



Chemical characterisation and analysis of the cell wall polysaccharides of duckweed (*Lemna minor*)

X. Zhao^a, G.K. Moates^a, N. Wellner^a, S.R.A. Collins^a, M.J. Coleman^b, K.W. Waldron^{a,*}

^a Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, United Kingdom

^b School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, United Kingdom

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ABSTRACT

Duckweed is potentially an ideal biofuel feedstock due to its high proportion of cellulose and starch and low lignin content. However, there is little detailed information on the composition and structure of duckweed cell walls relevant to optimising the conversion of duckweed biomass to ethanol and other biorefinery products. This study reports that, for the variety and batch evaluated, carbohydrates constitute 51.2% (w/w) of dry matter while starch accounts for 19.9%. This study, for the first time, analyses duckweed cell wall composition through a detailed sequential extraction. The cell wall is rich in cellulose and also contains 20.3% pectin comprising galacturonan, xylogalacturonan, rhamnogalacturonan; 3.5% hemicellulose comprising xyloglucan and xylan, and 0.03% phenolics. In addition, essential fatty acids (0.6%, α -linolenic and linoleic/linoelaidic acid) and *p*-coumaric acid (0.015%) respectively are the most abundant fatty acids and phenolics in whole duckweed.

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1. Introduction

The *Lemnaceae* family, members of which are commonly known as duckweeds, contains the smallest flowering plants. These plants float on the surface of water and have thin leaves attached to a simple root (Landolt & Kandeler, 1987). This morphology enables duckweed to obey Kleiber's 3/4-power rule ($G \propto M^{3/4}$) (Niklas & Enquist, 2001) in that duckweed exhibits very much higher specific growth rates than other larger aquatic or terrestrial plants, with doubling times of between 48 h and 96 h depending on species (Zuberer, 1982). Consequently, duckweeds potentially can produce high biomass yields, with high proportions of protein and carbohydrate (Landolt & Kandeler, 1987). Zhao et al. (2012) summarised earlier studies demonstrating the higher productivity of duckweeds compared to other energy crops. Duckweed can also be used for the decontamination of wastewater, absorbing problematic minerals as it grows (Jayaweera, Kasturiarachchi, Kularatne, & Wijeyekoon, 2008; Tripathia & Srivastava, 1991).

Abbreviations: AIR, alcohol insoluble residue; CWM, cell wall material; DM, dry matter; FA, fatty acid; FDM, freeze dried and freeze milled.

* Corresponding author at: The Biorefinery Centre, Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, United Kingdom.

Tel.: +44 01603 255385; fax: +44 01603 507723; mobile: +44 07787 851393.

E-mail address: keith.waldron@ifr.ac.uk (K.W. Waldron).

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Many studies report that duckweed is a protein rich resource for animal feed (e.g. Leng, Stambolie, & Bell, 1995). However, recent research also shows that duckweed is a good feedstock for bioethanol production due to its high proportion of starch (Cheng & Stomp, 2009). We have shown previously (Zhao et al., 2012) that the cell wall material of duckweed can be simply and efficiently saccharified. Ethanol and other biofuel products, such as gas, oil and biochar, can be produced, the latter through pyrolysis (Muradov, Fidalgo, Gujar, & T-Raissi, 2010) and thermolysis (Campanella et al., 2011). However, the lack of information on the chemical composition of duckweed, for example cell wall composition, whole duckweed fatty acid and phenolic components, hinders the exploitation of duckweed as a useful industrial feedstock of biofuel and other products, potentially of high value, by biorefining.

The cell walls of plants generally consist of three main groups of polysaccharides – cellulose, hemicellulose, and pectins (Brett & Waldron, 1996, chap. 2). Cellulose forms the main structural component of the cell wall and is a relatively homogenous polymer of β -1,4-linked glucose. The non-crystalline matrix phase of the cell wall consists of a variety of polysaccharides, proteins and phenolic compounds (Brett & Waldron, 1996, chap. 2). The pectic and hemicellulose polysaccharides are heterogeneous in nature and comprise polymers of different sugar compositions. Golovchenko, Ovodova, Shashkov, and Ovodov (2002) investigated the pectic polysaccharides of *Lemna minor* and analysed the monosaccharide compositions of the pectin. However there is little research

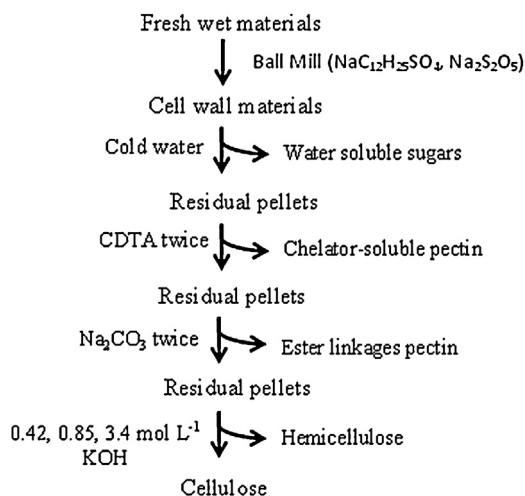


Fig. 1. The process of the preparation and sequential extraction of cell wall materials.

giving a comprehensive understanding of duckweed cell wall polymer composition. This paper describes the chemical composition of duckweed cell wall polymer groups, fatty acids and phenolics, and assesses the proportions of cellulose, hemicelluloses and pectic polysaccharides and investigates their monosaccharide composition.

2. Materials and methods

2.1. Preparation of plant material

L. minor plants were collected from a pond in Norwich, UK (52.622295 N, 1.221894 E), cleaned with tap water and then rinsed with distilled water. The cleaned, fresh wet biomass was packed in aluminium foil in long flat-thin packages and preserved at -40°C . Material was treated by various means depending on the experiment being conducted. The frozen duckweed material was dried using a freeze drier (Freeze Dryer 3.5, Birchover Instruments Ltd., UK). The freeze-dried materials were ground by freeze-milling in liquid nitrogen (Spex Freezer-Mill 6700, Spex Industries Inc., USA) to reduce the plant material to a powder. The freeze-dried and freeze-milled (FDM) duckweed was used for general chemical analysis. For the sequential extraction (see Fig. 1) of polysaccharides of duckweed cell wall, the original fresh biomass was treated by a rotary ball-mill process (ball mill jar, Capco Test Equipment Ltd., Ipswich, Suffolk, UK) to disrupt plant structure and remove starch (Parr, Waldron, Ng, & Parker, 1996). The fresh duckweed (200 g) was prepared as cell wall materials (CWM) by ball-milling and filtering on $25\text{ }\mu\text{m}$ nylon mesh to separate starch from the CWM. The ball-mill jar was filled with ceramic beads (750 g of 2.54 cm diameter beads, 500 g of 1.91 cm diameter beads and 500 g of 1.27 cm diameter beads) to break down plant tissues.

2.2. Moisture determination

The moisture content of the original fresh materials (1 g in triplicate) was determined using a Mettler Toledo LP16 Infrared Dryer balance (Mettler Toledo Ltd., Beaumont Leys, Leicester, UK).

2.3. Ash test

The total mineral nutrients were established by determination using the total ash method (Sluiter et al., 2008). The FDM duckweed

(1 g in triplicate) was placed in a crucible and dried in an oven (105°C) for 16 h. The dry weights were recorded. The resulting samples were then charred in a muffle furnace (Vulcan 3-550, Jencons Scientific Ltd., Leighton Buzzard, Beds., UK), starting at 200°C with a gradual temperature increase to 350°C . Finally, the samples were ashed at 500°C for 16 h.

2.4. Lignin determination

Acid insoluble lignin was measured by the gravimetric Klason lignin method. The FDM duckweed (0.1 g in triplicate) was hydrolysed in 1.5 mL 72% (w/w) H_2SO_4 at 30°C for 1 h, before adding 1.5 mL water and hydrolysing at 100°C for 2.5 h. The acid insoluble lignin was recovered on a sintered glass funnel (Porosity[®] 4, VWR, Lutterworth, Leics., UK) and dried in an oven (50°C). The resulting sample was burned in a muffle furnace to determine Klason lignin and the weight was corrected for ash content. Phenolic acids were measured by HPLC as described below.

2.5. Starch assessment

The starch content was measured using the standard method from the Total Starch Assay Procedure (Megazyme, 2011). FDM duckweed (30 mg in triplicate) was dispersed in 80% (v/v) ethanol (200 μL). After boiling for 5 min with 2 mL of dimethyl sulphoxide (DMSO), the samples were hydrolysed using α -amylase (3 mL, 300 U) whilst boiling for a further 6 min. The hydrolysed samples were cooled to 50°C by adding sodium acetate buffer (4 mL, 0.2 mol L^{-1} , pH 4.5) and then hydrolysed with amyloglucosidase (0.1 mL, 20 U) at 50°C for a further 0.5 h. The resulting sample (0.1 mL) was assessed by colourimetric assay using glucose oxidase-peroxidase-4-aminoantipyrine (GOPD) reagent (3 mL) at 510 nm.

2.6. The investigation of the lipid fraction

Duckweed was also cultured under lab conditions according to Zhao et al. (2012). Aliquots at various time points during a 28-day starvation period were collected and observed with a light microscope to understand the variation in lipid content when duckweed is starved of nutrients.

2.7. Sequential extraction of cell wall polysaccharides

The composition of the duckweed cell wall including cellulose, hemicellulose and pectin was evaluated by sequentially extracting the CWM as described by Stevens and Selvendran (1984). The process is illustrated in Fig. 1. CWM (2 g) was stirred in cold water (deionised water) at room temperature for 2 h and the supernatant containing the water-soluble components was recovered. The residual pellet was then extracted with CDTA (0.05 mol L^{-1} , pH 6.5, prepared in deionised water) at room temperature for 6 h following by CDTA (0.05 mol L^{-1} , pH 6.5, prepared in distilled water) extraction at room temperature for 2 h, recovering chelator-soluble polysaccharides. All further extractants were prepared using degassed ultrapure water. Next, the pellet was extracted with $0.05\text{ mol L}^{-1}\text{ Na}_2\text{CO}_3$ ($0.02\text{ mol L}^{-1}\text{ NaBH}_4$) at 4°C for 16 h to solubilise pectins with weak ester linkages followed by $0.05\text{ mol L}^{-1}\text{ Na}_2\text{CO}_3$ ($0.02\text{ mol L}^{-1}\text{ NaBH}_4$) at room temperature for 2 h to remove strong ester linkage pectin. Finally, the pellet was extracted using increasing concentrations (0.42 , 0.85 and 3.4 mol L^{-1}) of KOH ($0.02\text{ mol L}^{-1}\text{ NaBH}_4$) to solubilise hemicelluloses. The supernatants were filtered using GF/C filter paper (Whatman[®] glass microfibre filter, Whatman International, Maidstone Kent, UK) and neutralised to pH 5. Salts in the aqueous and solid pellets were

removed by dialysis: the samples were placed into Visking dialysis tubing (Scientific Instrument Centre Ltd., Colden Common, Winchester, Hants., UK) in 5 L flasks of distilled water. Chloroform (several drops, Fisher Scientific Ltd., Loughborough, UK) was added to prevent the growth of microorganisms. The dialysis water was changed twice daily and the process was carried out for 10 days. The resulting samples were freeze dried as described above for subsequent analysis.

2.8. Analytical methods

2.8.1. Gas chromatography (GC) analysis of alditol acetates

The monosaccharide compositions of the various samples were evaluated by GC analysis of alditol acetates (Blakeney, Harris, Henry, & Stone, 1983). Ball milled and distilled water washed CWM, sequentially extracted soluble materials and residual pellets were freeze dried for sugars analysis. Samples were prepared as alditol acetates as described in Zhao et al. (2012) and then were analysed by GC on a Perkin-Elmer Autosystem XL GC system with a RTX-225 (Restek, Bellefonte, USA) column and flame ionisation detector (Perkin-Elmer, Seer Green, Bucks., UK). 2-Deoxyglucose was added as the internal standard.

2.8.2. Gas chromatography (GC) analysis for fatty acid methyl esters (FAME)

Lipids extracted using a Soxhlet extraction were prepared for identifying FAME components and assessed by a GC method. The lipid samples (in triplicate) were purified with 0.5 mL dry toluene containing 0.1 mg butylated hydroxytoluene (BHT) with vortex mixing. Methylation reagent (1 mol L⁻¹ of methanol containing 2% (v/v) H₂SO₄) was added and the samples were vortex mixed again. The tubes were tightly capped and heated at 50 °C overnight with occasional mixing. After heating, the samples were cooled down to room temperature and neutralised by 1 mL mixed solution of 0.25 mol L⁻¹ KHCO₃ and 0.5 mol L⁻¹ K₂CO₃. The resulting solutions were mixed with 1 mL hexane and centrifuged to separate FAMES into upper phase which were transferred to clean Pyrex® culture tubes. 200 µL of prepared samples were transferred into vials and evaluated using a Hewlett Packard 5890 GC system (Hewlett-Packard Limited, Bracknell, England, UK) with BPX 70 column (SGE Analytical Columns, New Addington, Croydon, UK). Methyl heptadecanoate (Sigma Aldrich, Gillingham, UK) was added as the internal standard and the retention time of 31 FAME components had been specifically identified for the BPX 70 column by using a commercial standard (SGE Analytical Columns, New Addington, Croydon, UK).

2.8.3. FT-IR analysis of the identification of polysaccharides

FTIR-ATR spectra were measured with a BioRad FTS175 Fourier (Bio-Rad Laboratories Inc., USA) transform infrared spectrometer equipped with a MCT detector and a GoldenGate (Specac) single reflection diamond ATR accessory. Five aliquots from each sequentially extracted sample were loaded on the ATR crystal and pressed down with the clamp. For each, 64 scans at a resolution of 4 cm⁻¹ in the region 4000–800 cm⁻¹ were averaged and referenced against a spectrum of the empty crystal.

2.8.4. Phenolic acid assessment

Cell wall-bound phenolic acids (*p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, protocatechuic acid, vanillin, vanillic acid, *p*-hydroxyphenyl acetic acid, *trans*-*p*-coumaric acid, caffeic acid, *trans*-ferulic acid and chlorogenic acid) of dry cell wall material were evaluated by HPLC and the samples were prepared using the method reported by Parr, Waldron, Ng, and

Parker (1996). *p*-Hydroxybenzoic acid, protocatechuic acid, *p*-hydroxybenzaldehyde, vanillin, vanillic acid, *p*-hydroxyphenyl acetic acid, *trans*-*p*-coumaric acid, *trans*-ferulic acid and chlorogenic acid were obtained from Sigma (Sigma-Aldrich Company Ltd., Dorset, UK); caffeic acid was obtained from Fluka (Sigma-Aldrich Company Ltd., Seelze, Switzerland). The resulting samples were redissolved in 50% (v/v) methanol (1 mL) and filtered using a filter disc (0.2 µm, PVDF) for HPLC analysis with a Phenomenex Luna 5 µm (250 mm × 4.0 mm) column and a Perkin Elmer Diode Array Detector (UV). *Trans*-cinnamic acid was added as an internal standard. The phenolic acids were identified by comparing their retention time relative to the internal standard and the spectra with those of the authentic standards.

2.8.5. Quantification of lipids by using Soxhlet extraction

The Soxhlet extraction apparatus was originally designed for extracting the lipid fraction in solid samples (Laurence & Christopher, 1989). Sample (2 g) was contained in a cellulose extraction thimble capped with glass wool. Hexane was used to extract lipids from the sample in a solvent refluxing process for a 6 h period. The solvent containing the lipids was concentrated in a rotary evaporator under vacuum (Rotavapor R-114, BÜCHI UK Ltd., Oldham, UK). The residual pellet was re-extracted for 6 h and solvent was concentrated and combined with the first extract. The lipid content was measured gravimetrically.

2.8.6. Microscopy

Ball milled biomass was observed by microscopy (BX60, Olympus, Japan) to assess the extent of starch removal during the ball mill process. The materials were stained with Lugol's solution (20 kg m⁻³ KI with iodine (0.2 kg m⁻³)) to highlight the presence or absence of starch. The fresh healthy plants and starved plants were immersed in CDTA (50 mM Na₃H CDTA and 5 mM Na₂S₂O₅, pH 7) solution for 2 days to separate the plant cells, following by staining with Nile Blue (0.01% w/v, Raymond A Lamb, UK) for 5 min at room temperature. The samples were observed under blue Bertrand lens of a fluorescent microscope (BX60, Olympus, Japan, 20×) (Wang, Chi, Song, Wang, & Chi, 2012).

3. Results and discussion

3.1. Evaluation of duckweed composition (FDM)

The overall chemical composition of *L. minor* was assessed and some of them are reported in the first time. The dry matter content of the total freeze-dried duckweed was 8.5% (w/w DM) – which falls within the range of 3–14% reported by Landolt and Kandeler (1987). Of this dry matter, carbohydrate is the predominant component and accounts for up to 51.2% (w/w DM), of which starch contributes 19.9% (w/w DM). Ash accounts for 12.2% – Landolt and Kandeler (1987) reported that ash equivalents could constitute from 12% to 27.6% of dry matter. Zaher, Begum, Hoq, Begum, and Bhuiyan (1995) give a value for ash content of 12% of dry matter for *L. minor* cultured in ponds in Bangladesh. The ash value is attributed to large amounts of calcium oxalate stored in a crystal form in duckweed plants (Landolt & Kandeler, 1987). However, only 12.0% of protein was present in the materials used in this study which is lower than other published protein data for duckweed, such as 14% (w/w of dry matter) in Zaher et al. (1995) and 31% (w/w of dry matter) in Shireman, Colle, and Rottmann (1977). A small amount of lipid (3.1%, w/w DM) was also measured in this material. Low lipid content (1.8–9.2%) was also reported by Landolt and Kandeler (1987). A low level of Klason lignin (2.4%, w/w DM) is present in *L. minor* while a trace of cell wall-bound phenolic acids (0.03%, w/w CWM) was detected.

3.2. Analysis of duckweed polysaccharides

The total monosaccharide composition of *L. minor* freeze dried material (FDM) was investigated and the results are tabulated in Table 1. The high proportion of carbohydrate (51.2%) indicates the potential of using duckweed as a feedstock for biofuel production. The predominant monosaccharide is glucose (331.3 g kg⁻¹ DM) followed by uronic acid (96.1 g kg⁻¹ DM). Other less abundant monosaccharides include xylose (46.4 g kg⁻¹ DM), galactose (16.3 g kg⁻¹ DM), arabinose (8.0 g kg⁻¹ DM), mannose (7.1 g kg⁻¹ DM), rhamnose (4.4 g kg⁻¹ DM) and fucose (2.3 g kg⁻¹ DM). The large amount of uronic acid and small amount of xylose indicates that duckweed biomass contains significant amounts of pectin and less hemicellulose. The results of the 1 mol L⁻¹ H₂SO₄ hydrolysis indicate an approximate starch content of up to about 20% (w/w of DM) in fresh duckweed although it should be noted that about 10% of the cellulose is likely to have been hydrolysed by 1 mol L⁻¹ H₂SO₄. Of the quantifiable monosaccharides found in total duckweed, glucose, galactose and xylose are reported as fermentable sugars (Delgenes, Moletta, & Navarro, 1996) and, together, these three sugars account for 77.0% of total sugars (394 g kg⁻¹ of DM). The high proportion of fermentable sugars with a low level of lignin supports the rationale that duckweed could potentially be a useful feedstock for biofuel production.

3.3. Evaluation of lipid content

As noted above, total lipid (3.1%, w/w of DM) was assessed by using Soxhlet extraction. Lipid was further evaluated using fluorescence microscopy (see Fig. 2b–e). Under blue light, lipid equivalents appear yellow and chlorophyll and starch granules appear red. In fresh, healthy duckweed, lipid appears to be stored mainly in epidermal cells rather than the palisade cells although few lipids were observed in the healthy plant (Fig. 2b and d). Fig. 2c shows the same palisade tissue observed under sunlight as a reference. After duckweed had undergone 28 days of nutrient-starvation, more lipid equivalents were formed and stored in epidermal tissues (Fig. 2e). It is notable that abundant lipid equivalents are stored and chlorophyll and starch granules had disappeared in epidermal tissues of dead plants (Fig. 2f). These images of the long-term nutrient-starved duckweed imply that more lipids could be released from cytoplasmic membrane or chloroplasts and starch is consumed when the plant is dying due to lack of nutrition. Duckweed growing in nutrient deficient conditions might be a good method for biorefining lipid products from duckweed but it is not beneficial for converting duckweed sugars to ethanol due to the reduced starch levels.

FAME components were assessed by GC and the fatty acid profile is tabulated in Table 2. Fatty acids only account for 0.8% (w/w of DM), but contain a high proportion of the essential fatty acids (EFA), e.g. linoleic and α -linolenic acid. Of the total fatty acid, saturated FA accounts for 27.4% (equivalent to 6.9% of total lipid). Unsaturated FA contributes 72.6% (equivalent to 18.3% of total lipid) – however, due to the low overall amounts of fatty acids present, the quantity of unsaturated FA remains lower than most other vegetables (Holland, Buss, & Unwin, 1991). Palmitic acid as the predominant saturated FA compound accounts for 22.4% (w/w of total FA) and only 1.9%, 1.7%, 1.0% and 0.5% (w/w of total FA) of myristic, stearic, eicosanoic and lauric acids respectively were detected. Of the unsaturated FAs, α -linolenic acid was detected as the primary compound (33.4%), followed by linoleic/linoelaidic acid (25.2%). These unsaturated FAs are well known types of EFA and are potentially high-value byproducts. Other unsaturated FAs with nutritional and medicinal value are relatively low, e.g. stearidonic acid (3.6%), oleic acid (3.0%), γ -linolenic acid (1.8%) and eicosatrienoic acid (1.8%).

Table 1
The monosaccharide compositions of FDM and CWM materials (units g kg⁻¹)

Sample	Hydrolysis	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	Totals
FDM	72% H ₂ SO ₄ 1 mol L ⁻¹ H ₂ SO ₄	4.4 ± 0.5 4.3 ± 0.2	2.3 ± 0.4 2.1 ± 0.2	8.0 ± 0.6 6.5 ± 1.0	46.4 ± 4.3 38.8 ± 1.8	7.1 ± 0.9 3.3 ± 0.3	16.3 ± 1.5 15.4 ± 0.5	331.3 ± 33.9 199.6 ± 6.3	96.1 ± 4.9 44.2 ± 3.5	512.0 ± 37.4 314.3 ± 11.1
CWM	72% H ₂ SO ₄ 1 mol L ⁻¹ H ₂ SO ₄	7.0 ± 0.4 6.1 ± 0.3	2.2 ± 0.3 2.1 ± 0.2	6.9 ± 0.3 6.8 ± 0.2	83.8 ± 0.9 75.1 ± 3.6	8.5 ± 0.5 3.1 ± 0.2	14.2 ± 0.3 13.2 ± 0.5	308.0 ± 5.3 31.6 ± 0.8	202.2 ± 22.6 86.5 ± 10.6	632.7 ± 27.8 224.5 ± 6.1

Abbreviations: Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; GalA, galacturonic acid.

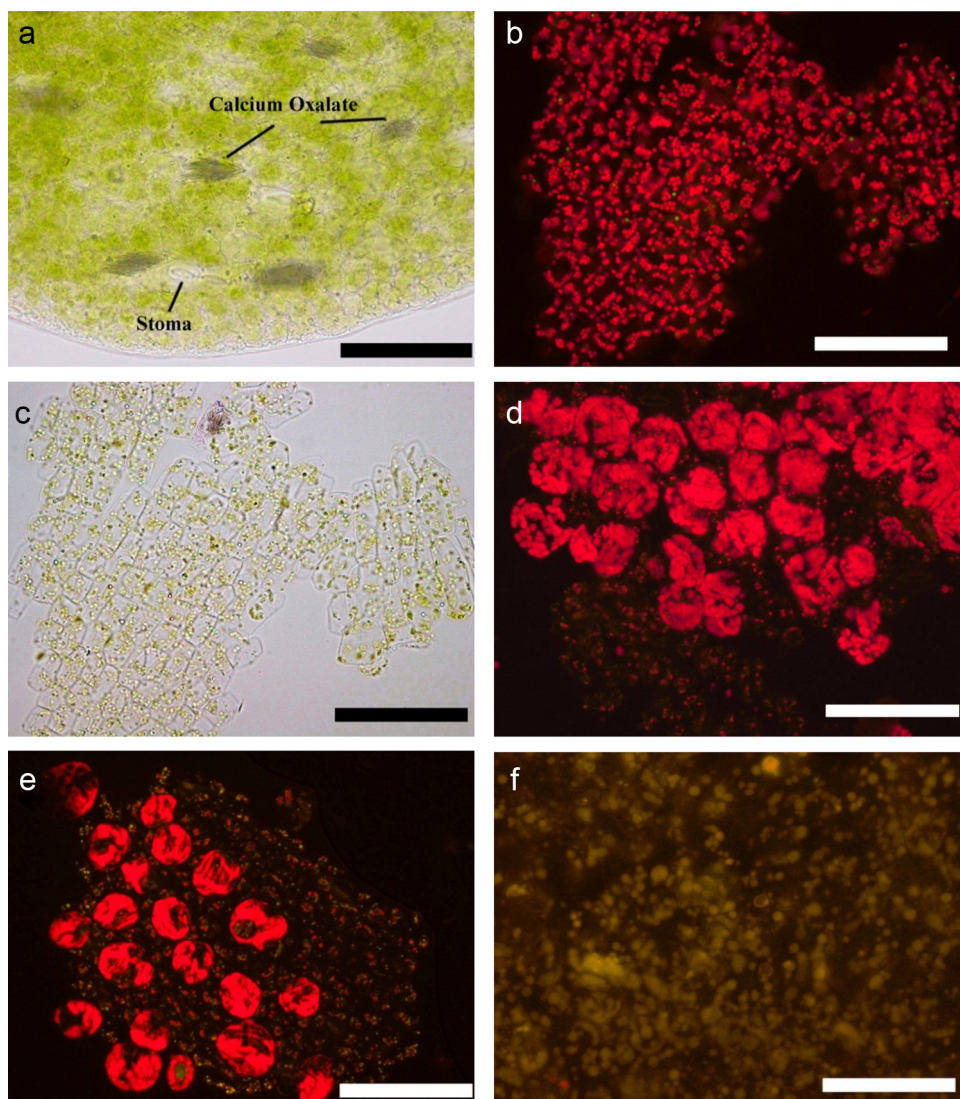


Fig. 2. Fluorescence micrographs indicating lipid fractions and the variation of lipid under nutrient-starved conditions (bars = 100 μm). (a) The leaf tissue of a healthy plant under sunlight, (b) palisade tissue of a healthy plant under blue light, (c) palisade tissue of a healthy plant under sunlight, (d) epidermal tissue of a healthy plant under blue light, (e) epidermal tissue of a healthy plant (Day 28) under blue light and (f) epidermal tissue of a dead plant (Day 28) under blue light.

Table 2
Fatty acid composition of *L. minor*.

Fatty acids	C:D ^a	n-x	% (w/w of FA)	% (w/w of lipid)
Total FA			0.8 $\pm$ 0.12 (of DM)	25.2
Saturated FA			27.4	6.9
Lauric acid	12:0		0.5 $\pm$ 0.03	0.1
Myristic acid	14:0		1.9 $\pm$ 0.02	0.5
Palmitic acid	16:0		22.4 $\pm$ 0.13	5.6
Stearic acid	18:0		1.7 $\pm$ 0.1	0.4
Eicosanoic acid	20:0		1.0 $\pm$ 0.13	0.3
Unsaturated FA			72.6	18.3
Palmitoleic acid	16:1	n-7	3.0 $\pm$ 0.03	0.8
Vaccenic acid	18:1	n-7	1.0 $\pm$ 0.12	0.3
Oleic acid (ω -9)	18:1	n-9	3.0 $\pm$ 0.12	0.7
Linoleic/linoelaidic acid (ω -6)	18:2	n-6	25.2 $\pm$ 0.04	6.4
α -Linolenic acid (ω -3)	18:3	n-3	33.4 $\pm$ 0.21	8.4
γ -Linolenic acid (ω -6)	18:3	n-6	1.8 $\pm$ 0.07	0.4
Stearidonic acid (ω -3)	18:4	n-3	3.6 $\pm$ 0.09	0.9
Eicosadienoic acid (ω -6)	20:2	n-6	0.5 $\pm$ 0.04	0.1
Eicosatrienoic acid (ω -3)	20:3	n-3	1.0 $\pm$ 0.12	0.3

^a C:D represents the ratio of carbon and double bonds while n-x represents the position of the first double counted from methyl end.

Table 3
Phenolic compositions of *L. minor* cell wall ($\mu\text{g kg}^{-1}$ DM) following sequential extraction

Fractionation	Protocatechuic aldehyde	<i>p</i> -OH-benzaldehyde	Truxillic acid (CA)	Ferulic acid	<i>p</i> -Coumaric acid	Total
CWM	22.5 $\pm$ 10.6	10.7 $\pm$ 7.1	43.8 $\pm$ 5.4	20.1 $\pm$ 10.4	153.0 $\pm$ 21.2	250.1 $\pm$ 20.6
Cold water	0	13.7 $\pm$ 0.2	51.3 $\pm$ 0.1	26.4 $\pm$ 0.3	33.4 $\pm$ 0.2	125.2 $\pm$ 0.7
CDTA 1st	0	9.5 $\pm$ 1.9	44.5 $\pm$ 1.5	39.2 $\pm$ 3.5	23.8 $\pm$ 0.9	117.0 $\pm$ 6.7
CDTA 2nd	0	6.6 $\pm$ 0.1	44.7 $\pm$ 0.2	29.6 $\pm$ 0.2	8.6 $\pm$ 0.1	89.5 $\pm$ 0.4
Na ₂ CO ₃ 1st	10.0 $\pm$ 0.3	23.2 $\pm$ 0.1	44.6 $\pm$ 0.5	113.4 $\pm$ 0.6	79.5 $\pm$ 0.2	284.1 $\pm$ 3.7
Na ₂ CO ₃ 2nd	45.8 $\pm$ 0.5	32.1 $\pm$ 0.1	36.6 $\pm$ 0.1	34.4 $\pm$ 0.1	123.0 $\pm$ 0.1	271.8 $\pm$ 0.9
0.42 mol L ⁻¹ KOH	12.8 $\pm$ 0.2	21.0 $\pm$ 0.1	27.3 $\pm$ 0.2	55.1 $\pm$ 0.6	334.6 $\pm$ 1	450.8 $\pm$ 3.8
0.85 mol L ⁻¹ KOH	11.1 $\pm$ 0.2	23.1 $\pm$ 0.1	27.6 $\pm$ 0.2	36.7 $\pm$ 0.1	361.2 $\pm$ 0.4	460.0 $\pm$ 0.6
3.4 mol L ⁻¹ KOH	0	17.0 $\pm$ 0.1	47.6 $\pm$ 0.1	86.4 $\pm$ 0.2	97.2 $\pm$ 0.1	248.2 $\pm$ 0.3
Pellets	17.0 $\pm$ 0.5	4.6 $\pm$ 5.0	21.2 $\pm$ 6.1	0	17.0 $\pm$ 6.0	42.8 $\pm$ 12.7

3.4. Compositional analysis of phenolics

Phenolic acids are the main low molecular weight components of lignin and also play a crucial role in the linkage among hemicellulosic polysaccharides (Brett & Waldron, 1996, chap. 2). The phenolic acids are therefore worthy of investigation even though the lignin content of *L. minor* is relatively low (2.4%, w/w of DM). Cell wall-bound phenolics account for 0.03% (w/w of CWM), and this mainly involves the five types of phenolic acids tabulated in Table 3. The

proportions of lignin and phenolic acids are much lower than in other well-known monocotyledonous energy crops: lignin levels in switchgrass and wheat straw are 15–29% and 15–20%, respectively (Harris & DeBolt, 2010; Theander & Aman, 1982) and phenolics in wheat straw represent approximately 1% (Waldron, 2010). *p*-Coumaric acid is the most abundant (0.15 g kg⁻¹ CWM), accounting for 60% of phenolics, followed by truxillic acid (0.04 g kg⁻¹ DM), protocatechuic aldehyde (0.02 g kg⁻¹ CWM) and ferulic acid (0.02 g kg⁻¹ CWM). 0.01 g kg⁻¹ CWM *p*-OH-benzaldehyde was also

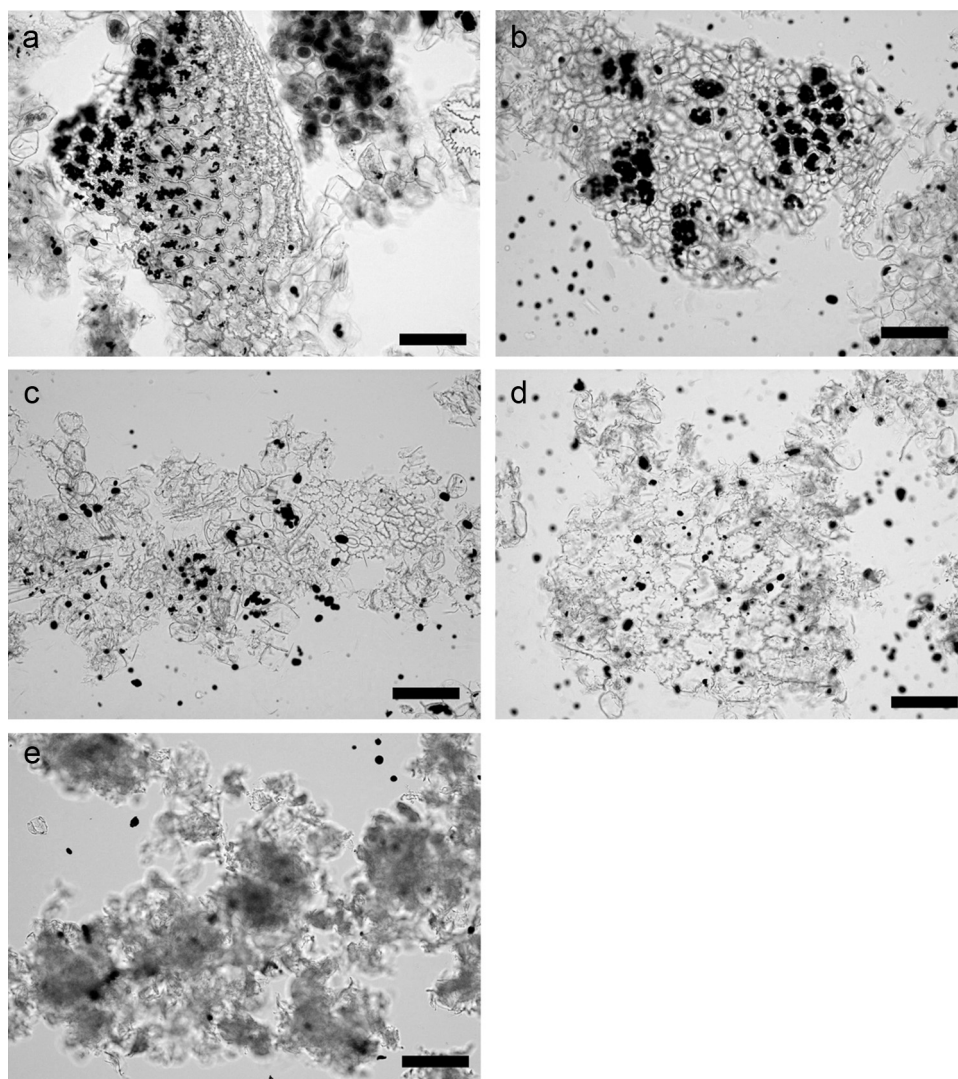


Fig. 3. The extent of starch removal during the ball-milling process observed by microscopy (bars = 100 μm). (a) Blender milled material, (b) 1 h, (c) 2 h, (d) 3.5 h and (e) distilled water washing residues.

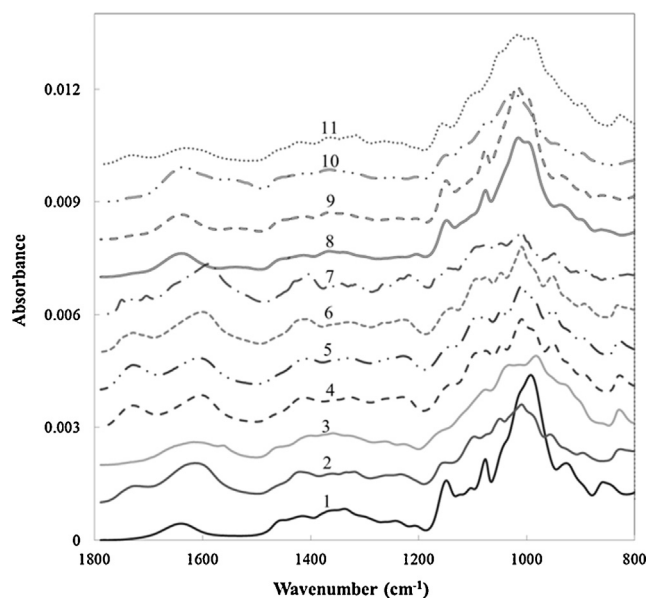


Fig. 4. FT-IR spectra of cell wall polysaccharides of sequential extraction – extracting in cold water (3), 1st CDTA (4), 2nd CDTA (5), 1st Na_2CO_3 (6), 2nd Na_2CO_3 (7), 0.42 mol L^{-1} KOH (8), 0.85 mol L^{-1} KOH (9), 3.4 mol L^{-1} KOH (10) and cellulose pellets (11). (1) and (2) represent starch and CWM respectively.

detected. Coumaric acid and ferulic acid are common phenolic components – indeed, they are the most abundant phenolics present in wheat straw (Merali et al., 2013).

3.5. Analysis of sequentially extracted polysaccharides

The materials used for the sequential extraction were prepared using a ball milling method to rupture cells and permit the release and removal of non-cell wall carbohydrate such as starch. The extent of starch removal was evaluated by microscopy (see Fig. 3a–e). Starch granules are conspicuously present in duckweed cells (Fig. 3a). In the ball milling process, starch granules were gradually released from disrupted tissues (Fig. 3b–d) and dispersed into the liquor phase during the distilled water wash and filtration (Fig. 3e). Thus, the majority of the starch was washed away after 3.5 h of ball milling and washing with distilled water. The monosaccharide composition of CWM analysed by GC (Table 1) also demonstrated the extensive starch removal and this was further demonstrated by the changes in the FTIR spectrum (Fig. 4). Spectra 2 (CWM) has much less intensity around 1150, 1080 and 990 cm^{-1} compared with spectra 1 (FDM). On a dry matter basis, the levels of all of the monosaccharides except glucose and the overall carbohydrate increased in CWM. This is in contrast to the monosaccharide concentrations in FWM and is probably due to the removal of intracellular proteins as well as starch. Only 31.2 g kg^{-1} DM glucose that could be released by hydrolysis in 1 M sulphuric acid was detected in CWM. This would suggest that any glucose detected in the fractions from the sequential extraction is likely to be of cell wall origin.

Starch-free CWM (12.8%, w/w of fresh wet material; 2 g) was sequentially extracted. In the fractionation process (see Table 4), 18.8% (w/w) of dry mass was extracted by CDTA solution and only 1.7% (w/w) was extracted by Na_2CO_3 solution. It is notable that KOH extracted only a small amount (3.5%, w/w) of dry mass (see Table 4). Over 62% (w/w) of the dry mass remained in the residual pellets (see Table 4). As Brett and Waldron (1996, chap. 2) described, CDTA and Na_2CO_3 extraction remove pectic polysaccharides by chelation of calcium and de-esterification respectively whilst alkaline extraction removes hemicellulose by breaking strong ester linkages and

Table 4
The concentration of polysaccharides and dry mass recovery of fractions following sequential extraction.

Fraction	Rha (g kg^{-1})	Fuc (g kg^{-1})	Ara (g kg^{-1})	Xyl (g kg^{-1})	Man (g kg^{-1})	Gal (g kg^{-1})	Glc (g kg^{-1})	GalA (g kg^{-1})	Total sugars (g kg^{-1})	Recovered mass (g)	% of extracted mass
CWM 72%	7.0 ± 0.4	2.2 ± 0.3	6.9 ± 0.3	83.8 ± 0.9	8.5 ± 0.5	14.2 ± 0.3	308.0 ± 5.3	202.2 ± 22.6	632.7 ± 27.8	2000	100
CWM 1 mol L^{-1}	6.1 ± 0.3	2.1 ± 0.2	6.8 ± 0.2	75.1 ± 3.6	3.1 ± 0.2	13.2 ± 0.5	31.6 ± 0.8	86.5 ± 10.6	224.5 ± 6.1	–	–
Cold water	2.7 ± 0.3	3.4 ± 0.4	7.8 ± 1.2	315.9 ± 26.9	1.6 ± 0.3	22.3 ± 2.6	13.6 ± 1.6	44.5 ± 8.4	411.7 ± 26.1	23.9 ± 13	1.2 ± 0.7
CDTA 1st	7.9 ± 0.3	1.7 ± 0.1	5.5 ± 0.2	77.5 ± 10.5	6.0 ± 0.9	7.8 ± 0.8	1.1 ± 0.1	562.1 ± 28.8	669.5 ± 32.9	337.6 ± 38	16.9 ± 1.9
CDTA 2nd	6.7 ± 1.3	2.0 ± 0.4	6.1 ± 2.2	63.8 ± 16.9	3.5 ± 0.6	7.2 ± 3.1	2.9 ± 1.5	282.0 ± 60.4	374.3 ± 81.7	37.4 ± 18	1.9 ± 0.9
Na_2CO_3 1st	7.6 ± 0.2	4.2 ± 0.3	15.1 ± 0.3	27.7 ± 0.1	9.2 ± 1.5	20.0 ± 0.1	5.5 ± 0.4	516.2 ± 1.1	605.7 ± 1.2	27.2 ± 8	1.4 ± 0.4
Na_2CO_3 2nd	7.2 ± 0.4	5.1 ± 0.5	20.1 ± 1.2	14.5 ± 3.2	12.6 ± 0.4	21.3 ± 1.1	21.9 ± 1.3	274.8 ± 3.4	377.5 ± 0.2	6.3 ± 1	0.3 ± 0.1
0.42 mol L^{-1} KOH	3.3 ± 0.1	1.9 ± 0.3	15.3 ± 1.5	15.0 ± 2.1	3.0 ± 0.2	10.8 ± 2.0	559.2 ± 14.5	32.1 ± 2.9	640.7 ± 9.1	39.7 ± 6	2.0 ± 0.3
0.85 mol L^{-1} KOH	2.4 ± 0.1	8.6 ± 0.5	35.8 ± 0.9	253.6 ± 12.2	4.4 ± 0.2	84.5 ± 4.2	398.9 ± 20.7	24.4 ± 1.3	812.6 ± 38.2	19.3 ± 3	1.0 ± 0.1
3.4 mol L^{-1} KOH	2.5 ± 0.3	5.6 ± 0.4	48.1 ± 2.4	115.9 ± 8.9	11.3 ± 0.6	56.4 ± 0.9	213.2 ± 5.9	32.6 ± 2.1	485.5 ± 7.2	10.6 ± 7	0.5 ± 0.3
Insoluble residue	7.5 ± 0.4	2.4 ± 0.1	6.8 ± 0.2	94.4 ± 5.3	9.6 ± 0.3	15.0 ± 0.8	457.2 ± 25.3	145.4 ± 7.1	738.2 ± 30.6	1241.0 ± 3	62.1 ± 0.2
Total recovery	–	–	–	–	–	–	–	–	–	1742.8 ± 4	87.1 ± 0.2

Abbreviations: Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; GalA, galacturonic acid.

hydrogen bonds. The residual matter is predominantly cellulose in conjunction with a significant quantity of highly cross-linked pectic polysaccharides and xylan-containing hemicelluloses. Looking at the GC sugar results in conjunction with the weights of the recovered fractions, it can be seen that cellulose is the major component of CWM and accounts for 43.7% of cell wall polysaccharide. Approximately 20% of the cell wall polysaccharide is pectin which is lower than the 30.1% of pectin in *L. minor* CWM reported by Kindel, Cheng, and Ade (1996). The major pectic polysaccharides were extracted by CDTA and 1st Na₂CO₃ extraction, which suggests pectic polysaccharides are predominantly bound with weak ester linkages (Brett & Waldron, 1996, chap. 2). Only a small amount of hemicellulose (approximately 3.5%) was found in *L. minor* CWM. We found no literature reporting hemicellulose in duckweed cell wall. These results together are consistent with duckweed cell walls as being predominantly primary walls.

FT-IR provides more specific profiles of cell wall polysaccharides as strong auxiliary evidence to identify the pectic and hemicellulosic polysaccharides. Even after the extensive washing to remove starch during the milling, further small amounts of material are solubilised with distilled water. The cold water extracted fraction (Table 4) contained 411.7 g kg⁻¹ of carbohydrate which is high in xylose (315.9 g kg⁻¹) suggesting the presence of xylan polysaccharides, possibly complexed with pectic polymers as found in asparagus (Waldron and Selvendran, 1992). The presence of fucose implies the presence of water soluble xyloglucans (Jacobs, Palm, Zacchi, & Dahlman, 2003). FT-IR (Fig. 4, Spectrum 3) also shows peaks at 1078 and 1043 cm⁻¹ corresponding to xyloglucan as described by Kacuráková, Capeka, Sasinková, Wellner, and Ebringerová (2000). The major carbohydrates from the CDTA and Na₂CO₃ extractions consisted of galacturonic acid – the hydrolysed residues of galacturonan that form the back bone of pectin. Traces of xylose, galactose, arabinose and mannose were also extracted in the CDTA and Na₂CO₃ extractions, which are the main components of side chains of xylogalacturonan and rhamnogalacturonan. Ovodova, Golovchenko, Shashkov, Popov, and Ovodov (2000) noted a similar proportion of pectic monosaccharides in *L. minor*. Accordingly, FT-IR Spectra 4–7 (Fig. 4) had a characteristic shape which contains many specific band peaks corresponding to pectic polysaccharides. Homogalacturonan is represented by strong specific bands at 1010 and 1090 cm⁻¹ (Kacuráková et al., 2000). The specific bands at 1045 and 1074 cm⁻¹ indicate pectic polysaccharide mixtures including rhamnogalacturonan, arabinan and arabinogalactans (Kacuráková et al., 2000). In addition, Hart and Kindel (1970) reported that apiogalacturonan was identified in *L. minor* and apiose are the major components of duckweed pectin that would be measured in the further study. Glucose and xylose were the major monosaccharide constituents of polymers extracted in KOH. A trace of arabinose, galactose and galacturonic acid were also present in the KOH fractions. The presence of large amounts of glucose in both the 0.42 and 0.85 mol L⁻¹ KOH extracts were probably due to the gelatinisation and solubilisation of small amounts of residual starch in KOH. Han and Lim (2003) reported that corn starch was dissolved extensively in 1 mol L⁻¹ NaOH with vigorous agitation at room temperature. The FT-IR spectra (Fig. 4, Spectra 8–9) of these fractions were dominated by the solubilised starch. The overall band pattern is very similar to that of the initial starch (Fig. 4, Spectrum 1), but the increased peak at 1022 cm⁻¹ and the smaller shoulder at 1045 cm⁻¹ clearly indicate a much more amorphous structure (van Soest, Tournois, de Wit, & Vliegthart, 1995). The 3.4 mol L⁻¹ KOH extract mainly contained xyloglucan and xylan and might include glucan, as indicated by the FT-IR spectrum (Fig. 4, Spectrum 10). The bands at 1130–60 cm⁻¹ were dominated by the glycosidic linkage (C–O–C) from xylan, xyloglucan and glucan (Kacuráková et al., 2000). Moreover, the small bands at 930–40 cm⁻¹ are likely to be

from glucomannan and galactoglucomannan (Kacuráková et al., 2000).

3.6. Assessment of sequentially extracted phenolics

Phenolics play an important role in the linkage of hemicellulosic and pectic polysaccharides. Phenolic acids were therefore extracted along with cell-wall polysaccharides in the sequential extraction (see Table 3). Their distribution could be determined by combining the phenolic acids mass recovery from the HPLC phenolic results (Table 3) with the weights of recovered fractions (Table 4). *p*-Coumaric acid was mainly extracted by alkali extraction (Na₂CO₃ and KOH) while truxillic and ferulic acids were only extensively extracted by CDTA solution. In contrast, protocatechuic aldehyde was only solubilised in Na₂CO₃ and low concentration KOH solution. However, *p*-OH-benzaldehyde was found in all fractions. These data indicate that protocatechuic aldehyde and *p*-coumaric acid are the important linkage compounds in *L. minor* hemicelluloses while truxillic acid, ferulic acid and *p*-coumaric acid were proven to have a role in the linkage of pectic polysaccharides. Ferulic and *p*-coumaric acids were found to contribute to xyloglucan and arabinoxylan oligosaccharides respectively (Ishii & Hiroi, 1990). In addition, phenolic compounds were detected in the residual pellets of sequentially extracted duckweed. Merali et al. (2013) also detected large amounts of several phenolic acids retained in the residual pellet of sequentially extracted wheat straw.

3.7. Further discussion

The high proportion of fermentable sugars (394 g kg⁻¹ of DM) in combination with the high productivity of duckweed suggests that duckweed is an ideal feedstock for biofuel production. Detailed investigation of the cell wall composition implies that duckweed cell wall comprises of cellulose (43.7%), pectin (20%) and small amounts of hemicellulose (3.5%) and lignin, which further reduce the cost of bioethanol production from duckweed. Waldron (2010) stated that the difficulty of pretreating highly lignified biomass. It should be noted that starch and cell wall composition may show variation between duckweed species and batches cultured under different conditions. This case study provides some fundamental background of duckweed sugars compositions using *L. minor* as a model system and indicates that duckweed is potentially an ideal biofuel resource. The selection of the preferred duckweed species for biofuel production requires a further screening of all duckweed species and optimisation of their individual growing conditions. In addition, the identification of fatty acids and pectic polysaccharides as high value by-products of bioethanol production potentially reinforce the nutritional and medicinal value of duckweed. However, the high moisture content of fresh duckweed is an unavoidable issue in the biorefining of duckweed.

4. Conclusions

This study provides a detailed examination of the chemical constituents of duckweed (*L. minor*). Duckweed biomass contains a high proportion of fermentable sugars (including glucose, 33.1%, w/w DM) and a low amount of lignin (3%, w/w DM). The results of the fractionation shows that 20% pectin is extracted by CDTA and Na₂CO₃ solutions consisting of galacturonan with small amounts of xylogalacturonan, rhamnogalacturonan, only 3.5% hemicellulose is extracted by KOH solution predominantly consisting of xyloglucan and xylan, and the insoluble residue is rich in cellulose. EFA (α -linolenic and linoleic/linoelaidic acid) and *p*-coumaric acid are the most abundant fatty acids and phenolics of *L. minor* respectively. The profiles of cell wall structure will play an important role in the enzymatic saccharification and fermentation of duckweed biomass

to ethanol, such as, the selection of enzymes (cellulase) and yeast (*Saccharomyces cerevisiae*).

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