Development of a chromatographic method for the analysis of carbohydrates in Posidonia Coriacea in artificial cultures

Olivia J. Oldfield
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DEVELOPMENT OF A CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF CARBOHYDRATES IN *POSIDONIA CORIACEA* IN ARTIFICIAL CULTURES.

OLIVIA JAYNE OLDFIELD

Faculty of Communications, Health and Science

EDITH COWAN UNIVERSITY

December 2002
USE OF THESIS

The Use of Thesis statement is not included in this version of the thesis.
This thesis is dedicated to my father, Douglas Charles Oldfield (1951 – 1995), who taught me a love of nature and has inspired me to make a difference in life.
ABSTRACT

Carbohydrates in *P. coriacea* leaves were analysed to determine if, when in artificial culture, they were unable to replenish their carbohydrate stores once the seed starch reserve was consumed. While no evidence was found to suggest that this was happening, other findings were made.

Soluble carbohydrates determined in *P. coriacea* were sucrose, fructose, glucose, trehalose, *myo*-inositol and mannitol. However, mannitol was only found in *in situ* plants sampled in June. This may be due to high epiphyte coverage elevating plant stress and therefore mannitol levels. *P. coriacea* grown in tissue culture has soluble carbohydrate levels up to 20-fold higher than plants maintained in aquaria or grown *in situ*. The average concentration (± 1.0 SE) of the soluble carbohydrates in *P. coriacea* *in situ* plants were: sucrose 2.54 (0.93) mg g⁻¹ fwt, glucose 0.42 (0.06) mg g⁻¹ fwt, fructose 0.70 (0.05) mg g⁻¹ fwt, trehalose 0.40 (0.01) mg g⁻¹ fwt, *myo*-inositol 0.05 (0.00) mg g⁻¹ fwt and mannitol 0.31 mg g⁻¹ fwt.

*P. coriacea* leaves generally had < 40 mg starch g⁻¹ fwt. For the experiment duration there was no significant variation of starch levels except in seawater tissue culture seedlings sampled in August and September, where starch levels were higher. Between environments there was a significant difference in starch levels measured in June, August and September. In June and August the *in situ* plants had significantly higher starch levels than seawater tissue culture plants, but levels were significantly lower than the seawater tissue culture plants in September.

For the analysis of soluble carbohydrates a chromatographic method was developed specifically for highly sensitive detection, plus simplicity for routine analysis. Method development compared a variety of methods used for the extraction, preparation and chromatographic analysis of soluble carbohydrates. An HPLC method
employing a polyamine column, acetonitrile/water mobile phase and evaporative light scattering detection, was developed. While this method was simple and robust, detection limits were not low enough to allow analysis of sugars from limited seagrass material of 0.1 g for each sample.

A GC method was developed for the analysis of soluble carbohydrates in seagrasses maintained in an artificial culture. It consisted of an 80 % ethanol extraction at room temperature, derivatisation of carbohydrates with BSTFA and analysis on a BP-1 column with FID detection. This method was simple, robust and sensitive for the analysis of plant soluble carbohydrates. This method only required 0.1 g of leaf for each sample. The detection limits were 90 μM L⁻¹ fructose, 40 μM L⁻¹ glucose and sucrose, and 20 μM L⁻¹ trehalose, mannitol and myo-inositol.
DECLARATION

I certify that this thesis does not, to the best of my knowledge and belief:

(i) incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education;

(ii) contain any material previously published or written by another person except where due reference is made in the text; or

(iii) contain any defamatory material

(signature)

6 December 2002
ACKNOWLEDGEMENTS

My many thanks go to Mary Boyce and Ian Bennett for their continual support throughout this project. My knowledge, understanding and experience are certainly the better for it.

To the Centre for Ecosystem Management, for providing funds for purchasing equipment used for this project.

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To Colin, my fiancé, your support through the last four years has been wonderful.

To my sister, Tammy, for your encouragement ... or discouragement!

To my mother and family, who remind me of what really matters in life.

And to my uni peers Julia Wilson and Michael Burt for collecting the seagrass plants for me, and Bryan Duff and Janelle Atkinson who gave me a reason to laugh. This year certainly would not have been the same without you.
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ABBREVIATIONS

ACN: Acetonitrile
ANOVA: Analysis of variance
BSA: N,O-bis(trimethylsilyl)acetamide
BSTFA: N,O-bis(trimethylsilyl)trifluoroacetamide
C_i: Inorganic carbon
dwt: dry weight
ELSD: Evaporative light scattering detector
FID: Flame Ionisation Detector
fw: fresh weight
GC: Gas chromatography
h: plate height
HMDS: Hexamethyldisilazane
HPLC: High performance liquid chromatography
I_k: Point of light saturation
LOD: Limit of detection
MeOH: Methanol
MOPS: 3-(N-morpholino) propanesulfonic acid
MS: Mass spectroscopy
M+S: Murashige and Skoog Basal Medium
n: Number of theoretical plates
P_i: Inorganic phosphate
P_max: photosynthetic production at saturating irradiance
PAD: Pulsed amperometric detector
PDA: Photodiode array detector
RI: Refractive index detector

%RSD: Relative standard deviation

TMCS: Trimethylchlorosilane

TMS: Trimethylsilyl

TMSI: Trimethylsilylimidazole

$t_r$: Retention time

UV-Vis: Ultra violet – visible light
CHAPTER 1: Introduction

1.1 Posidonia coriacea and Tissue Culture

"Posidonia seagrasses, are the most important marine angiosperms in temperate Australian coasts." (Kuo & Kirkman, 1996)

1.1.1 Ecological Importance of Posidonia coriacea

In Australia there are approximately 30 different species of seagrass that reside in estuaries, island lagoons and sheltered marine bays (Kirkman, 1997). *P. coriacea* is an Australian seagrass with long leathery leaves measuring approximately 7 x 1000 mm (Cambridge, 1999; Kuo & Cambridge, 1984). It forms patchy meadows of 25 - 40% coverage, compared to *P. sinuosa* that forms closely knit meadows with 75 - 100% coverage (Kirkman & Kuo, 1990; MacArthur & Hyndes, 2001). Major stands of *P. coriacea* meadows are found along the Western Australian coastline between Shark Bay and Geographe Bay, and also along the South Australian coastline between Roe Plain and Backstairs Passage, including Spencers Gulf and St. Vincent Gulf (Kuo & Cambridge, 1984). These meadows occupy depths of 1 to 30 m, in open ocean or rough water sublittoral habitats (Campey et al., 2000). Among their foliage are found a variety of animals, including eight species of fish from the Odacidae family – *Siphonognathus radiatus*, *S. beddomei*, *S. argyrophanes*, *S. attenuatus*, *S. caninus*, *Odax acroptilus*, *Neodox balteatus* and *Haletta semifasciata* (MacArthur & Hyndes, 2001).
Kirkman and Kuo (1990) found that while *P. coriacea* grew in disturbed meadows the seedling and rhizome spread was slow and was often associated with other seagrasses, particularly *Amphibolis* species. Unvegetated areas are colonized when seeds are carried into the area by their positively buoyant fruit, which then releases the negatively buoyant seed (Orth, 1999). Seeds settling in vegetated areas rarely establish themselves due to the difficulty in establishing in the dense root mats of *Posidonia* meadows and predation from marine organisms (Kirkman & Kuo, 1990; Kuo & Kirkman, 1996; Orth, 1999). Seeds that do manage to become established often have problems lasting through the turbulent winter weather (Cambridge, 1975). Growth of *P. coriacea* meadows from rhizome extension is estimated to be 0.1 to 0.5 m year⁻¹ (Kendrick et al., 1999).

1.1.2 Changes in Seagrass Cover

An area of concern has been the decline of seagrass meadows of Cockburn Sound, Western Australia, where species such as *P. sinuosa, P. australis, P. coriacea, Amphibolis griffithii* and *A. antarctica* are found (Cambridge & McComb, 1984). *P. coriacea* is predominately found on the exposed western side of Parmelia Bank and Success Bank regions of Cockburn Sound (Cambridge & McComb, 1984; Kendrick et al., 1999). Since 1955 industrial activity has been prevalent in this area, with the discharge of cooling waters from an oil refinery, blast furnace and power station, construction and dredging for an access jetty, and discharges from sewage-treatment, and nitrogen and phosphorous fertilizer plants (Cambridge & McComb, 1984). Seagrass meadow loss and growth in Cockburn Sound has been monitored by a number of researchers since 1954 (Cambridge & McComb, 1984; Kendrick et al., 1999; Kendrick
et al., 2000). Between 1954 and 1978 the meadow area was reduced from approximately 4,200 to 900 ha (Cambridge & McComb, 1984).

The suggested reason for the original decline in seagrass was that the seawater, enriched with nutrients from the surrounding activities, provided the appropriate conditions for elevated growth of epiphytes. This impaired the ability of the seagrasses to photosynthesise and led to extensive loss of seagrass meadows. While increased numbers of phytoplankton have also affected the depth to which seagrasses can occupy, such blooms did not occur until after the major seagrass loss (Cambridge et al., 1986). Studies measuring the effect of epiphytes on photosynthesis have shown their effect on seagrass decline to be possible (Silberstein et al., 1986). Additionally, localized loss in Cockburn Sound has been brought about by altered temperature, salinity, sedimentation and water movement, oil refinery effluent and grazing by sea urchins (Cambridge et al., 1986).

The major loss of seagrass, in areas such as Cockburn Sound, has led to interest in developing protocols for revegetation. In Cockburn Sound this has included the mechanical transfer of plants, harvested from established meadows, to revegetation sites using a specially designed seagrass planting machine (Paling et al., 2001). Recently, a method has been developed for sterilizing the fruits of Posidonia sp., which has subsequently opened up an avenue for the multiplication of these species using tissue culture (Henry, 1998). By producing plantlets on mass through tissue culture, there is a greater possibility of developing a procedure with less impact on existing meadows (Bird et al., 1993).
Attempts at culturing seagrasses started in the 1950's. Initial trials were unsuccessful but further attempts were more fruitful (McMillan, 1980). At first planlets survived less than a year, but with growing knowledge of the optimum culture conditions seagrass longevity increased to over two years (McMillan, 1980). In 1988 Balestrix et al. (1998) reported the first instance of in vitro germination and seedling development of a Posidoniaceae member, *P. oceanica*, a species endemic to the Mediterranean Sea. However, seedling survival after 10 months was less than 75 % and all plants eventually died (Balestrix et al., 1998).

Initially seagrass cultures used natural seawater as their starting medium but the seagrasses did not survive beyond one year. A change to using artificial seawater saw cultures surviving beyond two years (McMillan, 1980). It is thought that the initially shorter survival period is due to natural seawater having a lower quality from variations caused by human activities (McMillan, 1980). The use of artificial seawater also allows researchers greater manipulation of salt concentration. For example, Bird et al. (1993) found that when culturing *Ruppia maritima* a salt concentration between 0 and 10 % can increase rhizome and node growth, plus root production and growth. Additionally, experiments by Balestrix et al. (1998) indicated that a different salt concentration was required for seed germination and vegetative growth.

A carbon source is also necessary for the plantlets' survival (Woodhead & Bird, 1998). Organic carbon sources provide an energy and increase osmotic pressure (Loques et al., 1990). Different carbon sources target different growth responses. For example, in *R. maritima* bicarbonate stimulates elongation of roots while sucrose stimulates root production (Bird et al., 1993; Woodhead & Bird, 1998). Bird et al.
(1996) have also noted that light-limited seagrasses do not grow in bicarbonate-based media, but they do grow in sucrose-based media. Theoretically, this is because sucrose, unlike bicarbonate, supplies reduced carbon that can meet the energy needs when growth is stimulated (Bird et al., 1996). Other soluble carbohydrates (glucose, mannitol and myo-inositol) have also been used in tissue culture (Loques et al., 1990). Axenic cultures of *P. oceanica* showed little difference in growth when similar molar concentrations were used of either sucrose or glucose (Loques et al., 1990).

1.1.4 Biology

*P. coriacea* belongs to the species complex *P. ostenfeldii*, which also includes *P. ostenfeldii*, *P. denhartogii*, *P. robertsoniae* and *P. kirkmanii* (Kuo & Cambridge, 1984). *Posidonia* seeds are available only in the early Australian summer months. The seed appears to have no dormancy period and would therefore be considered viviparous, except that the seedling is not dependent on the parent plant (Kuo & Kirkman, 1996).

The large reserves of starch used for seedling growth are in the parenchyma cells of the seeds' hypocotyl (Kuo & Kirkman, 1996). In *P. coriacea* most of this starch is hydrolysed in the first three months, when the seedling possesses leaves up to 8 cm long and the initial pair of roots are developing (Hocking, 1981; Kuo & Kirkman, 1996). After four to six months the amount of nutrients supplied from the reserve to the seedling is not substantial (Kuo & Kirkman, 1996). After ten to twelve months, *P. coriacea* seedlings in artificial culture die. This is thought to be a result of the starch reserve being consumed by the eighth month and the seedlings being unable to replenish their carbohydrate stores (Kuo & Kirkman, 1996).
1.1.5 Factors that Affect Photosynthesis

As the starch reserve in the seed is exhausted, it is necessary for the plant to continue to supply carbohydrates for various metabolic processes. A major source of carbohydrates comes from the photosynthesis reaction that utilizes inorganic carbons, such as CO₂ (James & Larkum, 1996). There are many factors that influence photosynthesis, these include leaf age, light availability, pH and temperature (Alcoverro et al., 1998; Invers et al., 1997; Ruiz & Romero, 2001; Seddon & Cheshire, 2001).

Leaf age is a major cause of variation in photosynthetic performance, with leaf senescence following the loss of plastid integrity and the reallocation of nutrients (Alcoverro et al., 1998). The maximum leaf age is species specific: *P. oceanica* leaves may survive for 300+ days, while *Zostera marina* leaves only survive for 50 days (Alcoverro et al., 1998). While there is no relationship between leaf age and compensating irradiance (where net oxygen release is zero) or saturating irradiance, there is a relationship with $P_{\text{max}}$ (photosynthetic production at saturating irradiance) (Alcoverro et al., 1998). In *P. oceanica*, the latter pattern showed a decrease in $P_{\text{max}}$ with leaf age from 50 days onwards (Alcoverro et al., 1998).

A reduction in available light for seagrass photosynthesis has many repercussions. For example, in *P. sinuosa* there were decreases in the standing crop, leaf density, shoot density, leaf length and primary productivity (Gordon et al., 1994; Neverauskas, 1988), similar effects were also found for *P. oceanica* (Ruiz & Romero, 2001). Long term reductions in light, resulting from epiphyte growth in high nutrient waters, have led to mass seagrass meadow deaths (Cambridge et al., 1986). Gordon et al. (1994) found that even after *P. sinuosa* was returned to natural light conditions, following six months shading, the meadows showed signs of permanent damage.
The ability of a seagrass to survive long periods of light deprivation is thought to be related to either its ability to store large quantities of carbohydrates, or to tolerate the phytotoxic end products of anaerobic root respiration (Longstaff et al., 1999). For example, starch is stored as a source of energy to be utilised when photosynthesis is inhibited. However, when light deprived, Halophila ovalis survives off sugars and not the starch reserves (1999). The reason for this is suggested to be the condition of root anoxia, where starch utilisation is inhibited in the roots (Longstaff et al., 1999). Phytotoxic end products (e.g. ethanol) might also accumulate to toxic levels during light deprivation and cause rapid die-off (Longstaff et al., 1999).

Photosynthetic activity is reduced in low and high light conditions. Shading of P. oceanica led to a reduction of Pmax, which further reduced carbon assimilation (Ruiz & Romero, 2001). The result of light limitation is a decrease in the leaves total carbohydrate reserves and plant death may result even when only 2/3 of the carbohydrate storage in the rhizome has been consumed, as seen in Z. marina (Alcoverro et al., 1999). However, shoot growth may still continue in light-limited plants due to the shoot being the primary sink for reduced carbon and it may continue even until plant death (Alcoverro et al., 1999). In high light conditions it is necessary to reduce photosynthesis in order to protect the photosynthetic apparatus (Alcoverro et al., 1999).

Light requirements for photosynthesis are influenced by water temperature (Masini et al., 1995). For example, in P. sinuosa maximum gross photosynthesis almost doubles between 13 and 23 °C (Masini et al., 1995). When reduced temperatures are coupled with low light, the net photosynthesis may go into debit (Masini et al., 1995). While photosynthetic efficiency increases with temperature, there is no significant difference between 13 and 18 °C, however, there is a significant
increase in the point of light saturation ($I_k$) (Masini et al., 1995). When the optimal water temperature is exceeded there is a rapid decline in the rate of photosynthesis, due to the loss of enzyme activity involved in catalysing the photosynthesis reaction and changes in the transport of inorganic carbon (Seddon & Cheshire, 2001).

The water pH is important as it alters the ratio of HCO$_3^-$:CO$_2$ in the water, which in turn alters the available inorganic carbon for photosynthesis. Increasing pH decreases the CO$_2$ concentration, which also reduces the photosynthetic capacity as HCO$_3^-$ is relatively inefficient as a carbon source (Beer, 1989; Invers et al., 1997).

1.1.6 Carbohydrate Biosynthesis in Seagrasses

The photosynthesis reaction requires an inorganic carbon source (C$_i$). In most seagrasses the main point of entry for C$_i$ is through the leaves, as in terrestrial plants. However, for some species C$_i$ may also enter through the roots and/or rhizomes (James & Larkum, 1996). C$_i$ entering through the roots or rhizomes must subsequently be transferred to the leaves for photosynthetic fixation (Beer, 1989). Within the leaf the epidermis is the primary site of photosynthetic activity (Beer, 1989).

In terrestrial plants CO$_2$ is the only C$_i$ source available, but in seawater there are four forms of C$_i$ (James & Larkum, 1996). Only CO$_2$ and HCO$_3^-$ are thought to take part in seagrass photosynthesis, however, there is much discrepancy over how much, if any, HCO$_3^-$ is utilized (Beer, 1989; James & Larkum, 1996). James and Larkum (1996) suggest _P. australis_ and _P. oceanica_ utilize HCO$_3^-$ in up to 25 and 53 % of their C$_i$ demands respectively. If HCO$_3^-$ is utilized it may enter the plant by conversion via H$_2$CO$_3$ to CO$_2$, accomplished by acidification within the layer next to the leaf surface.
(Beer, 1989; James & Larkum, 1996), or it may be actively transported directly into photosynthesising cells (Touchette & Burkholder, 2000).

Photosynthetic fixation of $C_i$ occurs either via the $C_3$ or $C_4$ pathways; as yet there is no support for the crassulacean acid metabolism (CAM) pathway (Beer, 1989; Touchette & Burkholder, 2000). Improved analytical techniques have identified many seagrasses that were thought to be $C_4$ plants as $C_3$ plants. In $C_3$ plants CO$_2$ is the carbon form used by the enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), while in $C_4$ plants HCO$_3^-$ is used by phosphoenolpyruvate carboxylase (PEPC) (Beer, 1989). Nine of ten seagrass species studied have been identified as $C_3$ plants (Beer, 1989). One theory also suggests that $C_4$ photosynthesis could be an inducible response to low internal dissolved $C_i$ levels (Touchette & Burkholder, 2000).

In the $C_3$ pathway, carboxylation of carbon to ribulose 1,5-bisphosphate, a 5-carbon sugar, produces a product that splits into 3-phosphoglycerate (PGA), a 3-carbon molecule (Touchette & Burkholder, 2000). Rubisco is involved in this carbon fixation, but under high O$_2$ concentration, temperature, or light it can be used for photorespiration – a CO$_2$ releasing or carbon loss process (Touchette & Burkholder, 2000).

Starch and sucrose are biosynthesised in the plastid and cytosol respectively, from 3-PGA, triose-phosphate and dehydroxyacetone (Touchette & Burkholder, 2000). Sucrose is produced under high inorganic phosphate ($P_i$) levels, where more triose-phosphate is exported to the cytosol (Touchette & Burkholder, 2000). Starch production increases as a result of low $P_i$ levels and reduced exportation from the plastid of triose-phosphate (Touchette & Burkholder, 2000). The carbohydrate preferred for storage is species specific, one species may prefer sucrose while another may store starch, raffinose or stachyose (Touchette & Burkholder, 2000).
Sucrose biosynthesis involves the enzyme sucrose-phosphate synthase (SPS), which in Z. marina has been correlated with changes in CO₂ availability, photosynthesis, salinity and temperature (Touchette & Burkholder, 2000). In sink tissues, sucrose is hydrolysed into glucose by the enzyme invertase, or into uridine diphosphate-glucose (UDP-glucose) and fructose by sucrose synthase (Touchette & Burkholder, 2000). Sucrose synthase activity is suggested to increase plant survival during periods of carbon limitation by maximizing sucrose availability (Touchette & Burkholder, 2000).

Starch synthesis occurs under high levels of triose-phosphate, yielding fructose 1,6-bisphosphate (Touchette & Burkholder, 2000). Starch synthase is then utilised to transform adenosine diphosphate-glucose (ADP-glucose) into starch (Touchette & Burkholder, 2000).

Of the soluble carbohydrates in P. oceanica, generally 90 % is sucrose, with the balance including glucose, fructose and myo-inositol (Pirc, 1989). Other sugars found in seagrass include apiose, fucose, galactose, mannose, rhamnose, xylose and mannitol (Pirc, 1989; Touchette & Burkholder, 2000). Carbohydrate storage in the stem, leaf, root and rhizome is on average 95, 100, 135 and 275 mg g⁻¹ dry weight (dwt) respectively (Touchette & Burkholder, 2000). Generally, seagrasses with high compensation irradiance (the light intensity where O₂ production equals O₂ demand), such as Posidonia species, have total leaf soluble carbohydrates in excess of 50 mg g⁻¹ fresh weight (fwt) (Touchette & Burkholder, 2000). According to Drew (1983), the maximum sucrose, glucose and fructose levels in P. australis are 1-10 %, < 0.1 % and < 0.1 % dwt respectively. Starch levels in P. oceanica are < 1 % dwt (Pirc, 1989).
1.2 Methods of Sugar and Starch Analysis

1.2.1 Introduction to Carbohydrate Analysis

Methods used for analysing plant carbohydrates involve three steps: extraction of carbohydrates from the plant tissue using solvents, preparation of the extract for analysis and the analysis of the extract. The techniques for extraction and preparation are dependent on whether soluble or insoluble carbohydrates are to be analysed. Analysis of carbohydrates is usually by chromatographic and spectrophotometric methods.

A generalised definition of chromatography is '... a method, used primarily for separation of the components of a sample, in which the components are distributed between two phases, one of which is a stationary while the other moves. The stationary phase may be a solid, liquid supported on a solid, or a gel. The stationary phase may be packed in a column, spread as a layer, or distributed as a film ... The mobile phase may be gaseous or liquid.' (Robards et al., 1994).

Two common methods used for chromatographic soluble carbohydrate analysis are high performance liquid chromatography (HPLC) and gas chromatography (GC). The HPLC technique is ideal for non-volatile and thermally unstable compounds. The GC technique is ideal for volatile compounds and also for when sensitive detection is required. Spectrophotometric methods are used for the analysis of insoluble carbohydrates.
1.2.2 Extraction of Carbohydrates from Leaves

Extraction of carbohydrates from plant tissue involves the extraction of the soluble carbohydrates followed by insoluble carbohydrates. There are numerous methods in the literature for each, of which some are presented here. Extraction of soluble carbohydrates has been carried out at a range of temperatures between 0 and 100 °C and in a number of solvents. The insoluble carbohydrates are then 'gelled' at high temperature before the addition of acid, which hydrolyses the starch into its individual sugar units.

Prior to extraction, plant material is ground into a fine powder to maximise the extraction of the analytes into the extracting solvent. The complexity of the extract methods reported for the extraction of soluble carbohydrates, varies from a single hot water extract used by Pirc (1985), to dialysis methods, as used by Kleinschmidt et al. (1998).

However, for soluble carbohydrates, the most commonly used methods involve an alcohol/water extraction, using methanol or ethanol, but even here the method details can vary significantly. Holligan & Drew (1971) used three hot 80 % ethanol extracts over 24 hours, followed by two hot ethanol washings. Morvai & Molnár-Perl (1990) completed extractions at both 25 °C and by refluxing with 80 % ethanol or methanol for 20 minutes, followed by several washings. Kahane et al. (2001) incorporated calcium carbonate into an ethanol extraction medium to prevent a reduction in pH. Chapman & Horvat (1989) completed one extraction in 25 mL 75 % ethanol for 10 minutes at room temperature, with the total sugar yield being approximately 100 %, while Lo Bianco and Rieger (1999) only used 5 mL 80 % methanol. Bleton et al. (1996) combined 15
mL methanol with 0.4 mL acetyl chloride to form a methanolic HCl solution and methanolysis was carried out for 24 hours at 80 °C.

Combinations of methanol (MeOH), chloroform (CF) and water have also been used for soluble carbohydrate extractions. Adams et al. (1992) extracted soluble carbohydrates with MeOH:CF:water (12:5:3). Following this, water was added to the extract to form two phases and the MeOH:water phase was desalted using ion exchange columns in the H⁺ and OH⁻ forms. Naidu (1998) completed extractions on ice, using 5 mL MeOH:CF:water (12:5:3), adding an additional 5 mL water to form two phases. A second extraction was found to only provide an additional 2 % of analyte (Naidu, 1998).

Perchloric acid has also been used for the extraction of soluble carbohydrates. A general method is given by Gutmann & Wahlefeld (1974), where 5 mL 5 % perchloric acid was added to 1.0 g plant tissue, followed by a second extraction with 1 mL 5 % perchloric acid plus 1 mL water. An altered version of this is given in Adams et al. (1999) where 0.1 g plant tissue was extracted twice using 5 mL 5 % perchloric acid at 4 °C, followed by the precipitation of perchlorate by adjusting the solution to pH 3 – 3.5 with 1 M K₂CO₃. Using two extractions with the perchloric acid method extracted at least 96 % of analytes (Gutmann & Wahlefeld, 1974).

Like soluble carbohydrates, there are various methods for extracting starch, some of which are specific for reducing sugars, glucose, or hexose sugars (Macrae et al., 1974). In analysing starch content in P. corticaceae, Pirc (1985) gelatinised the starch at 80 °C in 1.1% HCl for 15 minutes. The starch was then neutralised with 1 M NaOH before adding the enzyme amylloglucosidase to degrade the starch to glucose. Buysse and Merex (1993) also used HCl, but in this case the starch was hydrolysed by boiling for 3 hours in 3 % HCl.
In a method described by Quarmby & Allen (1989), starch in the plant material was firstly gelatinised in a small amount of boiling water for 15 minutes. After cooling, 60% perchloric acid was rapidly added. This method was also used by Longstaff and Dennison (1999) in the analysis of starch in seagrasses. A modified version of Hansen and Møller's method (Burke et al., 1996) has used cold (0 °C) 30% perchloric acid for starch hydrolysis.

1.2.3 High Performance Liquid Chromatography

High performance liquid chromatography involves the analysis of semi- to non-volatile compounds. In analytical mode, typically a 5–50 μL sample is injected into a liquid mobile phase, which is pumped under high pressure through a column approximately 4 mm × 10–30 cm. The column is generally packed with 5 μm particles coated with the stationary phase. Separation of analytes takes place due to the relative interaction of the analyte with the mobile and stationary phases. Separated components are then passed by a detector, which gives a signal response based on the physical property of the solute or mobile phase. The signal is recorded on a data system (e.g. computer workstation) for analysis of the results. HPLC does not necessarily require preparation of the plant sample for analysis but it may be used to obtain lower detection limits. A variety of column and detector combinations may be used for analysis but there are restrictions. For example, a column may have specific mobile phase requirements that are not compatible with a particular detector. This section reviews the detectors and columns used for HPLC carbohydrate analysis.
1.2.3.1 HPLC Detectors

The main detectors used for soluble carbohydrate analysis with HPLC are the refractive index detector (RI), ultra violet-visible detector (UV-Vis), electrochemical detectors and evaporative light scattering detector (ELSD). Their detection limits vary over several orders of magnitude and where these are not low enough derivatisation of the analyte may be an option. Derivatisation involves attaching a chemical to the analyte to which the detector is highly sensitive.

The RI detector is sensitive to analytes with a different refractive index than the mobile phase. When using a Ca(II) column and a purely aqueous mobile phase, detection limits for non-derivatised soluble carbohydrates were 100 mg L$^{-1}$ and the reproducibility of the RI detector response was $<$ 3.0 % relative standard deviations (%RSD) (Lee & Coates, 2000; van de Merbel et al., 1992). van de Merbel et al. (1992) compared the performance of the carbohydrate detector capabilities of the UV-Vis and RI detection with and without post-column derivatisation; derivatisation increased sensitivity by 100-fold.

When using UV-Vis for detection of sugars, the detector is normally set at 190 nm where maximum absorbance occurs. Sugars do not possess a chromophore and therefore poorly absorb UV-Vis light. Above and below 190 nm the absorbance drops dramatically. The detection limits for carbohydrates in a pure aqueous mobile phase, using UV-Vis detection coupled with a Ca(II) column, is 1000 mg L$^{-1}$ (van de Merbel et al., 1992). Sensitivity is increased by using a mobile phase of water containing a low concentration of calcium ethylenediaminetetraacetate (CaEDTA), this has given detection limits of approximately 10 ng L$^{-1}$ for glucose and fructose (Naidu, 1998).

Sensitivity is also increased by post-column derivatisation, with p-aminobenzoic acid hydrazide derivatives giving detection limits of 1 $\mu$g L$^{-1}$ for glucose
and fructose when using UV-Vis detection at 410 nm (van de Merbel et al., 1992). Another derivatising agent is 1-phenyl-3-methyl-5-pyrazolone (PMP), where derivatised sugars are analysed at 245 nm. Glucose-PMP derivatives gave detection limits of about 0.2 ng L\(^{-1}\) and reproducibility of the detector response was 1.9 \%RSD at the 2 ng L\(^{-1}\) level (Honda et al., 1989).

The equipment for post-column derivatisation is not always available and the strict conditions required by some derivatising agents means that only a small percentage of carbohydrate derivatives are formed (Honda et al., 1989). It therefore may be more viable to use pre-column derivatisation. For example, Nojiri et al. (2000) used a method to analyse sugar alcohols. Briefly, 10 % \(p\)-nitrobenzoyl chloride, in pyridine solution, was added to anhydrous samples, which was incubated for 60 – 90 minutes at 50 °C, before being evaporated to dryness. The residue was taken up in chloroform and passed through a silica Sep-Pak cartridge to eliminate excess reagent. Solvents were evaporated off and the residue was re-dissolved in acetonitrile for HPLC analysis. Derivatives were detected with UV-Vis detection at 260 nm. While low detection limits for carbohydrates can be achieved with UV-Vis, this is only when used in conjunction with derivatisation. Derivatisation for HPLC can be a long process and is not convenient when analysing a large number of samples.

One way to avoid derivatisation and still achieve low detection limits, is by using pulsed amperometric detection (PAD). This method employs an electrode that generates a current specific for oxidization of sugars, which leaves other components in the sample undetected (Hanko & Rohrer, 2000). Electrodes that have been used include the Au working electrode (Adams et al., 1993; Corradini et al., 2001; Hanko & Rohrer, 2000) and the Ag/AgCl electrode (Gailing et al., 1998; Guraya et al., 2001).
PAD electrodes normally work using a triple-pulse waveform, for example +0.05 V for 0.00 to 0.40 s, then +0.75 V from 0.41 to 0.60 s, then -0.15 V from 0.61 to 1.00 s, with integration between 0.20 and 0.40 s (Hanko & Rohrer, 2000). While the triple-pulse waveform initially gives good detection, it has been found that over several months the detector response decreases (Hanko & Rohrer, 2000). This degradation is avoided by using a quadruple-pulse waveform described by Hanko & Rohrer (2000).

A method used by Lee and Coates (2000), employing PAD for analysis of myo-inositol, glucose, fructose and sucrose gave detection limits between 45 to 300 ng. The detection limit when using the CarboPac MA1 column with PAD, was approximately 1 ng for sugar alcohols and monosaccharides and 4 ng for sucrose (Hanko & Rohrer, 2000). The detection limits for glucose when using a sulfonated mono-disperse resin-based column (H⁺ form) was 5 ng (Corradini et al., 2001). Honda et al. (1989) used PMP derivatives, a C₁₈ column and PAD to achieve detection limits for glucose at 18.0 pg.

While the Au and Ag/AgCl electrodes are widely used for electrochemical detection in carbohydrate analysis, a more recently introduced electrode is the nickel-chromium alloy (Ni-Cr) electrode. The Ni-Cr electrode, however, does not require continual pulsing as adopted in PAD, instead it uses a constant potential amperometric detection (Mora & Marioli, 2001). The proposed mechanism of the electrode is that sugars are oxidized by an electro-catalytic process involving Ni(II)/N(II) oxy/hydroxides (Mora & Marioli, 2001). This electrode has given very low detection limits of 90 pg for glucose, without the need for derivatisation (Mora & Marioli, 2001).

Methods employing electrochemical detection show good sensitivity, however, as it is only useful for species that can be oxidised or reduced, it is not a versatile detector. The technique also requires highly specific conditions: anion exchange
columns, electrolytic mobile phases and high pH. The equipment can also be complex, difficult to equilibrate and requires maintenance for high sensitivity.

A recently developed detector that overcomes many of the disadvantages of the detectors mentioned above is the ELSD. This is a universal detector with good sensitivity, no maintenance, is relatively easy to operate and it can work with a range of mobile phase solvents. Another advantage is that its baselines are stable and flat, unlike UV-Vis detection that is affected by non-isocratic mobile phases and RI detection that can give fluctuations due to pressure changes from injections. For detection, the ELSD evaporates the mobile phase in a drift tube and blows a stream of nitrogen gas over the non-volatiles, before measuring the amount of light scattering produced by semi- or non-volatile components in a sample. This method does not require sugars to be derivatised and the detection limits for fructose, glucose and sucrose are between 30 - 80 ng (Young, 2002).

1.2.3.2 HPLC Columns

The amino column is most commonly used for normal phase separation, in conjunction with an acetonitrile (ACN) and water mixture as the mobile phase (de la Cruz-Garela et al., 2001). Over time, the performance of the amino column may be lost due to Schiff's base formation. Further loss of resolution may come from bleeding of the amino groups from the column. To overcome this problem a polyamine column has recently been developed, which combines amine functional groups in a polymer resin coating. This provides the same selectivity as the amino column but with greater stability and longer life. This column can also be reconditioned to remove the Schiff's base formation.
The elution order for the amino and polyamine columns is monosaccharides, disaccharides and then sugar alcohols, with fructose, glucose, sucrose and myo-inositol resolved and eluted within 20 minutes (Lee & Coates, 2000). The reproducibility of retention times was < 3.0 %RSD (Lee & Coates, 2000). The ratio of ACN:H₂O can be altered depending on the resolution of eluants and their molecular weights (MW). High concentrations of water in the mobile phase will elute high MW sugars faster. If a sample contains sugars of both low and high MW it is possible to use a programmed run, starting off at a high ACN:H₂O ratio (e.g. 80:20 %) and ending at a low ACN:H₂O ratio (e.g. 50:50 %). This will resolve low MW sugars and reduce retention times of high MW sugars. However, programmed mobile phases such as this are not possible for use with RI detection where changes in the mobile phase would affect the refractive index.

Young (2002) combined the polyamine column with an ELSD detector to analyse carbohydrates in a variety foods and beverages. This combination gave short run times (sucrose eluting <15 minutes) with high sensitivity. Young was also able to produce run times that were shorter again by the addition of acetone into the ACN:H₂O mobile phase, which enhanced peak efficiencies (Young, 2002). A similar method was also used to analyse carbohydrates in onions (Kahane et al., 2001).

Another option for carbohydrate separation is to use cation exchange columns. These columns comprise of sulfonated styrene divinylbenzene resins and come in either lead (Pb(II)) or calcium (Ca(II)) forms. The mobile phase conditions are quite strict: water or water with an organic modifier (e.g. CaEDTA), high temperatures (80 – 90 °C) and low flow rates (< 0.6 mL min⁻¹). Soluble carbohydrates are separated principally by size exclusion (larger molecules elute earlier), but also by ligand exchange and partitioning effects (Lee & Coates, 2000; Naidu, 1998). Ligand exchange coordinates
the hydroxyl groups of sugars and polyols with the cations on the resin. The elution order is opposite that of the amino column – higher MW sugars elute first and low MW sugars last (Lee & Coates, 2000) and is therefore useful as a complimentary column for checking co-eluting peaks. It is important to note that samples should be free of cations (e.g. Ni(II), Fe(II), Co(II), or high concentrations of H+) as they may exchange with the bound ions on the column and result in an unpredictable stationary phase (Marko-Varga et al., 1990).

The Ca(II) column is often coupled with RI detection, but it may also be used with UV detection. Separation of plant sugars and sugar alcohols (including raffinose, trehalose, glucose, fructose, inositol and mannitol) has been achieved in a run time of less than 16 minutes on a Polyspher OA-KC column, however, not all peaks had baseline resolution (Kleinschmidt et al., 1998). The method employed by Naidu (1998), using a Ca(II) column, failed to resolve sucrose and trehalose.

Methods employing PAD can be coupled with anion-exchange columns, for example, the Dionex CarboPac PA10 column. As sugars are very weak acids (due to their -COOH group), at high pH they can be separated on a column as anions. The mobile phase of choice is most often NaOH, with its concentration affecting chromatographic separations and retention times. However, when low concentrations of NaOH are used for the mobile phase, detection sensitivity may be lost. Sensitivity may be increased by adding a solution of high concentration NaOH through a post-column delivery system (Gailing et al., 1998). In the method used by Lee and Coates (2000), the retention times for myo-inositol, glucose, fructose and sucrose were less than 10 minutes.

The Dionex CarboPac columns include the PA1, MA1 and PA10. These are filled with a pellicular anion exchange resin (i.e. particles with a large, spherical solid
core (e.g. 10 μm) with a thin covering layer (e.g. 0.3 μm) of resin. Pellicular particles are more efficient than large fully porous particles, because they offer a lower resistance even though they are of similar size (Robards et al., 1994). These columns are, however, restricted to operating at low pressure and low concentrations of organic modifiers. Adams et al. (1993) calculated the PA1 to have lower capacity factors for analytes than the MA1, that is, the PA1 retained less analyte in the stationary phase than the mobile phase.

The reproducibility of the retention times on the MA1 for all soluble carbohydrates was < 0.4 %RSD (Hanko & Rohrer, 2000). While these figures are good, the retention times for the sugars are quite long with sucrose eluting at 45 minutes on the MA1 column, however, it does elute slightly earlier on the PA1 column (Hanko & Rohrer, 2000).

Another ion exchange column is the sulfonated monodisperse resin-based column (H+ form). These columns separate components on the principle of ion exclusion and partition and while they prefer pure water as the mobile phase a low concentration of NaOH is required for PAD analysis (Corradini et al., 2001).

C18 columns can support a mobile phase of organic solvents and varying pH (Honda et al., 1989). Honda et al. (1989) used a mixture of 0.1 M phosphate buffer and ACN, to a concentration of 20 % (v/v), to separate PMP derivatives on a C18 column. While derivatisation provided excellent sensitivity for detection, the retention time for glucose was quite long (approximately 40 minutes). This could be reduced by using a higher concentration of ACN, for example 23 % (v/v) reduced the retention time to less than 20 minutes (Honda et al., 1989).
The retention times on a phenyl column, using a 67:33 % ACN:H₂O mobile phase, for glucose, sucrose and mannitol derivatives of \( p \)-nitrobenzoyl chloride were quite long at 15.3, 39.9 and 48.9 minutes respectively (Nojiri et al., 2000).

1.2.4 Gas Chromatography

When available, GC provides a viable alternative to HPLC if low detection limits are required. Typically, injections of 0.1 – 10 \( \mu \)L are made into an injection port 20 – 40 °C higher than the column temperature. An inert gas mobile phase is used to push the sample through a column where the analytes are separated by their relative interactions with the stationary phase. Separated components then pass a detector where they provide an electrical signal that is recorded on a data processor. Throughout this process the sample is kept at high temperatures (100 – 350 °C) so as to keep them in a gaseous phase. However, sugars are not volatile, so prior to injection they must be derivatised.

1.2.4.1 Sample Preparation

The derivatisation process replaces the hydrogen in functional groups of \(-\text{COOH}, \ -\text{OH}, \ -\text{NH}\) and \(-\text{SH}\). There are three types of derivatising agents: silylation agents, acylation agents and alkylation agents. For analysis of sugars the silylation agents are recommended due to their ease of use and high reactivity (Knapp, 1979). However, it is important for the sample, solvent and equipment used to be free of moisture when adding this agent due to the preference it has for reacting with the \(-\text{OH}\) group of water.

Silylation involves the replacement of the functional group hydrogen with a trimethylsilyl (TMS) group. This increases the volatility of the analyte by blocking
active proton sites and therefore reducing the dipole-dipole interactions. They react with functional groups in the order: alcohol hydroxyl > phenol hydroxyl > carboxyl > amine > amide. The general reaction scheme for these agents is:

\[
R - Y - H + R' - C = N - TMS \rightarrow R - Y - TMS + R' - C = N - H
\]

where \( Y \) is the functional group minus hydrogen (i.e. -COO, -O, -N and -S), \( R \) is an alkyl or aromatic compound and TMS is \( \text{Si}(\text{CH}_3)_3 \). While there are seven silylation agents only four of these are commonly used for analysis of carbohydrates, these are

- Hexamethyldisilazane (HMDS)
- \( \text{N,O-Bis(trimethylsilyl)acetamide} \) (BSA)
- \( \text{N,O-Bis(trimethylsilyl) trifluoroacetamide} \) (BSTFA)
- Trimethylsilylimidazole (TMSI)

Figure 1.1 Structures of four trimethylsilyl (TMS) derivatising agents used in the derivatisation of soluble carbohydrates for analysis by GC.
hexamethyldisilazane (HMDS), N,O-bis(trimethylsilyl)acetamide (BSA), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylsilylimidazole (TMSI). The structures of these agents are shown in Figure 1.1.

While HMDS is specifically recommended for carbohydrate analysis, it is a weak TMS donor and will therefore only react easily with hydroxyl groups (Knapp, 1979). To provide a faster and more quantitative reaction the catalysts trimethylchlorosilane (TMCS) or trifluoroacetic acid may be used. The general conditions required for derivatisation with HMDS are an anhydrous sample, taken up in HMDS and a catalyst and heated for 60 minutes at high temperature (e.g. 60 – 100 °C) (Knapp, 1979).

BSA forms TMS derivatives under mild conditions with most organic functional groups. It remains stable for at least 9 hours (Pirc, 1985). For derivatisation to occur an anhydrous sample is taken up in a BSA/pyridine mix and heated for 20 minutes at 60 °C (Knapp, 1979). However, some samples may require heating for up to 16 hours. One disadvantage of using BSA is that it can foul the flame ionisation detector by oxidizing to form silicon dioxide.

BSTFA is one of the most reactive of the silylating agents. It reacts more completely and produces more volatile derivatives than BSA due to the trifluoroacetyl group. Also, unlike BSA, the combustion by-product silicon trifluoride does not foul FID detectors. For analysis of molecules with secondary alcohols and amines, where the functional groups are hindered, a mixture with TMCS may be used to catalyse the reaction.

TMSI is highly recommended for carbohydrate analysis (Knapp, 1979). It is a selective TMS donor that will not react with hindered amines or amides, but it will react with alcohols and phenols. This agent may therefore be used if co-elution occurs
between an analyte and an amine or amide when using another silylating agent, as TMSI will only attack the hydroxyl sites and will therefore alter the retention times of the silylated amine or amide.

As derivatising agents will react with the –COOH and –OH groups of sugars, the isomeric form (i.e. chain or cyclic) of the monosaccharide at the time of derivatisation will be the final form of the compound. Due to the slight variations in the physical properties of the isomers, each isomer may give rise to separate chromatographic peaks. For example, at equilibrium fructose may have five isomers, an open chain form and four cyclic forms and may therefore give rise to five chromatographic peaks (Figure 1.2). The first two of the cyclic forms arise from the
rotation at the anomeric carbon (-COOH) to form either α-D-fructose or β-D-fructose, where the anomeric -OH group lies either cis or trans to the terminal -CH2OH group. The other two cyclic forms refer to the formation of a five-membered ring (furanose) or a six-membered ring (pyranose). All isomeric forms are interchangeable via the open chained form designated D-fructose (Figure 1.2).

When analysing a complex chromatogram it is not ideal for an analyte to produce multiple peaks. Converting the sugars to their oximes may restrict the number of chromatographic peaks produced for each monosaccharide. An oxime is formed at the double bond of the keto group of open chain sugars.

\[
R - \text{CH}^\delta+ + \text{H}_2\text{N} - \text{OH} \rightarrow R - \text{CH} = \text{N} - \text{OH} + \text{H}_2\text{O}
\]

By reacting the sugars with hydroxylamine hydrochloride (\text{NH}_2\text{OH}.\text{HCl}) in the presence of pyridine the C=O group is converted to C=N-OH. The sugar is therefore prevented from forming the cyclic structure, which minimizes the number of chromatographic peaks produced.

1.2.4.2 GC Columns

Separation of analytes by GC can occur on two types of columns, packed and capillary. Characteristically, packed columns are 2 – 4 mm x 1 – 6 m and capillary columns are 0.1 – 0.75 mm x 10 – 100 m. Packed columns contain a dense packing material coated with a thin liquid stationary phase. Capillary columns have an open-tubular form with the stationary phase coated on the wall of the column or a support, such as diatomaceous earth.
A common stationary phase on columns used in the analysis of carbohydrates is polydimethyl siloxane, with varying percentages of phenyl groups attached to alter the polarity of the phase. For example, the DB-1 column contains no phenyl groups, therefore the column is non-polar and quickly elutes polar analytes, such as sugars. Analysis of carbohydrates on a DB-1 column have been carried out by Adams et al. (1999), Chapman & Horvat (1989) and Chen et al. (1998). A DB-5 column contains 5 % phenyl, slightly increasing the polarity, which increases the retention time of sugar analytes. Analysis of carbohydrates on a DB-5 column have been carried out by Bleton et al. (1996), Dormaar & Willms (1990), Ha & Thomas (1988) and Lo Bianco et al. (1999).

Other stationary phase options include the MS and Dexsil packings. The MS capillary columns are a methyl polysiloxane with varying ratios of phenyl groups. The MS designation indicates a very low bleed column that makes the column suitable for use with the mass spectroscopy detector. An example is the HP-5 MS capillary column used by Lee et al. (2002) and Oddo et al. (2002). Dexsil contains methylsilicone with varying ratios of carborane. It has been used at 3 % (Janauer & Englmaier, 1978; Pirc, 1985) and 15 % ratios (Morvai & Molnar-Perl, 1990) in carbohydrate analysis.

1.2.4.3 GC Detectors

Separated carbohydrates in a sample are detected by either a flame ionisation detector (FID) or a mass spectroscopy (MS) detector. The FID measures an electrical response produced by the burning of organic compounds in a hydrogen/air flame of approximately 300 °C. At this temperature reduced carbons produce ions and electrons that conduct electricity, which is collected and amplified for measurement. Analysis of
soluble carbohydrate samples using FID have been carried out by Bleton et al. (1996), Lee et al. (2002) and Oddo et al. (2002).

The MS detector continuously analyses each chromatographic zone that elutes from the column, by separating the various ions and recording their mass to charge (m/z) ratios. MS is therefore useful for creating characteristic ion profiles for analytes, which can be used in the identification of analytes. GC-MS has been utilised for soluble carbohydrate analysis by Adams et al. (1999), Chen et al. (1998), Ha & Thomas (1988), Janauer & Englmaier (1978), Morvai & Molnár-Perl (1990) and Pirc (1985). Detection limits for fructose, glucose and sucrose, measured by GC-MS, were 2.9 – 12.4 ng for a 1 μL injection (Adams et al., 1999).

1.2.5 UV-Vis Spectrophotometry

There are several major methods used for the analysis of starch, which include the anthrone method, phenol-sulfuric acid method and the iodine method.

In the anthrone method the carbohydrates are hydrolysed in concentrated sulfuric acid to which anthrone is added. This produces a blue-green colour, believed to be the product of hydroxymethylfurfural and anthrone. This method has previously been used to quantify a range of carbohydrates, including monosaccharides, disaccharides and starch (Hodge & Hofreiter, 1962). In comparing six methods of starch estimation, MacRae et al. (1974) found the anthrone method gave consistently higher values than four of the alternative methods. The authors reasoned that the anthrone method is non-specific and measures total hexoses while the alternative methods only measure glucose.
There are some disadvantages to the anthrone method, which have led to the replacement of this method with others. The anthrone reagent is not stable in sulfuric acid (Dubois et al., 1956); a modified version of this method requires absorbencies to be measured at exactly 12 minutes after boiling as deviation in time can lead to a 10% error (Buysse & Merckx, 1993). Moreover the reagent is expensive (Dubois et al., 1956). The method is also sensitive to ethanol, a common extraction solvent for sugars and would therefore require evaporation of the solvent before commencement (Buysse & Merckx, 1993).

With the iodine method, as described by Quarmby & Allen (1989), the pH of the solution is altered to be favourable for the reaction. This is done by using phenol red indicator (pH 6.8 – 8.2), sodium hydroxide and acetic acid until the correct colour is obtained. Solutions of potassium iodide (KI) and potassium iodate (KIO₃) are then added, which turn the solution blue in the presence of starch. In this case KI acts as a reducing agent.

A third method of starch analysis is by the phenol-sulfuric acid method. After starch is extracted a hydrous phenol solution is added, followed immediately by concentrated sulfuric acid. The original method, as written by Dubois et al. (1956), required the end mixture to stand for twenty minutes, followed by up to 20 minutes in a 25 – 30 °C water bath, before measuring the absorbance. A more recent version of this method requires only a 15 – 20 minute standing time at room temperature (Buysse & Merckx, 1993). The colour produced by this method is said to be stable for several hours and the method is accurate to ± 2% (Dubois et al., 1956). The absorbance is dependent on the amount of phenol added, with absorbencies for sugars reaching a peak before falling again as the phenol concentration is increased (Dubois et al., 1956). This method is believed to be more applicable for routine analysis than either the anthrone
method or the iodine method as it is fast, easy and has low costs (Buysse & Merckx, 1993). It has recently been utilised in the determination of starch in the seagrasses *Halophila ovalis* and *H. pinifolia* (Longstaff & Dennison, 1999; Longstaff *et al.*, 1999).

1.3 Project Aims

The aim of this project was to investigate a possible cause to why *P. coriacea* was dying in artificial culture (*e.g.* tissue culture) 10 – 12 months after germination. As death occurs soon after the depletion of the seed starch reserves, the seedlings may be unable to replenish their carbohydrate stores. One way to determine if this is true is by analysing the soluble and insoluble carbohydrates in their leaves. However, the method for analysis would need to be suitable for limited amounts of plant material and low concentrations of soluble carbohydrates.

As far as the author is aware, there has been no comparison made to determine which chromatographic methods are the most appropriate for when plant material is limited and carbohydrate concentrations are low. Therefore, before analysing the soluble carbohydrates in *P. coriacea*, a chromatographic method with high sensitivity needed to be developed.
2.1 Introduction

Chromatographic analysis of plant soluble carbohydrates involves three steps: the extraction of soluble carbohydrates, preparation of the extract and chromatographic analysis. The methods for extraction, preparation and analysis are varied, ranging from cumbersome yet sensitive to simple but insensitive methods. While HPLC does not necessitate carbohydrate extracts to undergo further preparation for analysis, GC does as they must be transformed into volatile analytes to undergo chromatographic separation and detection.

Extraction methods vary in solvents and temperature used. Solvents utilised include water (Pirc, 1985), ethanol (Holligan & Drew, 1971), methanol (Bleton et al., 1996), perchloric acid (Adams et al., 1999) and chloroform (Adams et al., 1992). These are often used in combination. Quarmby and Allen (1989) have suggested that extractions in pure water may lead to the hydrolysis of sugars and therefore recommend using 80% ethanol. Temperatures used for extraction vary between 0 °C (Adams et al., 1999), room temperature (Morvai & Molnar-Perl, 1990) and 100 °C (Bleton et al., 1996). Quarmby & Allen (1989) suggest that high temperatures should be avoided if possible, as sugars may be lost. As far as the author is aware, no studies so far have made a comprehensive survey of the solvents and temperatures that are most appropriate for extraction of carbohydrates from plants.

Preparation of the extract for analysis can be as simple as filtering the extract for HPLC analysis, to more time consuming and multi-stepped derivatisation procedures.
For example, Adams et al. (1999), used Sep-Pak C_{18} cartridges to simplify the extract matrix, converted the sugars to their oximes and then converted the sugars and sugar-oximes to their TMS derivatives using a combination of sonication and heating.

While methods for carbohydrate analysis are varied, there appears to be little comparison of these methods. As far as the author is aware there has been no comparisons made of the GC DB-5 and BP-1 (equivalent to DB-I) columns, of the derivatising agents TMSI and BSTFA, or of the HPLC amino and polyamine columns in the analysis of carbohydrates. Additionally, while Young (2002) compared the UV-Vis and ELSD detectors for analysis of carbohydrates, no details of the efficiency of each method was given. The article also did not include all of the sugars that were likely to be found in seagrass material. Neither, as far as the author is aware, have the analytical methods of HPLC and GC undergone a comprehensive comparison in relation to analysis of soluble carbohydrates.

The aim for this project was to develop a reproducible and sensitive method for the routine analysis of extracts. Particular challenges included developing a method suitable for small amounts of plant material (0.1 g) and low analyte concentrations. Method development included:

(i) Selecting an appropriate extraction method;
(ii) Developing an easy yet robust sample preparation method;
(iii) Comparing the amino, polyamine, DB-1 and BP-1 columns on their elution orders, total run times and reproducibility of their retention times; and
(iv) Determining the sensitivity of two HPLC detectors, the UV-Vis and ELSD and the GC detector, FID.
2.2 Materials and Methods

2.2.1 Reagents and Solvents

Standard sugars, perchloric acid, pyridine, BSTFA 1% TMCS (99% purity), TMSI (98% purity), 3-methoxybenzoic acid (m-Anisic acid) and phenyl-β-D-glycoside obtained from Sigma Aldrich Pty. Ltd. (NSW, Australia). Absolute ethanol was obtained from ICI Instruments, (Vic., Australia) and made to 80% concentration with MilliQ water. Acetonitrile (ACN) and methanol (MeOH) were of HPLC grade, obtained from EM Science (New Jersey, USA). Hexane was of 95 % concentration with 0.01 % water, obtained from Mallinckrodt-Baker Inc. (New Jersey, USA). MilliQ water was of 18.2 MΩ cm⁻¹ purification.

2.2.2 Sugar Standard Preparation

A standard sugar-mix contained sucrose, D-glucose, D-fructose, D(+)-trehalose, myo-inositol, mannitol, sorbitol and the amino acid L-proline, unless otherwise stated. Proline was added in the sugar mix as it is a common amino acid found in plants that may interfere with analysis. Standard sugar-mix ‘A’ contained 2000 mg L⁻¹ of each sugar. Standard sugar-mix ‘B’ contained 10 mM L⁻¹ of each sugar. A set of four sugar-mix standards ‘C’ contained 50, 250, 450 and 650 μM L⁻¹ sucrose and fructose, 50, 100, 200 and 350 μM L⁻¹ glucose, and 20, 40, 60 and 80 μM L⁻¹ of each mannitol, sorbitol, inositol and trehalose. Standards were prepared in 80% ethanol, unless otherwise stated. Where stated, 78 μM L⁻¹ phenyl-β-D-glycoside (PβDG) was added as an internal standard (IS).
2.2.3 Extraction of Plant Soluble Carbohydrates

*P. coriacea* leaves were homogenised in liquid nitrogen using a mortar/pestle. Ground leaf tissue (100 mg) was placed in a 40 mL centrifuge tube and the sugars were extracted twice with 5.0 mL MilliQ or 80 % ethanol. The extractions were carried out using agitation at either room temperature (approximately 23 °C) or in a hot water bath at 80 °C. Each extract was centrifuged for 10 minutes at 10 000 rpm and the supernatant collected and filtered through a 0.45 μm filter cartridge (Millipore, Bedford, MA) into a sample tube.

Extraction methods were compared using GC, which required the derivatisation of the extracts. A 500 μL aliquot of extract was transferred to a 2.0 mL autosampler vial, evaporated to dryness using an air stream and taken up in 400 μL pyridine and 200 μL of BSTFA. Sealed vials were heated for 30 minutes at 60 °C and 1 μL injections were made onto a DB-5 column, using the chromatographic conditions described in section 2.2.5.

2.2.4 HPLC Analysis of Soluble Carbohydrates

HPLC analysis was performed using a Varian ProStar high performance liquid chromatograph (Varian Aust. Pty. Ltd., Australia), equipped with an autosampler (Varian model 400) fitted with a 10 μL injection loop, a solvent delivery module with degasser (Varian model 240), a photo diode array (PDA) UV-Vis detector (Varian model 330) set at 192 nm and an Alltech ELSD (Alltech model 2000). Columns used were an Alltech Prevail Carbohydrate ES 5μm 250 x 4.6 mm (Alltech Associates Aust. Pty. Ltd., Australia) and a Phenomenex Phenosphere 5μm NH₂ 80A 250 x 4.6 mm
(Phenomenex, Australia). These will be referred to as polyamine and amino columns respectively. Data was processed using the Star Chromatography Workstation version 5.52.

2.2.4.1 Optimising ELSD Conditions

The ELSD was optimised by varying the drift tube temperature and gas flow rate. Drift tube temperature was varied between 70, 80 and 90 °C using a gas flow rate of 2.0 L min⁻¹. Gas flow rates of 1.7, 2.0 and 2.3 L min⁻¹ were compared using a drift tube temperature of 70 °C. A 1000 mV full-scale voltage was used.

Standard sugar-mix ‘A’ was run in triplicate for each method on the amino column using Method A2 described in section 2.2.4.2. The mobile phase solvents were MilliQ and ACN. The gradient employed was a linear increase from 5 % MilliQ to 40 % MilliQ over 20 minutes and using a 5 min equilibration time. Flow rate was 1.5 mL min⁻¹.

2.2.4.2 Column Conditions

The chromatographic conditions for the amino column were:

(Method A1) MilliQ:ACN mobile phase with flow rate of 2.0 mL min⁻¹. The linear gradient employed was 5:95 % to 12:88 % over 16 min, linear increase to 28:72 % at 21 min, isocratic for 9 min, linear increase to 50:50% over 10 min and a 5 min equilibration time.

(Method A2) MilliQ:ACN mobile phase with flow rate of 1.5 mL min⁻¹. The linear gradient employed was 5:95 % to 57:43 % over 20 minutes and a 10 min equilibration time.
(Method A3) MilliQ:MeOH mobile phase with flow rate of 1.5 mL min\(^{-1}\). The linear gradient employed was 5:95 % to 40:60 % over 40 min and a 5 min equilibration time.

(Method A4) MilliQ:MeOH:ACN mobile phase with flow rate of 1.5 mL min\(^{-1}\). The linear gradient employed was 75:5:20 % to 40:40:20 % over 40 min and a 5 min equilibration time.

The chromatographic conditions for the polyamine column were:

(Method PA1) MilliQ:ACN mobile phase with flow rate of 1.0 mL min\(^{-1}\). The linear gradient employed was 20:80 % to 50:50 % at 20 min and a 10 min equilibration time.

(Method PA2) MilliQ:ACN mobile phase with flow rate of 1.0 mL min\(^{-1}\). The linear gradient employed was 24:76 % to 50:50 % at 18 min and a 10 min equilibration time.

(Method PA3) Adapted from Young (2002), using solvent mix (A) ACN:Acetone and (B) MilliQ, with mobile phase flow rate of 1.0 mL min\(^{-1}\). The linear gradient employed was 80 % A to 60 % A over 15 min and isocratic 60 % A over 5 minutes, with a equilibration time of 5 min.

2.2.5 GC Analysis of Soluble Carbohydrates

GC analysis was performed using a Varian model 3800 gas chromatograph (Varian Aust. Pty. Ltd., Australia), equipped with an autosampler (Varian model 8200), fitted with split/splitless capillary inlet system, a FID and a Varian Star Chromatography Workstation v 5.3 data processor. Injections of 2 µL were made in the splitless mode. Between injections the syringe was washed with ethyl acetate. Helium flow rate through column was 1 mL min\(^{-1}\). Nitrogen flow rate to the FID was 29 mL min\(^{-1}\). A DB-5 capillary column (30 m x 0.25 mm 0.25 µm i.d.; J & W Scientific, USA) and BP-1 capillary column (25 m x 0.22 mm 0.2 µm i.d.; SGE Australia Pty. Ltd.,...
Australia) were used. The DB-5 column was held at an initial temperature of 60 °C for 2 min, then increased at 20 °C min\(^{-1}\) to 150 °C, then at 6 °C min\(^{-1}\) to 300 °C and finally held at that temperature for 3.5 min. The BP-1 column was held at an initial temperature of 60 °C for 2 min, increased at 20 °C min\(^{-1}\) to 150 °C, then at 6 °C min\(^{-1}\) to 276 °C. The injector and detector temperatures were 250 and 300 °C, respectively.

2.2.5.1 Derivatisation Conditions Development

A 200 µL aliquot of standard sugar-mix B was evaporated to dryness in a 2.0 mL autosampler vial using an air stream. The residue was taken up in 400 µL pyridine and 200 µL BSTFA or TMSI. Sealed vials were heated at 60 °C for 30 minutes and injected onto the DB-5 column. An additional set of BSTFA derivatives were heated at 60 °C for 10, 30 or 60 min, and for 30 min at 60 and 80 °C.

2.2.5.2 Derivative Degradation

Three 900 µL aliquots of standard sugar-mix C were evaporated to dryness in a 2.0 mL autosampler vial using an air stream. Residue was taken up in 150 µL of pyridine and 150 µL of BSTFA. Sealed vials were heated for 30 min at 60 °C. Injections were made onto the DB-5 column. Derivatised samples were run continuously for 24 hours.

2.2.5.3 Sample concentration

Various methods of concentrating a standard sugar-mix were trialed.

(i) A 500 µL aliquot of standard sugar-mix B was evaporated to dryness in a 2.0 mL autosampler vial using an air stream. The residue was taken up
in 400 μL of pyridine and 200 μL of BSTFA. Sealed vials were heated for 30 min at 60 °C and injected onto the DB-5 column. The sample was re-evaporated using an air-stream, taken up in 100 μL 95 % hexane and injected onto the DB-5 column.

(ii) A 2.0 mL aliquot of standard sugar-mix B (in MilliQ) was evaporated to dryness in a round bottom flask using a rotary evaporator at 45 °C. The residue was taken up in 300 μL pyridine. A 200 μL aliquot was transferred to a 2.0 mL vial and 200 μL BSTFA was added. Sealed vials were heated for 30 min at 60 °C and injected onto the DB-5 column.

(iii) A 1200 μL aliquot of standard sugar-mix B was evaporated to dryness in a 2.0 mL autosampler vial using an air stream. The residue was taken up in 100:50, 200:100, 300:150 or 400:200 μL pyridine to BSTFA respectively. Sealed vials were heated for 30 min at 60 °C and injected onto the DB-5 column.

2.2.6 Statistics and Calculations

Data was analysed using a one-way ANOVA or a Student T-test, with significance at p < 0.05.

Detection limits (LOD) were calculated as follows:

\[ \text{LOD} = \left( \frac{C}{p_h} \right) \times 3(\text{noise}) \]

where \( C \) is the concentration of the analyte, \( p_h \) is the height of the analytes detector response and noise is the detector variation measured prior to a sample run.

The relative standard deviation (%RSD) was calculated as follows:
\[ \%RSD = \frac{SD}{\text{mean}} \times 100\% \]

where SD is the standard deviation.

The number of theoretical plates \( (n) \) was calculated as follows:

\[ n = 5.54 \left( \frac{t_r}{w_{1/2}} \right)^2 \]

where \( t_r \) is the retention time and \( w_{1/2} \) is the width of the peak at half height.

The theoretical plate height \( (h) \) was calculated as follows:

\[ h = \frac{L}{n} \]

where \( L \) is the length of the column (mm).

2.3 Results and Discussion

2.3.1 Extraction of Plant Soluble Carbohydrates

Sugars extracted at different temperatures were analysed by GC to determine the concentrations of each sugar in the extract. While there was more fructose, glucose and sucrose extracted at 80 °C \( (p < 0.05) \), the trace amounts of trehalose were extracted more easily at room temperature \( (p < 0.05) \) than at 80 °C (Figure 2.1). However, the sugar alcohols, mannitol and inositol, showed no significant difference in the amount of each extracted at either temperatures \( (p > 0.05) \) (Figure 2.1).

Extraction of soluble carbohydrates in MilliQ water was not significantly different \( (p > 0.05) \) to extractions in 80 % ethanol, except for sucrose \( (p = 0.042) \) where MilliQ extracted the greater amount. Sugars identified in the extracts were glucose, \textit{myo}-inositol and sucrose, however, trace amounts of fructose, mannitol and trehalose were suspected. To confirm the presence of these carbohydrates, a more concentrated sample was required. Therefore, further trials used 900 μL 80 % ethanol extractions
that were quickly evaporated with an air stream in 30 minutes and taken up in 150 μL of each pyridine and BSTFA. These trials identified fructose, glucose, sucrose, trehalose, mannitol and myo-inositol in the seagrass samples.

2.3.2 HPLC Analysis of Soluble Carbohydrates

2.3.2.1 Optimal ELSD Operating Conditions

The optimum operating conditions for the ELSD is dependent on the mobile phase solvents and the analyte. The ELSD requires the evaporation of the solvent in the drift tube, but its temperature must not be high enough to cause evaporation of the analyte. The gas flow rate for the nebulizer is also dependent on the mobile phase,

![Graph showing detector response ratio for sugars](image)

Figure 2.1 The effect of temperature on the amount of sugars extracted from *P. coriacea* leaves. (a) room temperature, (b) 80 °C. IS = internal standard. Error bars show ± 1.0 standard error. Conditions described in section 2.2.3, page 34.
with low flow rates increasing the size of the droplets of the column effluent and therefore the sensitivity of the detector, but high flow rates increasing evaporation of the mobile phase.

For an isocratic water mobile phase the drift tube temperature should be set at 115 °C and the gas flow at 3.2 L min⁻¹ (Alltech ELSD 2000 Operating Manual). For an isocratic acetonitrile mobile phase the drift tube is set at 70 °C and gas flow at 1.7 L min⁻¹ (Alltech ELSD 2000 Operating Manual). However, a mobile phase of both water and acetonitrile was required and the method was not isocratic. The ELSD drift tube temperature was heated at 70, 80 and 90 °C, but there was no significant difference in the sensitivity of the ELSD operated at these temperatures. However, there was a general trend for lower detection limits when using 90 °C. There was also no significant difference in the ELSD sensitivity when using a gas flow rate of 1.7, 2.0 and 2.3 L min⁻¹.

For the methods presented in section 2.2.4.2 (methods A1,2; PA1,2; page 34) that utilize a gradient program of acetonitrile and water, this study recommends using a 90 °C drift tube temperature and 2.0 L min⁻¹ gas flow rate so as to conserve gas. The full-scale voltage setting of either 0 – 10 mV or 0 – 1000 mV is dependent on the concentration of the analytes in the sample.

2.3.2.2 Optimal column conditions

Using methanol or acetone in the mobile phase, for the amino and polyamine columns respectively, resulted in the loss of all peaks, even when acetonitrile was added (section 2.2.4.2, methods A3,4). A gradient of water and acetonitrile programmed from 5:95 % to 50:50 % over 40 min (section 2.2.4.2, method A1) on the amino column resolved all peaks, with the last peak, proline, eluting at 32 minutes. However, the
reproducibility of the retention times for each sugar analysed was between 1.45 and 4.54 %RSD (Figure 2.2).

The retention times were shortened and the reproducibility was improved on the amino column by using a linear gradient of water:ACN from 5:95 % to 57:43 % over 20 minutes and doubling the equilibration time to 10 minutes (section 2.2.4.2, method A2). With this method proline eluted at 13.09 min and the reproducibility of the retention times were between 0.31 – 0.64 %RSD (Figure 2.2). Increasing the water ratio more rapidly shortened the retention time because high molecular weight carbohydrates have a strong affinity for the polarity of water. The reproducibility of these retention times was improved due to the longer equilibration time, which allowed more time for the mobile phase to become stable. The shortened retention times, however, resulted in a

![Graph](image)

Figure 2.2 Reproducibility of the retention times (%RSD) for sugars on the amino (A) and polyamine (PA) columns using methods A1, A2, PA1 and PA2. For method details see section 2.2.4.2. n = 3. Sorbitol/mannitol and fructose/proline peaks co-eluted in methods A2 and PA2 respectively.
loss of resolution between sorbitol and mannitol, which co-eluted. This is a disadvantage for samples containing both sorbitol and mannitol and where qualification of analytes in a sample is required.

Using the polyamine column, a mobile phase gradient of MilliQ and acetonitrile from 20:80 % to 50:50 % (section 2.2.4.2, method PA1) resolved all peaks and the reproducibility of the retention times were between 0.41 and 1.10 %RSD for each sugar (Figure 2.2). By starting the gradient at 24:76 %, increasing the water gradient rate and increasing the equilibration time, the reproducibility of the retention times were slightly improved, with each sugar being between 0.28 – 0.50 %RSD (Figure 2.2). The last peak to elute on the polyamine column was raffinose at 13.4 minutes and the retention time of proline was reduced, however, proline now co-eluted with fructose. The sugar alcohols, mannitol and sorbitol, were now also eluting separately before fructose and glucose, which on the amino column were the first to elute.

2.3.2.3 Column comparison

A comparison of the amino and polyamine column methods (section 2.2.4.2, methods A2 & PA2) showed the polyamine column to provide more reproducible retention times, shorter elution times and lower ELSD detection limits (Table 2.1). The retention time of the last eluted peak for the amino and polyamine columns was 13.09 and 11.40 min respectively. The polyamine column provided greater reproducibility of retention times than the amino column, at 0.19 – 0.50 %RSD and 0.31 – 0.64 %RSD respectively. This would be expected because of the stability of the stationary phase particles on the polyamine column. The gradual degradation in the amino column, due to bleeding of particles, is prevented in the polyamine as the amine groups are held on a polymer resin.
The differences in the stationary phase coatings also led to slightly differing detection limits. The detection limits of the ELSD when using the polyamine column were between 3.42 – 8.12 mM L\(^{-1}\) and when using the amino column were between 5.12 – 23.73 mM L\(^{-1}\) (Table 2.1). This may be due to the interactions between the sugar and the stationary phase of the polyamine column allowing less zone broadening and therefore greater peak heights.

Table 2.1  Retention times (reproducibility, %RSD), detection limits, number of theoretical plates and plate heights of a 2.0 g L\(^{-1}\) standard sugar-mix on the amino (A) and polyamine (PA) columns. Column conditions see section 2.2.4.2, methods A2 and PA2. n = 3. Detection limits measured using ELSD.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Retention time</th>
<th>Detection Limits</th>
<th>Theoretical Plates</th>
<th>Plate Height (h) (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(min) (%RSD)</td>
<td>(mM L(^{-1}))</td>
<td>(n)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>PA</td>
<td>A</td>
<td>PA</td>
</tr>
<tr>
<td>Fructose</td>
<td>7.39 (0.54)</td>
<td>8.21 (0.36)</td>
<td>23.73</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>7.78 (0.64)</td>
<td>8.99 (0.33)</td>
<td>13.60</td>
<td>8.12</td>
</tr>
<tr>
<td>Mannitol</td>
<td>1.80 (0.48)</td>
<td>7.48 (0.40)</td>
<td>-</td>
<td>7.14</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>1.80 (0.48)</td>
<td>8.00 (0.50)</td>
<td>-</td>
<td>5.25</td>
</tr>
<tr>
<td>Inositol</td>
<td>10.05 (0.50)</td>
<td>10.35 (0.19)</td>
<td>5.12</td>
<td>5.57</td>
</tr>
<tr>
<td>Sucrose</td>
<td>9.60 (0.52)</td>
<td>10.71 (0.28)</td>
<td>10.25</td>
<td>3.42</td>
</tr>
<tr>
<td>Trehalose</td>
<td>10.59 (0.47)</td>
<td>11.40 (0.35)</td>
<td>6.35</td>
<td>4.05</td>
</tr>
<tr>
<td>Proline</td>
<td>13.09 (0.31)</td>
<td>8.21 (0.36)</td>
<td>17.41</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^1\) co-eluting peaks.
Zone broadening is measured by calculating the number of theoretical plates ($n$) and plate height ($h$). Theoretically, $n$ and $h$ can only be calculated for columns when using an isocratic mobile phase, however, they are presented here to give a general idea of the efficiency of the amino and polyamine columns. The number of theoretical plates for each sugar on the amino column was between 6,800 to 15,300 plates and for the polyamine column values were between 8,200 and 19,200 plates (Table 2.1). Both of these columns were of the same length, internal diameter, particle size and a similar mobile phase gradient was used allowing a direct comparison to be made using the $n$ values, however, $h$ values are also given in Table 2.1.

The elution order for the amino column was monosaccharides, sugar alcohols, disaccharides and then amino acids. The elution order on the polyamine column differed with sugar alcohols eluting first, followed by monosaccharides, amino acids and disaccharides. When using these methods specifically, co-elution was evident between sorbitol and mannitol on the amino column, and between fructose and proline on the polyamine column. Sorbitol was also not fully resolved from the fructose and proline peak on the polyamine column. This co-elution can be avoided by slowing the rate of change in the water ratio of the mobile phase, however this results in longer retention times.

2.3.2.4 Detector comparison

The lower limits of detection for sugars analysed with the ELSD were between 20 and 200-fold lower than for the photo-diode array (PDA) UV-Vis detector (Figure 2.3). For example, the detection limits for glucose were 3054 mM L$^{-1}$ when using the PDA and 38 mM L$^{-1}$ when using the ELSD. Sensitive UV-Vis detection relies on compounds possessing a chromophore, which is absent in carbohydrates.
An HPLC chromatogram obtained from a standard solution containing selected sugars, sugar alcohols and proline, each at 1.0 g L\(^{-1}\) in MilliQ. (A) UV-Vis PDA detector response. (B) ELSD detector response. (a) fructose; (b) glucose; (c) mannitol; (d) sucrose; (e) myo-inositol; (f) trehalose; (g) raffinose; (h) proline. Conditions: 10 µL of standard sugar-mix containing each compound was injected onto the amino column. Column conditions: MilliQ:ACN mobile phase with flow rate of 1.5 mL min\(^{-1}\), and linear gradient from 5:95 % to 50:40 % over 25 minutes, with a 5 min equilibration time.
detection of sugars only occurs at 190 nm. Detection is improved on the ELSD as it is a universal detector for analytes that are sufficiently less volatile than the mobile phase. Additionally, as it does not rely on the optical characteristics of an analyte, it does not call for analytes to possess a chromophore for detection. However, when compounds possess a chromophore the sensitivity of the UV-Vis and ELSD detectors are similar, such as seen for proline (Figure 2.3). The UV-Vis and ELSD detection limits for proline were 134 and 94.7 mM L\(^{-1}\) respectively.

2.3.3 GC Analysis of Soluble Carbohydrates

2.3.3.1 Derivatisation Conditions

The FID peak response for TMSI sugar derivatives was less than when using BSTFA (Figure 2.4). For example, derivatisation of 10 mM L\(^{-1}\) glucose with BSTFA produced peak heights 4-fold greater than when TMSI was used. The FID detection limits for sugars derivatised with TMSI were between 23 – 211 \(\mu\)M L\(^{-1}\) and for sugars derivatised with BSTFA were between 18 – 99 \(\mu\)M L\(^{-1}\) (Table 2.2).

The lower sensitivity of the FID to TMSI sugar derivatives may be due to the low volatility of TMSI. In preparing TMSI-derivatives for analysis, excess TMSI is normally evaporated off and the residue is redissolved in hexane to improve volatility. Injection of TMSI derivatives in hexane also avoids fouling of the FID that should be avoided as it can contribute to a decrease in detection sensitivity. However, this additional step is time-consuming and it increases the chance of moisture contamination – the highest grade of hexane available is of 95 % purification and contains 0.01 % water. BSTFA was therefore used as the derivatising agent for the remainder of the study.
Table 2.2  Detection limits, number of theoretical plates and plate height for sugars derivatised with (a) TMSI and (b) BSTFA, for a 10 mM L\(^{-1}\) standard sugar-mix. Conditions: 200 μL of standard containing each compound, was evaporated and the residue taken up in 400 μL pyridine and 200 μL of derivatising agent. Sealed samples were heated for 30 min at 60 °C and 1 μL was injected onto the DB-5 column.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Detection Limits (μM L(^{-1}))</th>
<th>Theoretical Plates (n)</th>
<th>Plate Height (h) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a) (b)</td>
<td>(a)</td>
<td>(b)</td>
</tr>
<tr>
<td>Fructose</td>
<td>181 99</td>
<td>401,680</td>
<td>306,900</td>
</tr>
<tr>
<td>Glucose</td>
<td>211 45</td>
<td>665,348</td>
<td>381,993</td>
</tr>
<tr>
<td>Sucrose</td>
<td>27 19</td>
<td>450,475</td>
<td>272,825</td>
</tr>
<tr>
<td>Trehalose</td>
<td>24 21</td>
<td>266,918</td>
<td>229,952</td>
</tr>
<tr>
<td>Mannitol</td>
<td>45 20</td>
<td>471,900</td>
<td>161,885</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>74 16</td>
<td>815,231</td>
<td>114,501</td>
</tr>
<tr>
<td>Inositol</td>
<td>74 20</td>
<td>695,095</td>
<td>66,538</td>
</tr>
</tbody>
</table>
Figure 2.4  GC- FID chromatograms obtained of standard sugars and sugar alcohols derivatised with (A) TMSI and (B) BSTFA.  (a) fructose isomers; (b) glucose isomers; (c) mannitol; (d) sorbitol; (e) myo-inositol; (f) sucrose; (g) trehalose.  Conditions as for Table 2.2, with the following changes: standard sugarmix contained 60 μM L⁻¹ fructose, mannitol, sorbitol, myo-inositol, trehalose; 200 μM L⁻¹ glucose; 1000 μM L⁻¹ sucrose.

While BSTFA is a very aggressive reagent, heating is often used to ensure all analytes have undergone a complete reaction. A one-sample T-test showed that there was no significant difference between derivatising for 10, 30 or 60 minutes at 60 °C, or for 30 minutes at 60 or 80 °C.
2.3.3.2 Derivative Degradation

Over 24 hours there was little variation in the detector response to the derivatised sugars (Figure 2.5). This ensured the stability of the derivatives for when large numbers of samples were to be analysed. However, repeated injections from the same vial eventually exposed the sample to moisture from the injector needle’s washing solvent, which quickly reduced the detector response. Previous research has found BSA, a less aggressive agent than BSTFA, was stable for at least 9 hours (Pirc, 1985). As BSTFA is more aggressive, the derivatives were also expected to be more stable.

![Figure 2.5](image)

Figure 2.5 Variation in detector response over 24 hours for fructose, glucose and sucrose. Error bars show 1.0 ± SE for 24 hours, with standards run every 3 hours. IS = internal standard. Five standard sugar-mixes included: fructose and sucrose at 50, 250, 450, 650 μM L⁻¹; glucose at 50, 100, 200, 350 μM L⁻¹; IS: 78 μM L⁻¹ phenyl-β-D- glycoside. Conditions as for Table 2.2. Regression (R²) values: fructose = 0.9674, glucose = 0.9968, sucrose = 0.9841.
2.3.3.3 Sample Concentration

Samples that were derivatised, evaporated to dryness and then taken up in hexane gave no detector response. The 95 % hexane contained 0.01 % water and it is likely that the preference of the derivatising agent for water over the sugar hydroxy sites resulted in the reversal of the derivatisation. This method was not explored any further.

Concentrating steps that involved evaporation of ethanol solvent by air stream could be achieved in 30 minutes for six 1.0 mL samples, whereas the rotary evaporator evaporated only one 2.0 mL sample in 10 minutes (therefore six samples took 1 hour). The rotary evaporator also involved the use of additional glassware and therefore the quantitative removal of the sample residue from the glassware, as opposed to the air stream evaporator where evaporation and derivatisation all took place in the one vial. Additionally, transferral of the rotary-evaporated sample required extra solvent for a quantitative transfer to a vial.

A one-way ANOVA showed no significant difference between using pyridine:BSTFA ratios of 200:100, 300:150 and 400:200 μL. However, when using a ratio of 100:50 μL there was no detector response, perhaps because of moisture and the agent not being present in excess.

By increasing the injection volume the detection limits are easily lowered. Using a 2 μL injection doubled the detector response of 1 μL injections and was not found to leak sample from the injector port.

2.3.3.4 Internal Standards

Variation was found in the detector response and it was therefore necessary to find an internal standard. Four chemicals were trialed as internal standards: m-anisic acid, gallic acid, ferulic acid and phenyl-β-D-glycoside (PPDG). m-Anisic acid eluted
earlier than the sugars and the relationship with sugars was not linear (regression $R^2$ values between 0.67 and 0.98) and was therefore not useful. Gallic acid did come off in the region of the sugars, but it co-eluted with mannitol. Ferulic acid and $\text{P}\beta\text{DG}$ both eluted within the region of the sugars and neither co-eluted with any sugars.

Calibration lines were constructed using the ratio of increasing sugar concentration to the internal standard (ferulic acid or $\text{P}\beta\text{DG}$) and the ratio of the detector response to each sugar and the internal standard. Calibration lines using ferulic acid were not linear and $R^2$ values were as low as 0.31. The $R^2$ values for $\text{P}\beta\text{DG}$ were

![Calibration plot for sucrose using phenyl-$\beta$-D-glycoside as the internal standard (IS). Four standards contained 50, 200, 600 and 850 $\mu$M L$^{-1}$ sucrose and 78 $\mu$M L$^{-1}$ IS. Conditions: 900 $\mu$L standard was evaporated and the residue taken up in 150 $\mu$L pyridine and 150 $\mu$L BSTFA. Vials were heated for 30 min at 60 °C and 2 $\mu$L injections were made onto the BP-1 column.](image)

$y = 2.1255x - 0.1952$

$R^2 = 0.9989$
all above 0.98 %, showing good linearity (Figure 2.6). When using the DB-5 column, PβDG was found to co-elute with an unknown compound in seagrass samples, but when the BP-I column was used the retention times of both were slightly altered and co-elution was avoided.

2.3.3.5 GC-FID Detection Limits

The GC-FID method was able to detect micromoles of sugars in solution (approximately 20 – 90 μM L⁻¹). This translated to being nanograms of sugar detected in the 2 μL injections (Table 2.3). The reproducibility of the detector response was

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Detection Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(μM L⁻¹)</td>
</tr>
<tr>
<td>Fructose</td>
<td>91.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>41.4</td>
</tr>
<tr>
<td>Sucrose</td>
<td>42.0</td>
</tr>
<tr>
<td>Trehalose</td>
<td>23.7</td>
</tr>
<tr>
<td>Mannitol</td>
<td>23.6</td>
</tr>
<tr>
<td>Inositol</td>
<td>18.9</td>
</tr>
</tbody>
</table>

Table 2.3 Detection limits of the GC-FID when 2 μL injections of a standard sugar-mix were made. Standard sugar-mix contained: 450 μM L⁻¹ fructose and sucrose; 250 μM L⁻¹ glucose; and 80 μM L⁻¹ mannitol, inositol, trehalose. Conditions as for Figure 2.6.
moderate when working at low concentrations, between 0.1 and 5.1 %RSD. Calibration lines were linear between 50 – 500 μM for glucose, 50 – 850 μM for fructose and sucrose and 10 – 160 μM for mannitol, sorbitol, myo-inositol and trehalose, which covered the sugar concentrations found in the seagrass extracts.

Table 2.4 Analytical characteristics for the BSTFA derivatives of sugars and sugar alcohols when run on the (a) BP-1 and (b) DB-5 columns. %RSD = reproducibility of the retention time. Conditions: as for Table 2.3.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Retention time (min) (%RSD)</th>
<th>Theoretical plates (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a)</td>
<td>(b)</td>
</tr>
<tr>
<td>Fructose</td>
<td>11.8 (0.00) 18.2 (0.04)</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>12.9 (0.04) 19.6 (0.04)</td>
<td>126,101 381,993</td>
</tr>
<tr>
<td>Sucrose</td>
<td>23.7 (0.10) 20.4 (0.06)</td>
<td>60,576 111,926</td>
</tr>
<tr>
<td>Trehalose</td>
<td>24.5 (0.04) 32.8 (0.03)</td>
<td>336,650 229,952</td>
</tr>
<tr>
<td>Mannitol</td>
<td>13.7 (0.04) 20.4 (0.02)</td>
<td>84,584 161,885</td>
</tr>
<tr>
<td>Inositol</td>
<td>15.9 (0.04) 23.1 (0.02)</td>
<td>183,871 2,031,860</td>
</tr>
</tbody>
</table>

1 No n value calculated due to overlap of isomer peaks; t values from first peak.
2 Glucose values taken from first isomer peak.
2.3.3.6 Column Comparison

The retention times of the sugars on the BP-1 column were approximately 7 minutes earlier than on the DB-5 column. The reproducibility of the retention times was high; between 0.000 – 0.094 %RSD for the BP-1 column and between 0.018 and 0.064 %RSD for the DB-5 column (Table 2.3). Calculation of theoretical plates showed the DB-5 column to be more efficient with the separation of soluble carbohydrates, with most sugars, except trehalose, having twice the number of theoretical plates on the DB-5 than on the BP-1.

2.3.4 Sugar analysis methods compared: HPLC vs GC

The performances of the HPLC-ELSD and GC-FID methods were compared using the factors developed above. While the retention times were up to twice as long using GC, the GC method was highly efficient compared to the HPLC method (Table 2.5). The retention times on the BP-1 column were ten-fold more reproducible than the polyamine column (Table 2.5). This factor is important when identifying the constituents of a sample. As expected, the GC theoretical plate number (n) was also higher, indicating more interactions of the analytes with the column and therefore the greater definition of peak shape. However, when n is put into relation with the length of the column (i.e. plate height, h), carbohydrates separated on the BP-1 column have less interaction with the column, as the plate height is greater than that on the polyamine column (Table 2.5).

Importantly, the GC-FID had detection limits at least 80-fold better than the HPLC-ELSD. While the ELSD was able to detect mM L$^{-1}$ of analytes, the GC-FID was detecting µM L$^{-1}$ of analytes (Table 2.5). Analysis of a seagrass sample using HPLC-
ELSD was only able to detect sucrose. Additionally, the HPLC chromatograms were complicated with other soluble plant extracts, such as organic acids and amino acids. These compounds can be removed from the sample extract by using cation and anion exchange cartridges, however this process leads to dilution of the extract. Chromatograms from the GC were not complicated and did not require the removal of organic acids or amino acids. The low detection limits allowed the determination of sucrose, glucose, fructose, trehalose, mannitol and myo-inositol in the seagrass leaf tissue.

Table 2.5 Some analytical parameters for the methods developed for (a) HPLC-ELSD coupled with the polyamine column and (b) GC-FID coupled with the BP-1 column. %RSD = Reproducibility of the retention times. HPLC conditions as for Table 2.1. GC conditions as for Table 2.3.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Retention time (min) (%RSD)</th>
<th>Theoretical Plates n</th>
<th>Plate Height h (mm)</th>
<th>Detection Limits (mM L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a)</td>
<td>(b)</td>
<td>(a)</td>
<td>(b)</td>
</tr>
<tr>
<td>Fructose</td>
<td>8.2 (0.36)</td>
<td>11.8 (0.00)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>9.0 (0.33)</td>
<td>12.9 (0.04)</td>
<td>10,546</td>
<td>126,101</td>
</tr>
<tr>
<td>Sucrose</td>
<td>9.6 (0.28)</td>
<td>23.7 (0.09)</td>
<td>8,116</td>
<td>60,576</td>
</tr>
<tr>
<td>Trehalose</td>
<td>10.6 (0.35)</td>
<td>24.5 (0.04)</td>
<td>19,168</td>
<td>336,650</td>
</tr>
<tr>
<td>Mannitol</td>
<td>8.4 (0.40)</td>
<td>13.7 (0.04)</td>
<td>8,150</td>
<td>84,584</td>
</tr>
<tr>
<td>Inositol</td>
<td>10.0 (0.19)</td>
<td>15.9 (0.04)</td>
<td>9,511</td>
<td>183,871</td>
</tr>
</tbody>
</table>
CHAPTER 3: Analysis of Carbohydrates in *Posidonia coriacea*

3.1 Introduction

Information available on *P. coriacea* is relatively scarce, as research has concentrated on more abundant seagrasses such as *P. australis*. *Ex situ* research on *P. coriacea* has been improved with the recent development of a technique for the sterilization of fruits that allows the species to be grown in tissue culture (Henry, 1998). However, the success of *P. coriacea* in tissue culture is limited, with seedlings dying 10 – 12 months after germination. The possible cause of death may be that the seedlings are unable to replenish their carbohydrate stores, as death occurs soon after the full depletion of the seed starch reserves (Kuo & Kirkman, 1992, 1996).

Generally, tissue cultures are provided with all the necessary conditions to grow. The tissue culture media, in which *P. coriacea* is maintained, contains sucrose which substitutes for carbohydrates that would otherwise be produced through *in situ* photosynthesis. A steady and controlled supply of light is also provided and the cultures are kept within a temperature-controlled room. Deaths of *P. coriacea* seedlings however, are not restricted to tissue cultures as they have also died when kept in small aquaria with no added nutrients and in large aquaria that mimic oceanic conditions (Lavery, P., *pers. comm.*).

One way to determine if placing *P. coriacea* in artificial cultures restricts their ability to replenish carbohydrate reserves, is to monitor the carbohydrates of cultured seedlings over time and compare their carbohydrate levels to those found in *in situ* plants. To gain a better understanding of which carbohydrates are present, it is
necessary to measure individual carbohydrates rather than the total carbohydrate content over a given period.

Few attempts have been made to determine what carbohydrates are found in Australian *Posidonia* species. Levels of sucrose, glucose and fructose in *P. australis* have been determined as 1-10, < 0.1 and < 0.1 % dwt, respectively (Drew, 1983). Pirc (1989) has determined the soluble carbohydrates found in the Mediterranean species *Posidonia oceanica*, where 90 % total soluble carbohydrate was sucrose, with glucose, fructose and myo-inositol making up the balance. Other soluble carbohydrates found in seagrasses include apiose, fucose, galactose, mannose, rhamnose, xylose, mannitol and a range of inositol isomers (Drew, 1983; Pirc, 1989; Touchette & Burkholder, 2000). These studies have been limited in the range of carbohydrates determined and in their detection limits.

The aim of this component of this project was to determine if *P. coriacea* plants maintained in artificial conditions were unable to replenish their carbohydrate stores. The specific aims were to:

(i) identify the soluble carbohydrates in *P. coriacea* leaves and determine the soluble and insoluble carbohydrate concentrations;

(ii) compare the soluble and insoluble carbohydrates of *P. coriacea* leaves, when the seagrass is grown in tissue culture, aquaria and *in situ* environments; and

(iii) to analyse the carbohydrates of *P. coriacea* grown in each environment, from May to October 2002 and determine if there is a pattern in the leaf carbohydrate concentrations over this duration.

Note that while analysis of the rhizome and roots would provide a better indication of carbohydrate loss, their growth is slow and their size on the tissue culture plants was small, therefore it was not possible to analyse them for this project.
3.2 Materials and Methods

3.2.1 Reagents and Solvents

Details of reagents and solvents are in section 2.2.1. Additionally starch, perchloric acid, phenol, concentrated sulfuric acid, Murashige and Skoog Basal Medium (M+S), 3-(N-morpholino) propanesulfonic acid (MOPS), potassium hydroxide and 6-furfurylaminopurine (kinetin) were purchased from Sigma Aldrich Pty. Ltd. (NSW, Australia).

3.2.2 Preparation of Standards for GC analysis

Standard sugar-mixes were prepared in 80% ethanol. Standards contained sucrose, D-glucose, D-fructose, D-trehalose, myo-inositol, mannitol and sorbitol. Five standards prepared contained: 50, 200, 450, 600 and 850 µM L⁻¹ fructose; 50, 150, 250, 350 and 500 µM L⁻¹ glucose; 50, 250, 450, 650 and 850 µM L⁻¹ sucrose; 10, 40, 80, 120 and 160 µM L⁻¹ mannitol, sorbitol, inositol and trehalose; and 78 µM L⁻¹ phenyl-β-D-glycoside as the internal standard.

3.2.3 Plant Material

Fruits from P. coriacea were collected in mid-December 2001 from Parmelia Bank, Western Australia. Fruits were sterilized by rinsing in sterile DI water for 20 min, 20 minutes in 2 % benzalkonium chloride (in 10 % ethanol), followed by 3 rinses in sterile seawater. The seeds were then placed into 30 mL culture tubes, with 10 mL
media consisting of $\frac{1}{2}$ strength M+S, 30 g L$^{-1}$ Ocean Nature® seasalts, 5.0 µM kinetin, 20 g L$^{-1}$ sucrose and 10 mM L$^{-1}$ MOPS in MilliQ, adjusted to pH 7.0 using 2 M L$^{-1}$ KOH. Cultures were kept in a constant temperature room of 21 °C, with a light regime of 16 h light / 8 h dark and a light intensity of 250 µM sec$^{-1}$ m$^{-2}$ for four weeks before transferring to fresh tissue culture media or aquaria.

Seagrasses in tissue culture were sub-cultured to fresh media every four to six weeks. Seagrasses in aquaria were sub-cultured in June and September. During sub-culturing, dead leaf material was removed and green leaf blades were cut back to approximately 50 mm. Removed green leaf material was kept at -85 °C until analysed.

3.2.3.1 Tissue Culture Plants

Uncontaminated seeds were transferred into 100 mL fresh media consisting of $\frac{1}{2}$ strength M+S, 10 mM L$^{-1}$ MOPS, 58 mM L$^{-1}$ sucrose and 5.0 µM L$^{-1}$ kinetin, in either filtered seawater or synthetic seawater made from 30 g L$^{-1}$ Ocean Nature® sea salts in MilliQ. The pH was adjusted to pH 7.0 using 2 M L$^{-1}$ KOH prior to autoclaving. Eighty plants were kept in each type of media.

3.2.3.2 Aquaria Plants

A further 120 seagrasses, sterilized and free of contamination, were divided evenly among three aquaria (300 x 400 x 250 mm) and kept in 24 L seawater at 21 °C with a light regime of 16 h light / 8 h dark and a light intensity of 250 µM sec$^{-1}$ m$^{-2}$. The seawater was changed and algal growth was removed every two months.
3.2.3.3 Field Plants

Seagrass shoots were collected from Parmelia Bank at depths of 4 – 6 m in June, August, September and October. Leaf material was kept at -20 °C until analysed. Before analysis epiphytes were removed with a razor blade.

3.2.4 Extraction of Carbohydrates

For each month, three replicates of leaf material (100 mg), harvested from each environment, were ground in liquid nitrogen using a mortar and pestle.Soluble carbohydrates were extracted in 80 % ethanol at room temperature using the method described in section 2.2.3. The solid residue in the centrifuge tube was retained for starch extraction.

Starch extracts were prepared using a method adapted from Quarmby and Allen (1989). To the seagrass residue, remaining after the soluble carbohydrates had been extracted, 2.5 mL MilliQ water was added. Sealed samples were placed in a 80 °C agitating water bath for 15 minutes to gel the starch and then removed and cooled for 20 minutes. Rapidly, 2.5 mL 60 % perchloric acid was added and the solution was left to stand for 20 minutes to hydrolyse the starch. Samples were centrifuged for 10 minutes at 13,000 rpm. The supernatant was made up to 50.0 mL with MilliQ water. To prepare the stock starch standard, 100 mg of starch and 2.5 mL MilliQ water was placed into a 30 mL centrifuge tube and treated the same as the samples. After extraction, starch standards of 4, 20, 40, 60 and 80 mg L⁻¹ were prepared from the stock solution.
3.2.5 Soluble Carbohydrate Analysis

In a fume cupboard, 900 μL aliquots of seagrass sugar extract or standard were transferred to 2.0 mL autosampler vials and evaporated to dryness using an air stream. The residue was dissolved in 150 μL of pyridine and 150 μL of BSTFA. Vials were sealed, vortexed for 10 seconds and heated for 30 minutes at 60 °C before GC analysis. Analytical conditions were as described in section 2.2.5 using the BP-1 capillary column.

3.2.6 Starch Analysis

Starch analysis was performed using the phenol-sulfuric acid method as described in Buysse and Mercx (1993). In a fume cupboard, 1.0 mL of sample, standard or blank (MilliQ) solution was transferred to a test tube, to which 1.0 mL of 20 % (w/v) phenol solution was added. Immediately, 5.0 mL concentrated H₂SO₄ was added by directing the stream onto the liquid surface. The tubes were immediately vortexed and left to stand for at least 20 minutes. The absorbance was measured using a Shimadzu UV-1601 UV-visible spectrophotometer set at 490 nm.

3.2.7 Statistical Analysis

Data was analyzed using one-way ANOVA and the Schefjes Post-Hoc test with significance at p < 0.05.
3.3 Results

3.3.1 Seagrass Plant Material

Over the period of the experiment there was a decline in the number of plants alive in tissue culture (Figure 3.1). There was rapid die-off especially for the seagrasses kept in the artificial seawater, where the original 80 plants were reduced to 13 plants over five months. The decline was not as rapid for the plants kept in filtered seawater where only 7 plants died over six months.

![Graph showing survival of P. coriacea in two tissue-culture media.](image)

**Figure 3.1** Survival of *P. coriacea* in two tissue-culture media. (a) plants in seawater tissue cultures. (b) plants in artificial seawater tissue cultures. Media conditions: ½ strength M+S, 10 mM L⁻¹ MOPS, 58 mM L⁻¹ sucrose and 5.0 μM L⁻¹ kinetin, in either filtered seawater or synthetic seawater made from 30 g L⁻¹ Ocean Nature® sea salts in MilliQ, adjusted to pH 7.0 using 2 M L⁻¹ KOH.
The amount of leaf material removed from each plant also decreased, with all plants averaging < 20 mg of material available per month, therefore necessitating the pooling of plant material for the extraction of carbohydrates (Figure 3.2). The first month of sub-culturing was May 2002 and hence the amount of leaf material removed was that which had been growing since germination in December 2001. In the last month of sub-culturing the whole leaf was removed so as to accumulate the maximum amount of material, which is shown in the slight increase in the months of September and October for the artificial and filtered seawater cultures respectively in Figure 3.2.

Plants kept in the aquaria were generally unhealthy and were only sampled in the months of June and September. Collection of in situ seagrass material was only possible for the months of June, August, September and October. Estimation of
epiphyte coverage on in situ plants was 50 – 75% in June, 25 – 50% in August and 10 – 20% for September and October. The dry weight of in situ plants was determined to be approximately 50% of their wet weight.

3.3.2 Analysis of Soluble Carbohydrates

3.3.2.1 Identified Soluble Carbohydrates

Soluble carbohydrates identified in P. coriacea leaves were fructose, glucose, sucrose, trehalose, mannitol and myo-inositol. Those available in greatest quantities were fructose, glucose and sucrose, whereas only trace levels were found of trehalose, mannitol and myo-inositol. Mannitol was only detected in the in situ plants from Parmelia Bank for the month of June. Sorbitol was not detected in any samples.

The percentage soluble carbohydrate composition was generally higher in the tissue culture plant leaves than in those grown in aquaria or in situ (Figure 3.3a-e). In the tissue culture plants, fructose and glucose made up < 1.0 % fwt, sucrose was generally < 1.0 % fwt, trehalose < 0.1 % fwt and myo-inositol was < 0.05 % fwt. Plants from the aquaria maintained similar soluble carbohydrate levels to in situ plants, with fructose and glucose being < 0.1 % fwt, sucrose < 1.0 % fwt, trehalose < 0.05 % fwt and myo-inositol < 0.01 % fwt. Mannitol levels in the in situ plants from June were approximately 0.03 % fwt. No significant difference was found between the sugar concentrations from individual leaves of in situ plants (p = 0.99).

3.3.2.2 Variation with Time

Over the duration of the experiment there was significant variation (p < 0.05) in the soluble carbohydrate concentrations of the seawater tissue culture plants. There was
Figure 3.3a,b Variations in soluble carbohydrate concentrations for *P. coriacea* seedlings maintained in seawater tissue culture (TC-SW), artificial seawater tissue culture (TC-ASW), aquaria and grown *in situ*. (a) fructose. (b) glucose. (c) sucrose. (d) trehalose. (e) myo-inositol. 1.0 SE = (a) < 1.28, (b) < 0.90.
Figure 3.3c,d Variations in soluble carbohydrate concentrations for *P. coriacea* seedlings maintained in seawater tissue culture (TC-SW), artificial seawater tissue culture (TC-ASW), aquaria and grown *in situ*. (a) fructose. (b) glucose. (c) sucrose. (d) trehalose. (e) myo-inositol. 1.0 SE = (c) 0.04 - 1.18, (d) 0.01 - 0.14.
Figure 3.3e Variations in soluble carbohydrate concentrations for *P. coriacea* seedlings maintained in seawater tissue culture (TC-SW), artificial seawater tissue culture (TC-ASW), aquaria and grown *in situ*. (a) fructose. (b) glucose. (c) sucrose. (d) trehalose. (e) myo-inositol. 1.0 SE = (e) < 0.01.

significant variation in the fructose concentration, with the lowest concentration in July (0.8 mg g\(^{-1}\) fwt) and the highest concentration in August (9.5 mg g\(^{-1}\) fwt) (Figure 3.3a). Glucose levels remained slightly more stable, with no significant difference between the months of May/June and September/October, however, levels were significantly greater in August at 10.6 mg g\(^{-1}\) fwt (Figure 3.3b). Sucrose levels also remained fairly steady, however, there was a significant difference between the months of June/July and July/August (Figure 3.3c). Levels of sucrose were not significantly higher in August, as opposed to the other sugars, but they were significantly higher in September where levels were more than twice that of August or October (Figure 3.3c). Trehalose levels
were significantly different throughout the experiment (ANOVA, p = 0.004), however a post-hoc test was not performed as the results did not meet the requirements (Figure 3.3d). Levels of inositol were only significantly higher in August (0.46 mg g⁻¹ fwt) (Figure 3.3e).

In the artificial seawater tissue cultures there was a significant difference in the soluble carbohydrate concentrations for May, June and July. However, there was not enough plant material to measure the significance for August or September. Fructose and glucose levels showed the same pattern of significant changes, with high concentrations in May and low concentrations in June (Figure 3.3a,b). Sucrose and trehalose levels increased significantly in June (Figure 3.3c,d). However, while sucrose levels in May were significantly lower than in July, there was no significant difference in trehalose concentrations for these months (p = 0.531). Inositol concentrations only increased significantly in July to 0.21 mg g⁻¹ fwt from 0.17 mg g⁻¹ fwt (Figure 3.3e).

For the aquaria plants there was no significant difference in soluble carbohydrate levels between June and September, except in June where glucose had significantly higher levels (p = 0.004) (Figure 3.3b). The probability values for fructose, inositol, sucrose and trehalose were p = 0.328, 0.643, 0.386 and 0.081, respectively.

Soluble carbohydrates in the in situ plants all significantly differed over the study period except for fructose (p = 0.095). Statistical analysis of fructose was limited as it was not detected in leaves from September and only one of the three replicates from June had detectable levels (Figure 3.3a). Levels of glucose were significantly lower in June and September than in August and October (Figure 3.3b). Sucrose levels were significantly different for all months sampled (Figure 3.3c), whereas trehalose only significantly increased in August (Figure 3.3d). Inositol levels were only
significantly different between June and August, with low levels in June and high levels in August (Figure 3.3e).

3.3.2.3 Variation with Environment

Seagrasses in seawater tissue culture had significantly higher levels of all sugars (fructose, glucose, sucrose, trehalose myo-inositol) than in situ plants in August and September (3.3a-e). Fructose, glucose, trehalose and myo-inositol levels were significantly different in all treatments for June, but sucrose levels were not different for the tissue culture plants (Figure 3.2c). An ANOVA also showed that all sugars were significantly different in each treatment in September, however, there was not enough plant material to meet Post-Hoc test requirements to find where this significance lay. Fructose, glucose, inositol and sucrose levels did not differ significantly between the tissue culture treatments in May or July (Figure 3.3a,b,c,e).

3.3.3 Analysis of Starch

Standards prepared using the phenol-sulfuric acid method were found to be stable for at least 2 hours and even after 12 hours there was only slight variation (about 0.002 absorbance units, AU). Calibration lines for 4 – 80 mg L\(^{-1}\) were linear, giving an \(R^2\) value of 1.000, between 0.040 – 0.860 AU.

No significant difference (p > 0.05) was found between the concentrations of starch in individual leaves from in situ plants. Figure 3.4 shows the variation of starch over the duration of the experiment for each environment.
3.3.3.1 Variation with Time

Over the duration of the experiment, starch levels did not significantly change in the *in situ* plants (*p* = 0.113) or tissue cultured plants in artificial seawater (*p* = 0.984) (Figure 3.4). The data from the aquaria grown plants was not analysed, as only two months data was available. The seagrasses maintained in seawater tissue culture had significantly different starch levels over the experiments duration. The subsets formed by the Scheffe Post Hoc test for seawater tissue culture plants were low starch levels in May/June/July/October, moderate starch levels in June/July/August/October and high starch levels in August/September (Figure 3.4).

![Graph showing starch concentration variations](image)

**Figure 3.4** Variations in starch concentration for *P. coriacea* seedlings maintained in seawater tissue culture (TC-SW), artificial seawater tissue culture (TC-ASW), aquaria and grown *in situ.*
3.3.3.2 Variation with Environment

For the months of May, July, August and October, there was no significant difference between the starch concentrations of plants from each environment (Figure 3.4). There were significant differences, however, for June (p = 0.041), August (p = 0.101) and September (p = 0.230). In June, the in situ plants had significantly higher starch levels than the seawater tissue culture plants (p = 0.045). Due to a lack of plant material, post-hoc tests were unable to be performed on the months of August or September.

3.4 Discussion

The type and levels of soluble carbohydrates found in in situ P. coriacea were similar to that found by Drew (1983) for P. australis. Levels of sucrose, glucose and fructose in P. australis were 1-10 %, < 0.1 % and < 0.1 % dwt respectively. This study found levels of sucrose, glucose and fructose in in situ P. coriacea to be 0.1 - 0.5, < 0.1 and < 0.1 % fwt respectively, with water content being approximately 50 %. Additionally, this study also found low levels of trehalose, myo-inositol and mannitol at concentrations approximately < 0.5 % fwt respectively.

P. coriacea in tissue culture contained significantly higher levels of soluble carbohydrates than found in aquaria or in situ. This difference may be due to the increased regularity of the light periods and the light intensity, however, the aquaria plants were also exposed to the same light conditions. The difference between the aquaria and tissue culture media were the inclusion of micro- and macro-nutrients and most importantly, sucrose into the tissue culture media. It is likely that the addition of
extra nutrients and particularly sucrose in tissue culture media elevated levels of soluble carbohydrates in the leaf tissue.

The age of the *in situ* plants was unknown, but they were likely to be at least one year old. The lower levels of soluble carbohydrates found in the *in situ* plants may therefore be somewhat related to plant age, as older plants have lower carbohydrate levels (Pirc, 1985). It may also be related to decreased photosynthesis rates, as plants were collected over the winter months where increased turbidity, lower temperatures and cloud cover prevent maximum photosynthetic rates. Additionally, *in situ* plants were covered with epiphytes, with 50 – 75 % epiphyte coverage in June, which decreased to 10 – 20 % by September.

Alcoverro *et al.* (1999) mentions that under stress, seagrasses will preferentially mobilize stored carbohydrates to the leaf tissue to be utilized in shoot growth, which can be measured by increased carbohydrate levels in the leaves. This study found the carbohydrates of seawater tissue culture plants, which did not die off quickly, did not vary significantly from the artificial seawater plants that did die quickly (Figure 3.1). The effect mentioned by Alcoverro *et al.* (1999) was therefore not seen.

The significant variation in the soluble carbohydrate concentrations of tissue culture plants is not well understood due to a lack of knowledge on their use in seagrasses. The increase of fructose and glucose concentrations in August may be a genetic response to mobilise stored sugars for the initiation of shoot growth, which *in situ* plants may then have exploited for increasing photosynthesis rates before flower onset in September. While this response was not seen as clearly in the *in situ* plants, this may be due to the lower availability of intercellular carbohydrates and seawater C\textsubscript{i}. However, there was no concurrent increase of shoot growth of tissue culture plants in August (Figure 3.2). September then saw the decrease of fructose and glucose and an
increase of sucrose concentrations. Should there be some truth in the above hypothesis, perhaps a genetic response may also initiate an increased uptake of $\mathrm{C}_i$ for flower initiation.

In addition to fructose, glucose, sucrose and myo-inositol, the soluble carbohydrates found in $P. \text{oceanica}$ (Pirc, 1989), trehalose and mannitol were also identified. Trehalose was found in most of the seagrass extracts at trace levels. Mannitol, however, was only found in trace levels in the extracts from the in situ plants in June. Mannitol is believed to be an osmo-regulator, with increased levels in water stressed plants (Lewis & Smith, 1967). In higher plants mannitol is produced from glucose and is easily transported through vaso-structures to be utilised as carbohydrate reserves or for osmoregulation (Lewis & Smith, 1967). The presence of mannitol in June in situ plants may be due to the high epiphyte coverage and competition for light in that month. No mannitol was detected in the other months studied (detection limit = 8.6 ng/2 µL) and in these months the epiphyte coverage was decreasing. While epiphyte survival has been found to decrease with a decrease in light availability, $P. \text{coriacea}$ may be able to utilize this light more efficiently, therefore negating the need for mannitol.

Starch levels in $P. \text{coriacea}$ were approximately 1 - 8 % fwt. Starch levels in $P. \text{oceanica}$ were found to be about 1 % dwt (Pirc, 1989). Over the duration of this experiment the starch levels varied significantly in $P. \text{coriacea}$ grown in seawater tissue culture but not in the aquaria or in situ plants.

Starch is one of the major storage carbohydrates in seagrasses, along with sucrose (Touchette & Burkholder, 2000). When mobilized, it is broken down into its glucose units to be used for various activities, including formation of cell walls, proteins and tannins. While variation of starch levels was significant for the seawater tissue
cultures (ANOVA, p < 0.05), there was no general pattern to suggest that any of the treatments were responding to environmental factors, such as light availability, or physical factors, such as seed starch reserves.
CHAPTER 4: Concluding Remarks

This study was not able to provide evidence to suggest that \textit{P. coriacea} in tissue culture is unable to replenish carbohydrate reserves, however, they do contain significantly greater amounts of soluble carbohydrates than plants growing \textit{in situ}. Elevated production of soluble carbohydrates in tissue-cultured plants is believed to be related to the high concentration of sucrose provided in the media and the favourable conditions provided for growth. Further studies to elucidate this may involve using tissue culture or aquaria with/without additions of sucrose, or differing sucrose concentrations.

In addition to the soluble carbohydrates found in \textit{P. oceanica}, that is sucrose, glucose, fructose and \textit{myo}-inositol, this study also found trace levels of trehalose and mannitol in \textit{P. coriacea}. Mannitol, however, was only found in the \textit{in situ} plants from June and may be a response to stress. As there is greater availability of \textit{in situ} material, a greater sample mass (mass > 0.100g) could be used to see if mannitol is present in trace levels year round or only at particular times where the plant is under stress.

The significantly higher levels of sucrose in September and glucose and fructose in August, in the tissue culture plants is not understood. However, should it be a genetic response to prepare the plant for flowering in September, the increased sugar levels may be related to increases in the levels of enzyme used to synthesise these sugars. Enzyme levels could therefore be analysed in a future study.

Soluble carbohydrates in \textit{P. coriacea} were determined using a simple, yet robust GC-FID method developed in Chapter 2. This method used minimal volumes of solvent, maximising the potential to identify and quantify carbohydrates in samples when only small amounts of plant tissue is available. Preparation of extracts for GC
analysis was relatively quick, uncomplicated and quantitative. This method also provided low detection limits and highly reproducible retention times for soluble carbohydrates.
References


