 3.2 ±0.7 |
| Hexanoic acid (C6H12O2) | 5.1 ±0.3 | 5.1 ±0.3 | 7.6 | 12.8 ±1.8 |
| Heptanoic acid (C7H14O2) | 0.9 ±0.2 | 1 ±0.3 | 1.2 ±0.1 | 2 ±0.8 |
| Octanoic acid (C8H16O2) | 2.6 ±0.8 | 7 ±0.7 | 2.4 ±0.1 | 8.3 ±1.1 |
| Nonanoic acid (C9H18O2) | 2.8 ±0.4 | 4.3 ±1.1 | 4.2 ±0.1 | 10.4 ±1.1 |
| Decanoic acid (C10H20O2) | 2.4 ±0.9 | 18.5 ±1.7 | 1.7 | 15.9 ±2.1 |
| Dodecanoic acid (C12H24O2) | 9.2 ±0.6 | 128.1 ±12.3 | 5.1 ±0.2 | 124.4 ±13.8 |
| Tetradecanoic acid (C14H28O2) | 7.4 ±0.5 | 74.9 ±4.7 | 5.2 | 86.4 ±7.5 |
| Pentadecanoic acid (C15H30O2) | 5.9 ±1.2 | 7.5 ±0.9 | 8.5 ±0.2 | 16.9 ±2.1 |
| Hexadecanoic acid (C16H32O2) | 165.5 ±5.7 | 307.5 ±35.7 | 169 ±1.6 | 464.6 ±47.6 |
| Heptadecanoic acid (C17H34O2) | 4.2 ±0.3 | 15.5 ±7.9 | 8.7 ±0.1 | 13.4 ±0.4 |
| Octadecanoic acid (C18H36O2) | 18.6 ±1.2 | 94.3 ±13.1 | 15.4 ±0.1 | 88.5 ±7.6 |
| Nonanoic acid (C19H38O2) | 3.3 ±1.1 | 18 ±2.4 | 2.4 | 19.1 ±3.4 |
| Eicosanoic acid (C20H40O2) | 11 ±1 | 99.9 ±16.3 | 12.8 ±0.5 | 85.3 ±6.4 |
| Heneicosanoic acid (C21H42O2) | 3.7 ±0.4 | 8.1 ±0.7 | 7.1 ±0.1 | 10.6 ±2.1 |
| Docosanoic acid (C22H44O2) | 9.1 ±3.3 | 39.1 ±1.8 | 16.8 ±0.1 | 44.3 ±7.4 |
| Tricosanoic acid (C23H46O2) | 7.1 ±2 | 25.8 ±2.3 | 20.8 ±0.2 | 35.5 ±6.4 |
| Tetracosanoic acid (C24H48O2) | 7.4 ±2.7 | 35.3 ±5.8 | 29.5 ±0.4 | 60.7± 12.6 |
| Pentacosanoic acid (C25H50O2) | 2.7 ±0.5 | 13.9 ±1.8 | 16.8 ±0.1 | 24.4 ±2.6 |
| Hexacosanoic acid (C26H52O2) | 17.8 ±0.7 | 10.4 ±2.5 | 2.9 ±0.1 | 69.4 ±27.2 |
| Octacosanoic acid (C28H56O2) | TR | TR | TR | TR |
| Tricontanoic acid (C30H60O2) | TR | TR | TR | TR |
| Dotriacontanoic acid (C32H64O2) | TR | TR | TR | TR |
| Total saturated fatty acids | **287.5** ±**24.1** | **916.5 ±112.6** | **338.5 ±4** | **1196.1 ±154.7** |
|  |  |  |  |  |
| 9-hexadecenoic acid (C16H30O2) | 1.6 ±0.3 | - | 0.9 | 15.1 ±2.2 |
| 9-octadecenoic acid (C18H34O2) | 6.6 | 4.6 ±2.2 | 1.1 ±0.1 | 8.7 ±1.4 |
| 9,12-Octadecadienoic acid (C18H32O2) | 64.9 ±0.1 | 213.6 ±17.4 | 1.8 ±0.1 | 48.2 ±1.8 |
| 9,12,15-Octadecatrienoic acid (C18H30O2) | 90.1 | 87 ±10.1 | 1.7 ±0.1 | 27.1 ±4.9 |
| Total unsaturated fatty acids | **160.7 ±0.1** | **305.2 ±29.7** | **4.6 ±0.3** | **99.1 ±8.1** |
|  |  |  |  |  |
| Heptanedioic acid (C7H12O4) | - | - | 1.2 ±0.1 | - |
| Octanedioic acid (C8H14O4) | 0.9 ±0.2 | - | 0.9 ±0.1 | 3 ±0.2 |
| Nonanedioic acid (C9H16O4) | 10.2 ±4 | 5.1 ±1.7 | 21.5 ±0.5 | 26 ±7.7 |
| Decanedioic acid (C10H18O4) | 1 ±0.3 | - | 1.5 | - |
| Total saturated difatty acids | **12.1 ±4.5** | **5.1 ±1.7** | **25.1 ±0.7** | **29 ±7.9** |
|  |  |  |  |  |
| Docosanol (C22H46O) | 1.7 ±0.1 | 12 ±2.6 | 0.9 | 6.5 ±2.5 |
| Tetracosanol (C24H50O) | 1.4 ±0.1 | 9.1 ±3.7 | 2.3 ±0.2 | 13.6 ±3.7 |
| Hexacosanol (C26H54O) | 6.3 ±0.4 | 16.2 ±0.1 | 12.7 ±0.1 | 31.5 ±7.4 |
| Octacosanol (C28H58O) | 26.4 ±2.4 | 54.2 ±1.6 | 210.2 ±1.4 | 227.7 ±9.6 |
| Triacontanol (C30H62O) | 84.3 ±0.8 | 138.5 ±23.6 | 36.6 ±0.8 | 157.8 ±20.5 |
| Dotriacontanol (C32H66O) | 17.1 ±0.9 | 319.4 ±45.6 | 29.3 ±0.4 | 356 ±119.3 |
| Total saturated fatty alcohols | **137.2** ±**4.7** | **549.4 ±77.2** | **292 ±2.9** | **793.1 ±163** |
|  |  |  |  |  |
| Tetracosanal (C24H48O) | - | 14.3 ±3.4 | 1.8 ±0.1 | 7.2 ±1.2 |
| Hexacosanal (C26H52O) | 11.6 ±0.8 | 24.7 ±1 | 18.2 ±0.3 | 50.8 ±12.5 |
| Octacosanal (C28H56O) | 49.2 ±8.1 | 121.2 ±12.3 | 206.7 ±0.1 | 498.3 ±55.6 |
| Triacontanal (C30H60O) | 64 ±3.4 | 124.9 ±61 | 35.4 ±1.3 | 311.7 ±71.5 |
| Total saturated fatty aldehydes | **124.8** ±**12.3** | **285.1 ±77.7** | **262.1 ±1.8** | **868 ±140.8** |
|  |  |  |  |  |
| Pentacosane (C25H52) | 4.9 ±0.4 | 25.8 ±4.5 | 13.6 ±0.2 | 34.4 ±1.1 |
| Heptacosane (C27H56) | 11.2 ±0.1 | 82 ±5.1 | 15.8 ±0.2 | 175.7 ±10.1 |
| Octacosane (C28H58) | - | 14.2 ±2.4 | 2.9 ±0.2 | 20 ±2.4 |
| Nonacosane (C29H60) | 16.7 ±0.9 | 120.7 ±5.9 | 8.1 ±0.1 | 215.9 ±16.9 |
| Hentriacontane (C31H64) | 17.9 ±2.2 | 158.4 ±0.7 | 21.9 ±0.7 | 169.8 ±6.7 |
| Triatriacontane (C33H68) | 6.1 ±1.3 | 63.1 ±1.4 | 7.7 ±0.7 | 63.8 ±11.7 |
| Total hydrocarbons | **56.8 ±4.9** | **464.2 ±20** | **70 ±2.1** | **679.6 ±48.9** |
|  |  |  |  |  |
| Campesterol (C28H48O) | 73.5 ±1.2 | 117.9 ±19.1 | 25.7 ±0.5 | 165.3 ±15 |
| Stigmasterol (C29H48O) | 78.3 ±2.3 | 73.4 ±20.7 | 45 ±0.4 | 135.6 ±12.6 |
| β-Sitosterol (C29H50O) | 140.2 ±5 | 387.7 ±45.7 | 57.8 ±0.6 | 311.3 ±21.5 |
| Total sterols | **292 ±8.5** | **579 ±85.5** | **128.5 ±1.5** | **612.2 ±49.1** |
|  |  |  |  |  |
| Stigma-4-en-3-one (C29H48O) | 37.3 ±2.8 | 85.7 ±2.9 | 21.6 ±0.6 | 55 ±12.2 |
| Stigmastan-3,6-dione (C29H48O2) | 24 ±3.3 | 67.4 ±20.4 | 22.6 ±0.9 | 81.1 ±27.6 |
| Total steroid ketones | **61.3 ±6.1** | **153.1 ±23.3** | **44.2** ±**1.5** | **136.1 ±39.8** |
|  |  |  |  |  |
| Wax ester 38 (C38H78O2) | 3 ±0.2 | 64.6 ±20.4 | 1.4 | 4.5 ±0.3 |
| Wax ester 40 (C40H82O2) | 9.8 ±0.9 | 36.3 ±7.3 | 5.2 | 11.8 ±2.8 |
| Wax ester 41 (C41H84O2) | 0.9 ±1.5 | - | 0.6 ±0.2 | 4.5 ±1.1 |
| Wax ester 42 (C42H86O2) | 31 ±2.3 | 23 ±5.3 | 24.4 ±0.2 | 24.2 ±1.2 |
| Wax ester 43 (C43H88O2) | 6.9 ±0.6 | 14.9 ±4.3 | 10.7 | 16.6 ±5.8 |
| Wax ester 44 (C44H90O2) | 83.8 ±11.2 | 28.7 ±1.6 | 143.1 ±0.3 | 72.2 ±2.6 |
| Wax ester 45 (C45H92O2) | 8.4 ±1.2 | 14.3 ±0.3 | 9.4 ±0.1 | 14.5 ±0.4 |
| Wax ester 46 (C46H94O2) | 36.4 ±7.1 | 60.8 ±9.4 | 28 ±0.1 | 50.5 ±7.3 |
| Wax ester 47 (C47H96O2) | 5.7 ±1 | 12.9 ±0.9 | 4.1 | 12.6 ±2.1 |
| Wax ester 48 (C48H98O2) | 37.8 ±9.2 | 110.7 ±1 | 27.7 | 76.1 ±6.4 |
| Wax ester 49 (C49H100O2) | 5 ±1.5 | 11.9 ±1.5 | 3.8 | 10.4 ±1.5 |
| Wax ester 50 (C50H102O2) | 19.8 ±6.2 | 65.3 ±9 | 12.5 | 34.7 ±4.4 |
| Wax ester 51 (C51H104O2) | 3.9 ±1.6 | 8.6 ±2.8 | 4.1 | 7.8 ±2.1 |
| Wax ester 52 (C52H106O2) | 13.7 ±5.2 | 46 ±25.4 | 9.6 ±0.1 | 31 ±9.1 |
| Wax ester 53 (C53H108O2) | TR | 3.6 ±6.2 | 2.5 | TR |
| Wax ester 54 (C54H110O2) | 7.4 ±3.1 | 24.1 ±13.1 | 7.9 | 16.7 ±11 |
| Wax ester 55 (C55H112O2) | TR | TR | - | TR |
| Wax ester 56 (C56H114O2) | 11.5 ±1.1 | 31.4 ±10.4 | 11.5 ±1.7 | 19.6 ±8.5 |
| Wax ester 58 (C58H118O2) | TR | 17 ±1.4 | TR | 19.8 ±4.2 |
| Wax ester 60 (C60H122O2) | **-** | TR | **-** | TR |
| Total wax esters | **285 ±53.9** | **574.1 ±120.3** | **306.5 ±2.7** | **427.5 ±75.7** |
|  |  |  |  |  |
| 2-Pentadecanone-6,10,14-trimethyl (C18H36O) | 49 ±18.3 | 264.2 ±124.7 | 19.3 ±0.9 | 597.6 ±60.1 |
| Phytol (C20H40O) | 2.2 ±1.4 | 359.5 ±15.2 | 3.3 | 26.5 ±4.9 |
| Total ‘other’ compounds | **75.2 ±19.7** | **623.7 ±139.9** | **21.3 ±0.9** | **624.1 ±65** |

The identification and quantification of the various families of compounds constituting the waxes was conducted *via* GC and GC-MS (Figure S1). Table 1 illustrates the various lipophilic molecules present in the leaf and stem extracts of the two species. A large, diverse range of molecules were detected (varying in both structure and molecular weight); including long-chain hydrocarbons, fatty acids (saturated and unsaturated), *n-*policosanols, aldehydes, wax esters, sterols and steroid ketones. Although there is a similarity in the types of molecules found in each extract (as shown by GC-MS), quantification data indicates that there is a considerable difference in the quantities of specific compounds among each species.

Numerous odd-chained hydrocarbons (C25 – C31) were detected in all waxes from MS and MG. Octacosane (C28) was also identified in minute amounts. For all waxes, the predominant hydrocarbons were found to be nonacosane (C29) and hentriacontane (C31). *n-*Alkanes could be used in insecticides as they are known to act as semiochemicals in plant-insect interactions.32 In a previous study in which DCM was used to extract waxes from the bark and core of MG, the sole hydrocarbon detected was heptacosane indicating that scCO2 can extract a greater variety of *n-*alkanes.

A large range of saturated fatty acids was detected in each extract having chain lengths of C5 – C32. Even-chain length fatty acids were dominant. However, odd-chain fatty acids were identified albeit in significantly smaller quantities. In both the stem and leaf extracts, MG had the highest amount of saturated fatty acids. The most abundant fatty acid in all extracts was found to be palmitic acid (hexadecanoic acid), while stearic acid (octadecanoic acid), myristic acid (tetradecanoic acid) and lauric acid (dodecanoic acid) were also found in considerable amounts. Interestingly, unusually high quantities of lauric acid were found in the leaf extracts of both MS and MG (second highest amount in the leaf extracts following palmitic acid). Saturated fatty acids are utilised in a wide array of applications including soaps, detergents, lubricating grease and polishes.33 Previous work on the bark and core of MG has shown saturated fatty acids with chain lengths of C6 – C30, using DCM as the extraction solvent.29 These results are in good agreement with those obtained in in this current investigation.

All waxes contained small amounts of dicarboxylic acids; however these differed in type and quantity. Azelaic acid (nonanedioic acid) was the predominant compound which is a component of plant systemic immunity. Although dicarboxylic acids are quite polar molecules, it has been demonstrated that there is an increase in solubility of these compounds in CO2 once a cross-over pressure has been reached. The pharmaceutical properties of azelaic acid have been well documented, being used effectively in the treatment of comedonal and inflammatory acne.34 Azelaic acid is the only saturated dicarboxylic acid from miscanthus previously reported in the literature.29, 35 Figure 2 indicates the distribution of unsaturated fatty acids in the stem and leaf waxes of MS and MG. Two chain lengths were detected; C16 (C16:1) and C18 unsaturated fatty acids (C18:1, C18:2 and C18:3). It is interesting to note that there are significantly higher amounts of unsaturated fatty acids in the stem and leaf extracts of MS when compared to MG. In the leaf extracts of both species, linoleic acid (C18:2) was the major unsaturated fatty acid detected while in the stem of MS linolenic acid (C18:3) predominated.

**Figure 2. Unsaturated fatty acid content in all scCO2 mediated extracts from the various miscanthus samples. (MS – *miscanthus sinensis,* MG – *miscanthus giganteus*).**

It has been well documented that linoleic acid and linolenic acid have a hypocholesterolemic effect.36-38 Work has shown that diets rich in varied compositions of unsaturated fatty acids lowers the levels of serum cholesterol.39 Diets that are rich in α-linolenic acid have also been found to be effective in cardioprotection.40 Previous work has shown the presence of C16:1, C18:1 and C18:2 in the extracts from the bark and core of MG but no C18:3 was identified.29

The stem and leaf waxes of both MG and MS had long-chain fatty alcohols (even-chain length) ranging from C22 – C32. Figure 3 shows the three predominant fatty alcohols in the stem and leaf waxes; 1-octacosanol (C28), 1-triacontanol (C30) and 1-dotriacontanol (C32)

**Figure 3. Predominant policosanols in MS and MG (scCO2 extraction). (MS – *miscanthus sinensis,* MG – *miscanthus giganteus*).**

Interestingly, there is a substantial quantity of 1-octacosanol in the stem wax of MG when compared to all other extracts. In fact, the stem wax of MG was the only extract in which 1-octacosanol was the major fatty alcohol. In the leaf extracts of both species, the major alcohol was 1-dotriacontanol while the predominant alcohol in the stem extract of MS was found to be 1-triacontanol. In contrast to the leaves, 1-dotriacontanol was found in the lowest amounts in the stems of both species. The nutraceutical benefits of *n-*policosanols have been well established with particular relevance to cardiovascular health.41 The application of policosanol therapy has been found to have a beneficial effect in the treatment of conditions such as arteriosclerosis, intermittent claudication and hypercholesterolemia.41-43 In previously published work on extracts from the bark and core of MG, the predominant alcohol detected was 1-octacosanol (25 mg kg-1 of dry plant in the core, 81 mg kg-1 of dry plant in the bark) which is comparable to the stem wax composition of MG. Four other alcohols were detected albeit in minimal quantities.29 Therefore, this demonstrates the dominance of different chain lengths in varying parts of the plant.

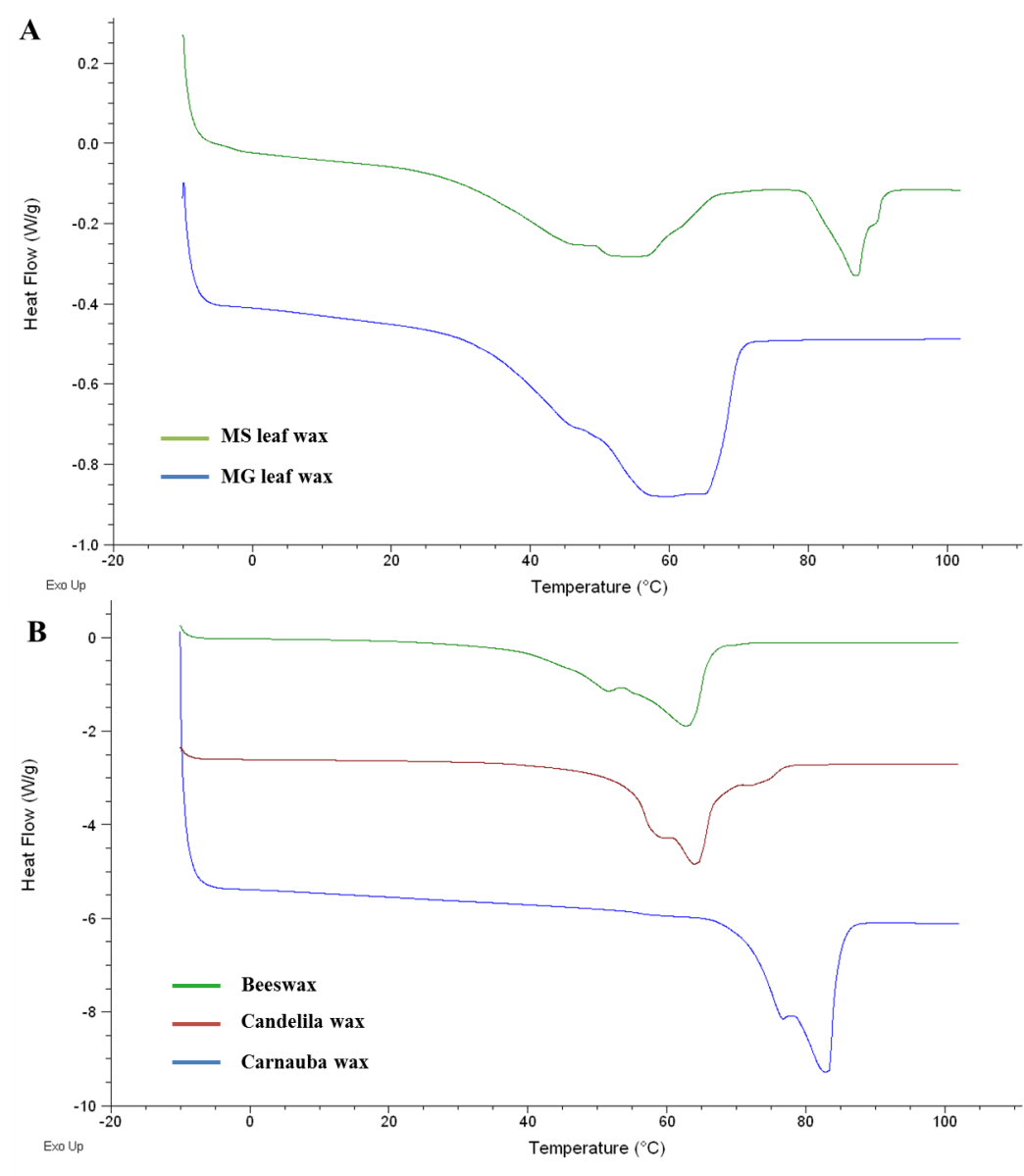
Long-chain fatty aldehydes were also present in the extracts, with chain-lengths varying from C24 – C30. The MG extracts had considerably higher amounts of aldehydes (69 ±0.48 mg g-1 of stem wax, 44 ±7.2 mg g-1 of leaf wax) than the MS extracts (29.8 ±2.7 mg/g-1 of stem wax, 18 ±4.9 mg/g-1 of leaf wax). Octacosanal (C28) was the major aldehyde identified in the extracts (stem and leaf) of MG while triacontanal was the most abundant aldehyde in the MS extracts. Long-chain aldehydes are utilised extensively in the food industry as food flavouring agents.44

The cyclic compounds detected in the miscanthus wax samples were phytosterols and steroid ketones. The major sterol identified in the extracts was β-sitosterol, with two other sterols (stigmasterol and campesterol) also present. A larger abundance of sterols were found in the stem extracts from both species (64 ±1.9 mg g-1 of MS stem wax MS, 34 ±0.4 mg g-1 of MG stem wax) than in the leaves (36 ±5.4 mg g-1 of MS leaf wax, 31 ±2.5 mg g-1 of MG leaf wax). Stigma-4-en-3-one and stigmastan-3,6-dione were the steroid ketones found in the waxes, with the former predominating. Consumption of phytosterol-enriched diets leads to a significant decrease in common cancers namely colon, breast, lung and prostate cancers.45-49 Phytosterols have also been shown to reduce the levels of plasma LDL-cholesterol.50 The phytosterol results are comparable to those obtained by DCM extraction, highlighting that β-sitosterol was the dominant sterol in the bark and core of MG.29

**Figure 4. Wax ester composition of MS and MG stems and leaves as extracted by scCO2. (MS – *miscanthus sinensis,* MG – *miscanthus giganteus*).**

A high abundance of wax esters (with chain lengths of up to C58 detected for the stem samples, C60 for the leaf samples) were present in the MS and MG waxes, as shown in Figure 4. The stem extracts (for both MS and MG) had larger amounts of wax esters (55 ±10 mg g-1 of MS wax, 71 ±0.6 mg g-1 of MG wax) than the leaf extracts (32 ±6 mg g-1 of MS wax, 18 ±3 mg g-1 of MG wax). Interestingly, C44 wax ester was the most abundant wax ester for both the MS and MG stem extracts. In the MG stem extract this comprised approximately 53% of the total wax ester composition (37.7 ±0.07 mg g-1 of MG stem wax). C48 wax ester was found to be the major wax ester in both of the leaf extracts. Significant value is given to wax esters due to their use in a vast array of industrial applications, some of which include hard wax polishes, plasticisers, lubriacants and coatings.51 No wax esters were indicated in previous studies*.*29

A number of additional lipophilic molecules were identified in the wax samples including phytol and 2-pentadecanone-6,10,14-trimethyl. In the case of the latter, higher quantities were found in the leaf waxes for both species when compared to the stems. Considerably higher amounts of phytol were found in the MS extracts (both leaves and stems) when compared to the MG extracts. Phytol is widely utilised in the fine fragrance industry as well as in other numerous applications, in the home and personal care market52

****

**Figure 5. DSC thermograms of: A) MS and MG leaf wax B) Beeswax, candelila wax and carnauba wax. (MS – *miscanthus sinensis,* MG – *miscanthus giganteus*).**

Figure 5A and 5B compare the DSC thermograms of the MS and MG waxes (extracted with scCO2) with waxes that are commercially available (beeswax, candelila wax and carnauba wax). Results indicate considerable differences between the DSC thermograms of each extract (MS and MG waxes) as well as with the commercially available waxes. The DSC thermogram of the MG stem extract was similar to that of the leaf extract with endothermic mimina at 68 oC and 66 oC respectively. These melting profiles are very similar to that of the candelila wax (64 oC). Interestingly, a contrast was observed between the MS extracts and the MG extracts in that there were two distinct melting regions in the former (whereas only one was observed in the MG extracts). In the first melting region, endothermic minima were observed at 60 oC and 67 oC for the stem and leaf waxes respectively while in the second melting point region a sharper peak was observed with a melting maximum at 87 oC for both the leaf and the stem waxes. In this study, the DSC traces obtained for the commercial waxes are similar to previously recorded data in literature.24 Since the melting profiles of the lipophilic extracts are different, this indicates that they could be used in different applications. The MS wax extracts from the stems and leaves have high-melting point waxy constituents (having higher melting profiles than carnauba wax (83oC)) which could enable them to be utilised in applications requiring this characteristic such as polishes for instruments and automobiles. However, future work involves looking at the fractionation and purification of these high-melting waxy constituents for applications testing. The waxes from the MG stem and leaves have melting profiles that make them useful for applications including food, pharmaceuticals and cosmetics.

In order to determine whether scCO2 extraction was an effective pre-treatment step in a biorefinery, it was important to see what effect the supercritical extraction has on the biomass. Saccharification was conducted on the MG leaves following scCO2 extraction (scCO2-treated) and compared to samples of untreated MG leaves (untreated) and the alcohol-insoluble residue (AIR-treated) of these MG leaves.

Saccharification of *M. x. giganteus*

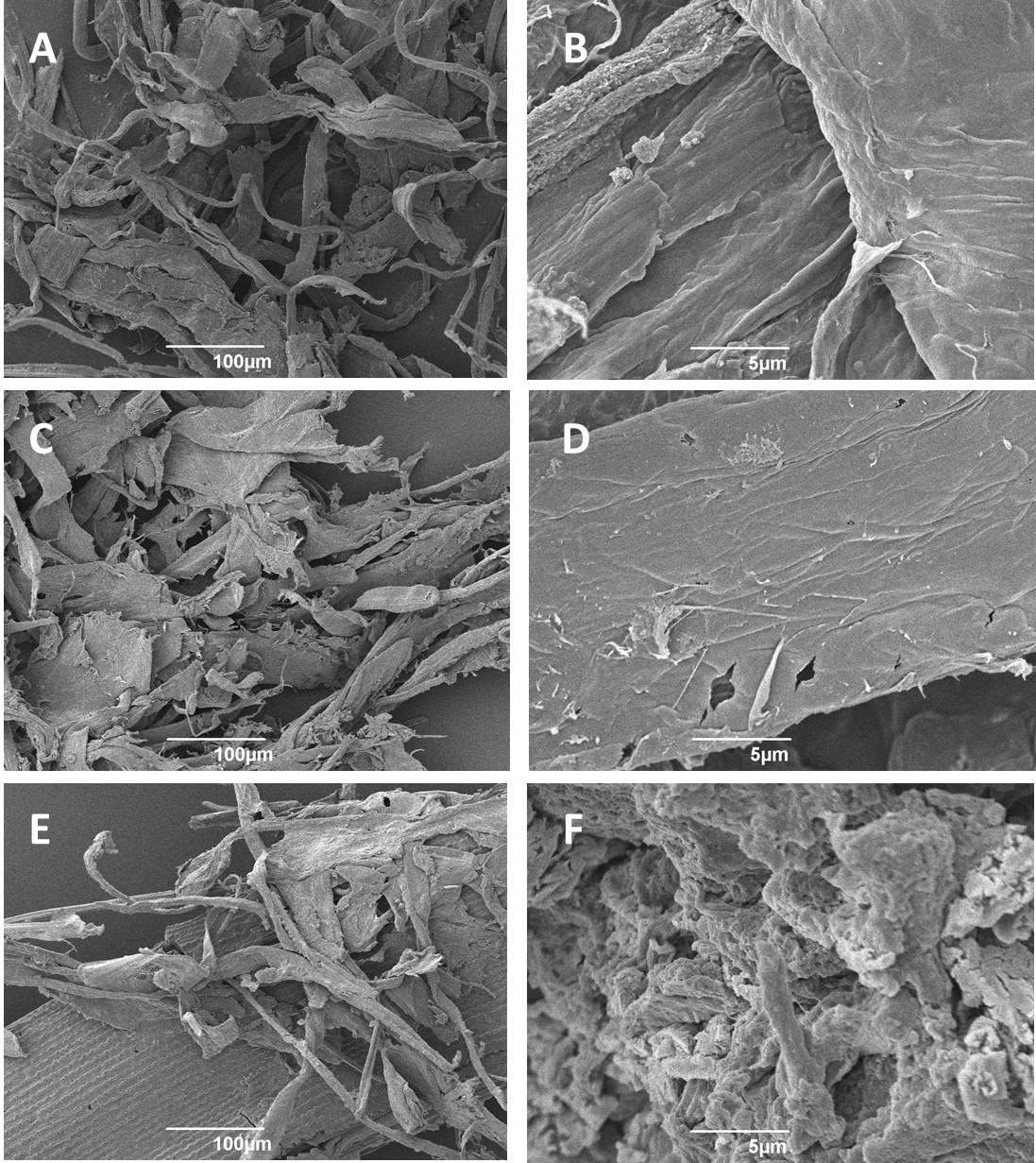
**Figure 6. Total sugars released from MG after NaOH pre-treatment and saccharification for the untreated, AIR-treated and ScCO2-treated samples compared to a control where no NaOH pre-treatment had taken place. (MG – *miscanthus giganteus*).**

Each biomass sample was subsequently subjected to 0.1 M NaOH treatment before enzymatic hydrolysis to increase the ease with which sugars were released. A control sample (control) of untreated MG leaves, which were not modified with NaOH, was also subjected to saccharification to assess the effectiveness of the NaOH treatment step. The results indicate that extraction of waxy components using scCO2 improved the saccharification efficiency of the biomass (Figure 6). 220 nmols of sugars mg-1 of material were produced for the scCO2 pre-treated sample compared to 180 nmols of sugars mg-1 of the untreated example (the AIR-treated sample produces essentially the same result as the untreated sample, within error). Thus, scCO2 extraction lead to an increase in sugar release of approximately 20% compared to the non-treated and AIR samples. These results suggest that in addition to the benefit of extracting valuable waxes, scCO2 also has a positive effect on the downstream processing of the biomass compared to untreated samples.

Previous work has already demonstrated the advantages of utilising scCO2 as a pre-treatment step for lignocellulosic biomass, improving sugar release.53-54 However, the role of scCO2 in this research was to provide a cheap and nontoxic method for pressurising the system, which was then rapidly released to explosively open up the biomass or as part of a static two solvent system to increase hydrolysis yields. Carrying out dynamic extraction not only helps to increase free sugar content for fermentation but also gives rise to a wide range of high value extractives. This demonstrates the applicability of scCO2 extraction as an important initial step in a miscanthus biorefinery.

Scanning Electron Microscopy (SEM) of miscanthus biomass following hydrolysis

SEM analysis of the biomass before and after extraction was conducted. SEM micrographs of the samples indicates that the overall structure of the materials is similar before and after supercritical extraction (figure 7 A and E). However, on further examination at X5000 magnification significant textural differences can be observed post extraction (figure 7 B, D and F), with supercritical treated surfaces being noticeably rougher. As such the supercritical extracted materials were the only samples where the xylem and phloem are clearly exposed (Supporting information Figure S2).

****

**Figure 7. SEM micrographs of A) Untreated MG (X250 magnification), B) Untreated MG (X5000 magnification), C) Ethanol extracted MG (X250 magnification), D) Ethanol extracted MG (X5000 magnification), E) Supercritical extracted MG (X250 magnification), F) Supercritical extracted MG (X5000 magnification). (MG – *miscanthus giganteus*).**

Economic study of scCO2 extraction of waxes from *M. x. giganteus*

In order to assess the viability of the extraction process, it is important to carry out an economic assessment based on the scCO2 extraction of waxes from miscanthus. This study was performed using a model by Turton *et al,* developed to estimate the cost of manufacture (COM) of chemicals.55 This model has been used previously to adequately estimate scCO2 extraction costs of essential oils, resin and fatty acids, as well as for waxes from other biomass.56-59 The COM was calculated using five main costs: the fixed capital investment (FCI), operational labour costs (COL), raw material costs (CRM), utility costs (CUT) and waste treatment costs (CWT) (eq. 1).

COM = 0.280FCI + 2.73*C*OL + 1.23(*C*RM + *C*WT + *C*UT) (1)

The COM for the extraction of waxes from miscanthus is based on a small commercial scCO2 plant normally used to extract spices, essential oils, natural pigments and nutraceuticals.56-59 The annual capacity of a facility this size is *ca.* 2000 tonnes year-1. The pressure and temperature of the extraction were taken to be 350 bar and 50 oC. The flow rate was taken to be 40 g min-1. A kinetic study (Figure S5 – supplementary information) was carried out in order to identify the ideal extraction time using the flow rate selected. It was found that 40 minutes gave rise to 83% of the total wax extracted and was found to be optimal from an economic perspective. Therefore, in the economic study, the extraction time was taken to be 40 minutes and not four hours (as in the lab-scale study). The results from the kinetic study are very similar to other studies in which the same flow rate was incorporated.58 Furthermore, CO2 is recycled on an industrial scale, meaning that limited CO2 is lost in the process. The supporting information contains a fully detailed breakdown of all the calculations and costs associated with the extraction process. Four types of calculations were estimated: (i) Cost of extracting from milled miscanthus straw (ii) cost of extracting from pelletised miscanthus straw (iii) cost of extracting from pelletised miscanthus leaves (iv) cost of extracting from pelletised miscanthus leaves followed by combustion of the miscanthus for electricity generation.

The COM for the scCO2 extraction from milled miscanthus straw was estimated to be around €160 kg-1 of miscanthus straw wax. However, modifying some parameters can lead to improvements in the COM. In this study, the COM was based on milled miscanthus. In industry, pelletised biomass is normally utilised leading to a threefold increase in biomass loading. A calculation using pelletised miscanthus straw was therefore carried out. Although the CRM and CUT costs increase when pelletised miscanthus straw is utilised, the total COM is reduced to approximately €96 kg-1 of miscanthus straw wax. From the results shown above it was found that there is a higher wax content in the miscanthus leaves when compared to the stem. The third calculation looked at the COM for scCO2 extraction of waxes from the leaves of miscanthus and it was found that the COM reduced significantly to *ca.* €24 kg-1 of miscanthus leaf wax (due to the wax yield being almost 3 times as much.)

Finally, as scCO2 extraction is a non-destructive technique, it can be viewed as an initial step in a biorefinery process, with the resultant biomass available for further valorisation. Any additional steps employed would thus lower the COM of the wax produced. One example of a downstream process following extraction is electricity generation. Herein, cost estimations for the scCO2 extraction of waxes from pelletised leaves and subsequent combustion for electricity generation was conducted. It was found that the COM further reduces to €6 per kg of miscanthus wax.

**CONCLUSIONS**

This work demonstrated the successful extraction of surfaces waxes from the stems and leaves of *miscanthus sinensis and miscanthus giganteus* using scCO2. Extractions of MG leaf exhibited the highest wax yields (1.96 ±0.03%), while the opposite was observed in the stems, whereby the largest quantities of wax were obtained from the MS stems (0.46 ±0.04%). A diverse range of molecules were detected including long-chain hydrocarbons, fatty acids (saturated and unsaturated), *n-*policosanols, aldehydes, wax esters, sterols and steroid ketones. These molecules may find use in a number of different applications including cosmetics, nutraceuticals, home and personal care products. Although there is a similarity in the types of molecules found in each extract, quantification data indicates that there is a considerable difference among each species in the quantities of specific compounds, as well as families of compounds. Furthermore, a positive effect was observed when using scCO2 extraction prior to the downstream processing of the miscanthus, thus illustrating its usefulness as a pre-treatment technique prior to saccharification. Enhanced plant digestibility was observed with the scCO2-treated material (ca. 20% increase in sugars released compared to non-treated MG leaves) which may be related to structural changes to the biomass particles, as evident at high magnification in SEM studies.

**ASSOCIATED CONTENT**

**Supporting Information**.   
Word document explaining the model for the economic analysis. (file type: Word document (.docx)

**AUTHOR INFORMATION**

Corresponding Author

Andrew J. Hunt. Department of Chemistry, University of York, Heslington, York, UK. YO10 5DD, Email: [andrew.hunt@york.ac.uk](mailto:andrew.hunt@york.ac.uk). Tel. +44 (0)1904 324456

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally. (match statement to author names with a symbol)

**ACKNOWLEDGMENTS**

We gratefully acknowledge funding through the European Commission's Directorate-General for Research within the 7th Framework Program (FP7/2007–2013) under the grant agreement no. 251132 (SUNLIBB).

**ABBREVIATIONS**

ScCO2, supercritical carbon dioxide; MG, *miscanthus x.* giganteus; MS, *miscanthus* sinensis; GC, gas chromatography; GC-MS, gas-chromatography mass spectrometry; DSC, differential scanning calorimetry; COM, cost of manufacture; FCI, fixed capital investment; COL, labour costs; CRM, cost of raw materials; CWT, waste treatment costs; CUT, utility costs.

**REFERENCES**

1. Budarin, V. L.; Shuttleworth, P. S.; Dodson, J. R.; Hunt, A. J.; Lanigan, B.; Marriott, R.; Milkowski, K. J.; Wilson, A. J.; Breeden, S. W.; Fan, J.; Sin, E. H. K.; Clark, J. H., Use of green chemical technologies in an integrated biorefinery. *Energy & Environmental Science* **2011,** *4* (2), 471-479.

2. Clark, J. H.; Budarin, V.; Deswarte, F. E. I.; Hardy, J. J. E.; Kerton, F. M.; Hunt, A. J.; Luque, R.; Macquarrie, D. J.; Milkowski, K.; Rodriguez, A.; Samuel, O.; Tavener, S. J.; White, R. J.; Wilson, A. J., Green chemistry and the biorefinery: a partnership for a sustainable future. *Green Chemistry* **2006,** *8* (10), 853-860.

3. Attard, T. M.; Watterson, B.; Budarin, V. L.; Clark, J. H.; Hunt, A. J., Microwave assisted extraction as an important technology for valorising orange waste. *New Journal of Chemistry* **2014,** *38* (6), 2278-2283.

4. Dodson, J. R.; Hunt, A. J.; Budarin, V. L.; Matharu, A. S.; Clark, J. H., The chemical value of wheat straw combustion residues. *RSC Advances* **2011,** *1* (3), 523-530.

5. Dodson, J. R.; Cooper, E. C.; Hunt, A. J.; Matharu, A.; Cole, J.; Minihan, A.; Clark, J. H.; Macquarrie, D. J., Alkali silicates and structured mesoporous silicas from biomass power station wastes: the emergence of bio-MCMs. *Green Chemistry* **2013,** *15* (5), 1203-1210.

6. Jiang, T.; Budarin, V. L.; Shuttleworth, P. S.; Ellis, G.; Parlett, C. M. A.; Wilson, K.; Macquarrie, D. J.; Hunt, A. J., Green preparation of tuneable carbon-silica composite materials from wastes. *Journal of Materials Chemistry A* **2015,** *3* (27), 14148-14156.

7. Hunt, A. J.; Budarin, V. L.; Breeden, S. W.; Matharu, A. S.; Clark, J. H., Expanding the potential for waste polyvinyl-alcohol. *Green Chemistry* **2009,** *11* (9), 1332-1336.

8. Clark, J. H.; Pfaltzgraff, L. A.; Budarin, V. L.; Hunt, A. J.; Gronnow, M.; Matharu, A. S.; Macquarrie, D. J.; Sherwood, J. R., From waste to wealth using green chemistry. *Pure and Applied Chemistry* **2013,** *85* (8), 1625-1631.

9. Greef J, M.; Deuter, M., Syntaxonomy of Miscanthus × giganteus GREEF et DEU. *Angewandte Botanik* **1993,** *67* (3-4), 87-90.

10. Zub, H. W.; Brancourt-Hulmel, M., Agronomic and physiological performances of different species of Miscanthus, a major energy crop. A review. *Agronomy for Sustainable Development* **2010,** *30* (2), 201-214.

11. Beale, C. V.; Long, S. P., Can perennial C4 grasses attain high efficiencies of radiant energy conversion in cool climates? *Plant, Cell & Environment* **1995,** *18* (6), 641-650.

12. Clifton-Brown, J. C.; Lewandowski, I., Water Use Efficiency and Biomass Partitioning of Three Different Miscanthus Genotypes with Limited and Unlimited Water Supply. *Annals of Botany* **2000,** *86* (1), 191-200.

13. Michel, R.; Mischler, N.; Azambre, B.; Finqueneisel, G.; Machnikowski, J.; Rutkowski, P.; Zimny, T.; Weber, J. V., Miscanthus × Giganteus straw and pellets as sustainable fuels and raw material for activated carbon. *Environ Chem Lett* **2006,** *4* (4), 185-189.

14. Mansouri, N.-E. E.; Salvadó, J., Structural characterization of technical lignins for the production of adhesives: Application to lignosulfonate, kraft, soda-anthraquinone, organosolv and ethanol process lignins. *Industrial Crops and Products* **2006,** *24* (1), 8-16.

15. Vega, A.; Bao, M.; Lamas, J., Application of factorial design to the modelling of organosolv delignification of Miscanthus sinensis (elephant grass) with phenol and dilute acid solutions. *Bioresource Technology* **1997,** *61* (1), 1-7.

16. Fernando, S.; Adhikari, S.; Chandrapal, C.; Murali, N., Biorefineries:  Current Status, Challenges, and Future Direction. *Energy & Fuels* **2006,** *20* (4), 1727-1737.

17. Lemke, D. W.; Thiede, M. C. Wax compositions and methods of preparing wax compositions. 0024281, 2010.

18. Kline *Global wax industry 2010: Market analysis and opportunities*; Kline & Company Inc.: 2011.

19. Deswarte, F. E. I.; Clark, J. H.; Hardy, J. J. E.; Rose, P. M., The fractionation of valuable wax products from wheat straw using CO2. *Green Chemistry* **2006,** *8* (1), 39-42.

20. Hunt, A. J.; Sin, E. H. K.; Marriott, R.; Clark, J. H., Generation, Capture, and Utilization of Industrial Carbon Dioxide. *ChemSusChem* **2010,** *3* (3), 306-322.

21. Arshadi, M.; Hunt, A. J.; Clark, J. H., Supercritical fluid extraction (SFE) as an effective tool in reducing auto-oxidation of dried pine sawdust for power generation. *RSC Advances* **2012,** *2* (5), 1806.

22. Subramaniam, B.; Rajewski, R. A.; Snavely, K., Pharmaceutical processing with supercritical carbon dioxide. *Journal of Pharmaceutical Sciences* **1997,** *86* (8), 885-890.

23. Sin, E. H. K.; Marriott, R.; Hunt, A. J.; Clark, J. H., Identification, quantification and Chrastil modelling of wheat straw wax extraction using supercritical carbon dioxide. *Comptes Rendus Chimie* **2014,** *17* (3), 293-300.

24. Athukorala, Y.; Mazza, G., Supercritical carbon dioxide and hexane extraction of wax from triticale straw: Content, composition and thermal properties. *Industrial Crops and Products* **2010,** *31* (3), 550-556.

25. Choi, Y.; Kim, J.; Noh, M.; Park, E.; Yoo, K.-P., Extraction of epicuticular wax and nonacosan-10-OL from Ephedra herb utilizing supercritical carbon dioxide. *Korean Journal of Chemical Engineering* **1996,** *13* (2), 216-219.

26. Athukorala, Y.; Mazza, G.; Oomah, B. D., Extraction, purification and characterization of wax from flax (Linum usitatissimum) straw. *European Journal of Lipid Science and Technology* **2009,** *111* (7), 705-714.

27. Attard, T. M.; McElroy, C. R.; Rezende, C. A.; Polikarpov, I.; Clark, J. H.; Hunt, A. J., Sugarcane waste as a valuable source of lipophilic molecules. *Industrial Crops and Products* **2015,** *76* (0), 95-103.

28. Attard, T. M.; Theeuwes, E.; Gomez, L. D.; Johansson, E.; Dimitriou, I.; Wright, P. C.; Clark, J. H.; McQueen-Mason, S. J.; Hunt, A. J., Supercritical extraction as an effective first-step in a maize stover biorefinery. *RSC Advances* **2015,** *5* (54), 43831-43838.

29. Villaverde, J. J.; Domingues, R. M. A.; Freire, C. S. R.; Silvestre, A. J. D.; Neto, C. P.; Ligero, P.; Vega, A., Miscanthus x giganteus Extractives: A Source of Valuable Phenolic Compounds and Sterols. *Journal of Agricultural and Food Chemistry* **2009,** *57* (9), 3626-3631.

30. Attard, T. M. Supercritical CO2 extraction of waxes as part of a holistic biorefinery. University of York, York, March 2015.

31. Gammons, R. J. Optimising the Pre-treatment Effects of Protic Ionic Liquids on Lignocellulosic Materials. University of York, York, September 2014.

32. Schiestl, F. P.; Ayasse, M.; Paulus, H. F.; Löfstedt, C.; Hansson, B. S.; Ibarra, F.; Francke, W., Orchid pollination by sexual swindle. *Nature* **1999,** *399* (6735), 421-421.

33. Hill, K., Fats and oils as oleochemical raw materials. *Pure and applied chemistry* **2000,** *72* (7), 1255-1264.

34. Fitton, A.; Goa, K., Azelaic Acid. *Drugs* **1991,** *41* (5), 780-798.

35. Villaverde, J. J.; De Vega, A.; Ligero, P.; Freire, C. S. R.; Neto, C. P.; Silvestre, A. J. D., Miscanthus x giganteus Bark Organosolv Fractionation: Fate of Lipophilic Components and Formation of Valuable Phenolic Byproducts. *Journal of Agricultural and Food Chemistry* **2010,** *58* (14), 8279-8285.

36. Shepherd, J.; Packard, C. J.; Patsch, J. R.; Gotto Jr, A. M.; Taunton, O. D., Effects of dietary polyunsaturated and saturated fat on the properties of high density lipoproteins and the metabolism of apolipoprotein AI. *Journal of Clinical Investigation* **1978,** *61* (6), 1582.

37. Ahrens Jun, E.; Insull Jun, W.; Blomstrand, R.; Hirsch, J.; Tsaltas, T.; Peterson, M., THE INFLUENCE OF DIETARY FATS ON SERUM-LIPID LEVELS IN MAN. *The Lancet* **1957,** *269* (6976), 943-953.

38. Horrobin, D. F.; Huang, Y. S., The role of linoleic acid and its metabolites in the lowering of plasma cholesterol and the prevention of cardiovascular disease. *International Journal of Cardiology* **1987,** *17* (3), 241-255.

39. Chan, J. K.; Bruce, V. M.; McDonald, B. E., Dietary alpha-linolenic acid is as effective as oleic acid and linoleic acid in lowering blood cholesterol in normolipidemic men. *The American journal of clinical nutrition* **1991,** *53* (5), 1230-1234.

40. de Lorgeril, M.; Renaud, S.; Salen, P.; Monjaud, I.; Mamelle, N.; Martin, J. L.; Guidollet, J.; Touboul, P.; Delaye, J., Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. *The Lancet* **1994,** *343* (8911), 1454-1459.

41. Marinangeli, C. P. F.; Jones, P. J. H.; Kassis, A. N.; Eskin, M. N. A., Policosanols as Nutraceuticals: Fact or Fiction. *Critical Reviews in Food Science and Nutrition* **2010,** *50* (3), 259-267.

42. Arruzazabala, M. L.; Carbajal, D.; Mas, R.; Garcia, M.; Fraga, V., Effects of policosanol on platelet aggregation in rats. *Thrombosis Research* **1993,** *69* (3), 321-327.

43. Arruzazabala, M. L.; Valdés, S.; Más, R.; Carbajal, D.; Fernández, L., COMPARATIVE STUDY OF POLICOSANOL, ASPIRIN AND THE COMBINATION THERAPY POLICOSANOL-ASPIRIN ON PLATELET AGGREGATION IN HEALTHY VOLUNTEERS. *Pharmacological Research* **1997,** *36* (4), 293-297.

44. Mottram, D. S., Flavour formation in meat and meat products: a review. *Food Chemistry* **1998,** *62* (4), 415-424.

45. De Stefani, E.; Boffetta, P.; Ronco, A. L.; Brennan, P.; Deneo-Pellegrini, H.; Carzoglio, J. C.; Mendilaharsu, M., Plant Sterols and Risk of Stomach Cancer: A Case-Control Study in Uruguay. *Nutrition and Cancer* **2000,** *37* (2), 140-144.

46. Shimizu, H.; Ross, R.; Bernstein, L.; Yatani, R.; Henderson, B.; Mack, T., Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. *British Journal of Cancer* **1991,** *63* (6), 963-966.

47. Mendilaharsu, M.; De Stefani, E.; Deneo-Pellegrini, H.; Carzoglio, J.; Ronco, A., Phytosterols and risk of lung cancer: A case-control study in Uruguay. *Lung Cancer* **1998,** *21* (1), 37-45.

48. Ronco, A.; De Stefani, E.; Boffetta, P.; Deneo-Pellegrini, H.; Mendilaharsu, M.; Leborgne, F., Vegetables, Fruits, and Related Nutrients and Risk of Breast Cancer: A Case-Control Study in Uruguay. *Nutrition and Cancer* **1999,** *35* (2), 111-119.

49. McCann, S. E.; Freudenheim, J. L.; Marshall, J. R.; Graham, S., Risk of Human Ovarian Cancer Is Related to Dietary Intake of Selected Nutrients, Phytochemicals and Food Groups. *The Journal of Nutrition* **2003,** *133* (6), 1937-1942.

50. Moghadasian, M. H.; Frohlich, J. J., Effects of dietary phytosterols on cholesterol metabolism and atherosclerosis: clinical and experimental evidence. *The American Journal of Medicine* **1999,** *107* (6), 588-594.

51. Gunawan, E. R.; Basri, M.; Rahman, M. B. A.; Salleh, A. B.; Rahman, R. N. Z. A., Study on response surface methodology (RSM) of lipase-catalyzed synthesis of palm-based wax ester. *Enzyme and Microbial Technology* **2005,** *37* (7), 739-744.

52. McGinty, D.; Letizia, C. S.; Api, A. M., Fragrance material review on phytol. *Food and Chemical Toxicology* **2010,** *48, Supplement 3* (0), S59-S63.

53. Zheng, Y.; Lin, H.-M.; Wen, J.; Cao, N.; Yu, X.; Tsao, G., Supercritical carbon dioxide explosion as a pretreatment for cellulose hydrolysis. *Biotechnol Lett* **1995,** *17* (8), 845-850.

54. Kim, K. H.; Hong, J., Supercritical CO2 pretreatment of lignocellulose enhances enzymatic cellulose hydrolysis. *Bioresource Technology* **2001,** *77* (2), 139-144.

55. Turton, R.; Bailie, R. C.; Whiting, W. B.; Shaeiwitz, J. A., *Analysis, Synthesis and Design of Chemical Processes*. Prentice Hall: New Jersey, 2013.

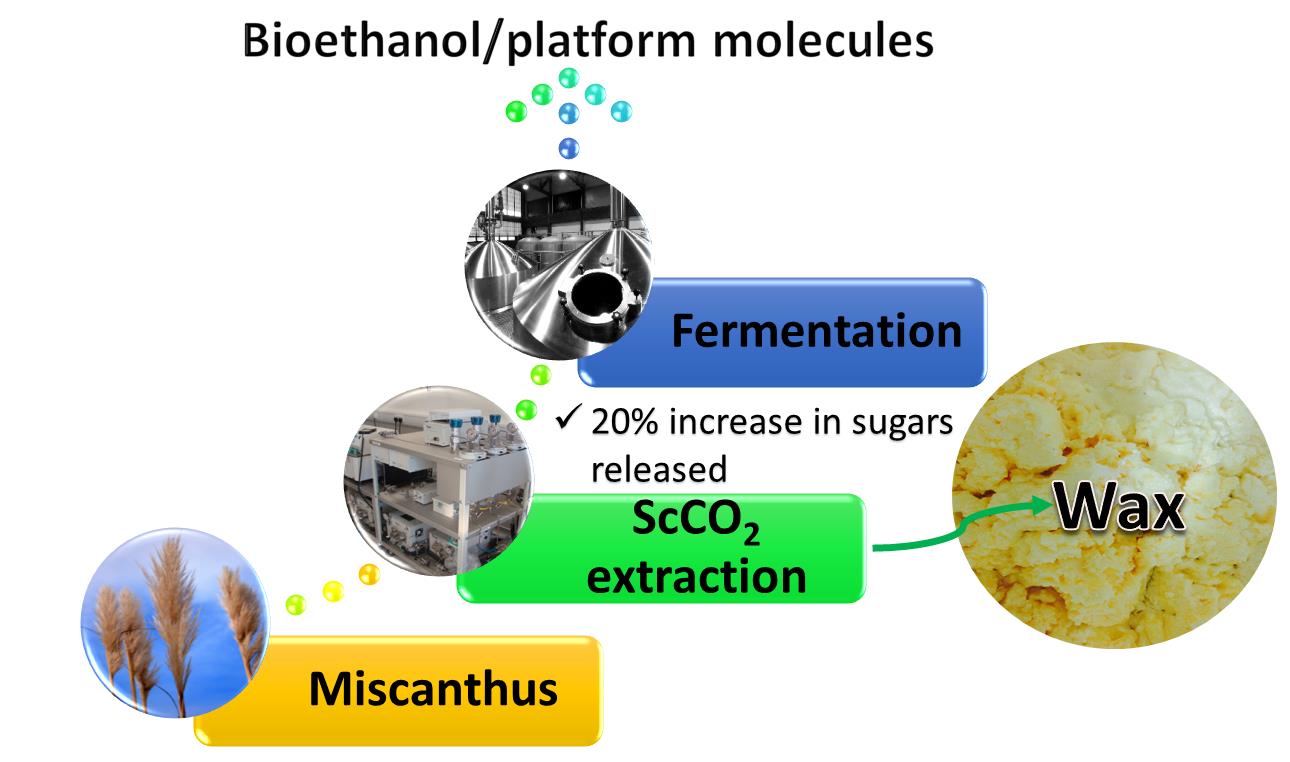
56. Rosa, P. T. V.; Meireles, M. A. A., Rapid estimation of the manufacturing cost of extracts obtained by supercritical fluid extraction. *Journal of Food Engineering* **2005,** *67* (1–2), 235-240.

57. Pereira, C. G.; Meireles, M. A. A., Economic analysis of rosemary, fennel and anise essential oils obtained by supercritical fluid extraction. *Flavour and Fragrance Journal* **2007,** *22* (5), 407-413.

58. Attard, T.; McElroy, C.; Hunt, A., Economic Assessment of Supercritical CO2 Extraction of Waxes as Part of a Maize Stover Biorefinery. *International Journal of Molecular Sciences* **2015,** *16* (8), 17546.

59. Attard, T. M.; Arshadi, M.; Nilsson, C.; Budarin, V. L.; Valencia-Reyes, E.; Clark, J. H.; Hunt, A. J., Impact of supercritical extraction on solid fuel wood pellet properties and off-gassing during storage. *Green Chemistry* **2016**.

For table of context only



Supercritical CO2 extraction as an effective pre-treatment step for wax extraction in a miscanthus biorefinery, Thomas M. Attard, C. Rob McElroy, Richard J. Gammons, John M. Slattery, Nontipa Supanchaiyamat, Claire Lessa Alvim Kamei, Oene Dolstra, Luisa M. Trindade, Neil C. Bruce, Simon. J. McQueen-Mason, Seishi Shimizu and Andrew J. Hunt

This work demonstrates supercritical CO2 extraction of waxes as an effective pre-treatment in a holistic miscanthus biorefinery.