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Pectic Methyl and Non-Methyl Esters and the Environmental Implications of Methanol Emissions from Plants

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Thesis submitted for the degree of Doctor of Philosophy

September 2007

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Abstract

Pectin methyl esterase (PME) enzymes are produced by bacteria, fungi and higher plants, and hydrolyse methyl ester groups present on the backbone of pectic polysaccharides found in the primary cell walls of plants.

Pectic polysaccharides are required for intercellular adhesion in dicotyledonous plants. To fulfil this function, they need to be cross-linked, covalently or non-covalently. The texture of plant-based foods is affected by the location and extent of these linkages, ultimately determined by chemical and subsequent structural modifications undergone by the methyl ester groups on the pectic polysaccharide backbone during growth, ripening, storage, cooking and processing. These processes result in spatial variations in cell separation and adhesion between the walls of adjacent cells. These modifications are due to the action of PME enzymes. It has been suggested that covalent intercellular linkages are formed by glycosidic bonds between xyloglucan and acidic pectins. However, the presence of non-methyl esters has been confirmed and these linkages are candidates for the role of intercellular adhesion. The correlation between the quantity of these non-methyl ester groups and the deterioration of potato tuber texture during the period of storage following harvest was explored in two potato cultivars using titrimetric techniques and atomic absorption spectrometry.

Pectic methyl ester groups in plant material are a major reservoir of methanol in the biosphere. PME enzymes are responsible for the cleavage of the ester bond between these methyl groups and the pectic polysaccharide backbone, resulting in the release of methanol and acidic pectin. Methanol from both anthropogenic and biogenic sources is an important precursor of the gaseous pollutant, tropospheric ozone. Thus the accurate quantification of methanol emitted to the troposphere from both growing and decaying plant material is essential as the global balance of gases in the Earth's atmosphere is continuously skewed as a result of anthropogenic activities. Particular principles and techniques used in the quantification of methyl ester groups in potato tubers were developed and utilised in order to quantify the amount of methanol contained in leaves from tree species native to Great Britain, in the form of pectic methyl esters, that could potentially be emitted to the troposphere as a consequence of the senescence of these leaves.

The potential impact on the quantity of methanol in the troposphere of the growth of certain crop species grown commercially in the UK was investigated. A modified bell jar headspace capture and purge method and thermal desorption technique, in conjunction

with gas chromatographic (GC) quantification, were developed and used to quantify methanol emitted from the developing tissues of plant species with significant agronomical importance, both in the UK and globally.

Results obtained from the determination of pectic methyl esters contained in mature leaves of tree species, native to Great Britain and growing in sites in Scotland, were extrapolated using available leaf litterfall and leaf area index (LAI) data to account for total area of trees of the same species growing across Great Britain and the globe. The results obtained following the quantification of methanol emitted from growing plant species of agricultural significance were extrapolated to ultimately estimate the quantity of methanol being emitted from these plant species on a global scale annually.

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Acknowledgements

My thanks firstly to my supervisor, Dr. Mike Jarvis, whose seemingly boundless breadth and depth of knowledge has been a constant source of inspiration. Thanks also for the welcome distraction of Wildlife Explorers and tales of all creatures great and small.

Thanks to all the staff and students in the Environmental Chemistry Section for the advice, laughs and occasional mountain expeditions. Thank you in particular to Michael Beglan, for invaluable help with the GC and unrivalled leaf-collecting skills.

Finally, thanks to all the special people in my life.

Author's Declaration

This thesis and the research described here are performed entirely by the author except where expressly stated.

Christine Jane Finlay

Chapter One - Introduction

1.1 Pectin Methylesterase Enzymes

The cell wall in plants comprises several intricate, structurally independent networks (Section 1.3), each dedicated to specific functions and subjected to various structural changes as the cell wall initially forms, matures and potentially decomposes. In the case of the pectic fraction of the cell wall, several families of enzymes are responsible for the observed alterations that pectic polysaccharides undergo as the cell wall develops. These enzymes, collectively known as pectinolytic enzymes or pectinases, are present in higher plants and in many microorganisms and include protopectinases, polygalacturonases, lyases and pectin esterases. The most abundant of these pectinases are the pectinesterase enzymes, sometimes referred to as pectin methyl esterases (PME's), which liberate acidic pectins and methanol by catalysing the de-esterification of the methyl ester groups present on the homogalacturonan pectin backbone (Figure 1.15).

Pectin methyl esterase (PME) enzymes allow plant cell walls to extend whilst maintaining their structural integrity and are subsequently responsible for the softening of some plant tissues during ripening and storage. Endogenous PME activity is also considered to be responsible for the abscission and senescence of plant material. Saprophytic organisms, particularly fungi, produce PME's that are responsible for a large proportion of the decomposition of plant material in the natural environment, contributing to the global balance of nutrients.

The mode of action of the PME and the subsequent pattern of methyl esterification in the pectin backbone of plant cell walls depends, to some extent, on the origin of the PME involved in catalysing the demethylation reactions, together with prevailing chemical conditions within the cell wall. Demethylesterification of pectic polysaccharides in a random fashion results in the release of protons that promote the action on endopolygalacturonase enzymes (Moustacas et al., 1991) which subsequently result in cell wall loosening. PME's that remove methyl groups in a block-wise pattern allow the cross-linking of divalent cations, particularly Ca^{2+}, between two adjacent free carboxyl groups

(Figure 1.14). This cross-linking results in a stable framework, contributing to the stiffening of the cell wall (Goldberg et al., 1996).

Initial hypotheses suggested that PME's originating from fungi, thought to be predominantly acidic PME's, resulted in a random pattern of demethylation. Alkaline PME's were thought to be responsible for the removal of methyl groups in a linear fashion. However, it has recently been demonstrated that the pattern of PME activity depends also on the pH of the cell wall and the initial degree of esterification of the pectic polysaccarides (Catoire et al., 1998; Denes et al., 2000).

1.2 Emissions of Volatile Organic Compounds from Plants

1.2.1 Introduction

Over the past 15 to 20 years it has been widely recognised that an extensive variety of volatile organic compounds (VOC's), including isoprene, terpenoid compounds, alkanes, alkenes, alcohols, esters and acids (Peñuelas and Llusià, 2001), are emitted to the atmosphere, not only from the fruit (Nursten, 1970) and seeds (Fisher et al., 1979) but also from the leaves (MacDonald and Fall, 1993; Nemecek-Marshall et al., 1995; Kirstine et al., 1998) of terrestrial flowering plants. The complex nature of the pathways via which these compounds are biosynthesised, controlled and emitted, together with their unstable chemical nature has, until recently, hampered research attempts aimed at identifying and quantifying these biogenic VOC's, or BVOC's. The release of BVOC's varies greatly, not only between plant species, but also depending on numerous spatial and temporal factors. This variability results from a complex network of interactions that may occur between the plant and the atmosphere or between the plant and other organisms. For example, VOC emissions have been shown to vary depending on factors such as injury, herbivorous predation, temperature, light and developmental stage (Monson et al., 1995; Lerdau and Keller, 1997).

Isoprene and terpenoid compounds, collectively known as isoprenoids, are produced in the melavonic acid pathway, constitute over 50% of the total volatile organic compounds emitted by plants (Guenther et al., 1995; Kesselmeier and Staudt, 1999) and have, as a result, been extensively studied.

1.2.2 Terpenoid Compounds

Terpenoid compounds constitute an enormously varied group of plant hydrocarbons, all derived from 5-carbon isoprene (C_5H_8) subunits (Figure 1.2) (McGarvey and Croteau, 1995). Approximately 5000 terpenoid compounds have been identified and structurally characterised to date. These include monoterpenes (C_{10}) (Figure 1.1), sesquiterpenes (C_{15}), diterpenes (C_{20}) and higher molecular weight species. Of these, only monoterpenes are sufficiently volatile to play an active role in plant-atmosphere interactions. They are chemically highly reactive and subsequently have a chemical lifetime of a maximum of only a few hours (Kesselmeier and Straudt, 1999).

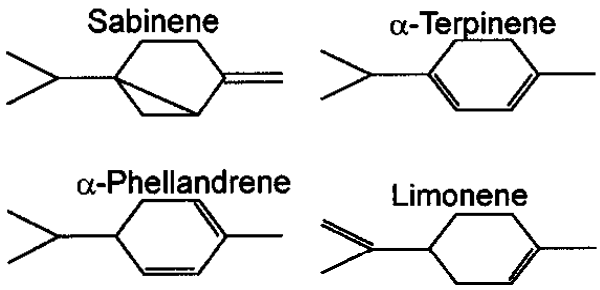


Figure 1.1 – The molecular structure of some common monoterpene compounds (C10)

The roles of terpenoid compounds vary enormously from hormonal roles to photosynthetic pigments, from attracting pollinating insects and repelling herbivorous invertebrates to enzyme regulation. Terpenoids are of commercial significance as they form naturally occurring essential oils, waxes and resins in large quantities that may be exploited (Dawson, 1994). It is generally accepted that monoterpenes and sesquiterpenes are synthesized in the plastids and cytosol respectively (Gleizes et al., 1983; McCaskill and Croteau, 1995) via either the mevalonic acid pathway (Spurgeon and Porter, 1981; Sharkey et al., 1991) or the novel melavonate-independent, or Rohmer pathway (Flesch and Rohmer, 1988; Rohmer et al., 1993).

Because monoterpenes are always present in leaves, their emission rates are thought to be dependent predominantly on ambient atmospheric temperatures. In certain regions, for example those extensively occupied by coniferous or non-isoprene emitting tree species, the emission of terpenoid compounds may be much greater than that of any other biogenic VOC (Geron et al., 2002).

1.2.3 Isoprene

Isoprene (C_5H_8), or 2-methyl-1,3-butadiene (Figure 1.2), is responsible for approximately 30% of the total biogenic VOC's emitted globally (Kesselmeier and Straudt, 1999). Much less is known about the biosynthesis and emission of isoprene than of other terpenoid compounds as its concentration is very low in the natural environment, generally <10ppb (Jacob and Wofsy, 1988). This is predominantly due to isoprene's high reactivity and subsequent very short atmospheric lifetime of only a few hours. Isoprene emissions from plants to the atmosphere have, however been estimated to be one of the most important biosphere-atmosphere interactions, with approximately 500 Tg yr^{-1} being emitted from plants globally (Wiedinmyer et al., 2006). It is known that isoprene is formed enzymically through the elimination of diphosphate and proton abstraction from dimethylallyl diphosphate (DMAPP) (Silver and Fall, 1991). Mounting evidence has subsequently led to the conclusion that isoprene synthesis is situated in chloroplasts, and that the reactions of isoprene synthesis are possibly compartmentalised and confined to the thylakoid membrane regions where the isoprene synthase proteins are embedded (Kuzma and Fall, 1993, 1994).

The precise function of isoprene in plants is as yet unknown, although it is speculated that the compound is responsible for thermotolerance in some plant species (Sharkey and Yeh, 2001). Some research has hypothesised that isoprene acts as an antioxidant in plants due to its very rapid reaction with ozone and hydroxyl radicals (Penuelas et al., 2005). It has however, been proven that isoprene emission is a sensitive indicator of electrical-based signals following the wounding of a plant (Loreto and Sharkey, 1993). Burning had the most profound effect on the emission rate of isoprene, with a reduction in isoprene emissions from a terminal leaflet of up to 75% after burning a lateral leaflet with a match (Loreto and Sharkey, 1993). Despite these findings, it has generally been found that isoprene emissions to the atmosphere are either relatively unchanged or greatly enhanced by the puncturing, smashing, cutting or burning of foliage (Monson et al., 1994; Loreto et al., 2006).

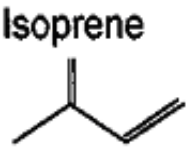


Figure 1.2 – The chemical structure of isoprene

Approximately 40% of the biogenic VOC's released globally are made up of non-terpenoid compounds such as hexene derivatives and methanol.

1.2.4 Methanol

1.2.4.1 Introduction

Methanol, or methyl alcohol, is a clear, colourless, volatile flammable liquid with mild alcoholic odour when pure. Commercial manufacturing processes involve the reaction of hydrogen with carbon monoxide or carbon dioxide at high temperature over a catalyst or by oxidation of hydrocarbons (Windholz et al., 1983). The term 'wood alcohol' is also used to refer to methanol and originates from the substance being produced from the destructive distillation of wood.

Methanol is toxic to mammals via breakdown by alcohol dehydrogenase enzyme in the liver to form formic acid and formaldehyde, both of which cause blindness through destruction of the optic nerve (Kavet and Nauss, 1990). However, methanol occurs naturally in humans, animals and plants (Axelrod and Daly, 1965) and has been found in blood, urine and saliva (Leaf and Zatman, 1952) as well as in expired air (Jones, 1990). It has been suggested that methanol is produced in the human intestine by enzymic reactions or possibly microflora (Axelrod and Daly, 1965). Protein carboxylmethylase enzyme, which methylates the carboxyl groups of proteins, is thought to be involved in methanol production in the digestive system (Kim, 1973). The chemical structure of methanol is shown in Figure 1.3.

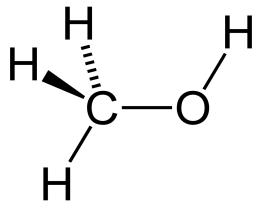


Figure 1.3 – The chemical structure of methanol

1.2.4.2 Sources of Atmospheric Methanol

Methanol, a biogeochemically active volatile organic compound (VOC) is, together with methane and isoprene, one of the most significant organic compounds found in the earth's atmosphere. The atmospheric lifetime of methanol, limited by the reaction with gaseous

hydroxyl radicals (HO), is approximately 19 days (Bey et al., 2001). In the free troposphere, the estimated lifetime of isolated methanol can vary between 69 hours (in the presence of daylight) and over a thousand hours (without daylight) (Jacob et al., 1989). Thus, under certain environmental conditions, methanol can be distributed globally from its source (Singh et al., 2001). Comparing these figures with those for the lifetime of isoprene, which reacts rapidly with HO and is removed from the atmosphere in only a few hours (Jacob et al., 1989), it is apparent that methanol is an extremely important organic constituent of the troposphere.

Methanol is introduced to the atmosphere via a great number of anthropogenic and biogenic processes. Anthropogenic activities resulting in methanol production include biomass burning, where the pyrolysis of methyl and methoxy groups found in lignin and hemicellulose is believed to be the methanol source (McKenzie et al., 1995). The biological decomposition of biological waste, sewage and sludges is also a source of methanol in the troposphere (Nielsen et al., 1993).

Global industrial methanol production in 1996 was estimated to be 24.3 Tg y^{-1}, with this figure increasing to approximately 27 Tg y^{-1} in 2000 (Crocco 1997). Methanol is used extensively in the manufacture of chemicals, for example paints, solvents, varnishes, paint thinners and certain anti-freeze solutions. Due to its relatively high flammability, methanol has been used as a fuel in open wheel racing cars and model engines. As alternatives to oil-based fuels are sought, the concentration of methanol in the atmosphere is likely to increase considerably. Demand for biodiesel is forecast to increase by 32% per year, rising from 30 million gallons in 2004, to 150 million gallons by 2008, and 350 million gallons by 2013. The main transesterification reaction for converting oil to biodiesel involves the reaction of methanol with the triglyceride oils contained in vegetable oils, animal fats, or recycled greases, forming fatty acid alkyl esters, or biodiesel, and glycerol.

Sources of naturally occurring methanol, or biogenic methanol, in the troposphere include volcanic gases, respiration of anaerobic microorganisms and insects (Holzer et al., 1977), and emissions from plant material (e.g. Owens et al., 1969; MacDonald and Fall, 1993). Table 1.1 lists some of the major sources of methanol in the troposphere, together with current estimates for the quantities of methanol contributed by each source (Galbally and Kirstine, 2000).

Methanol Source	Contribution from the Southern Hemisphere (Tg yr-1)	Contribution from the Northern Hemisphere (Tg yr-1)	Global Methanol Contribution (Tg yr-1)
Higher Plants	38	62	100 (37-212)
Biomass Burning	5	8	13 (6-19)
Atmospheric production	9	10	19 (14-24)
Anthropogenic	1	3	4 (3-5)
Decay of dead plant material	5	8	13 (5-31)
Ocean sources	-	-	<0.1
Total	58	91	149 (83-260)

Table 1.1 – Current estimates for the quantities of methanol contributed to the global troposphere by various sources (from Galbally and Kirstine, 2000)

Despite the fact that methanol, to some extent, is present in the troposphere as a result of certain anthropogenic activities, the data in Table 1.1 highlights that the vast majority of the methanol present in the troposphere originates from higher plants.

1.2.4.3 The History of the Quantification of Methanol Emissions from Plants

In 1955, Went first suggested that VOC's may be emitted from vegetation (Went, 1955). Isoprene emissions from plants were also detected in the 1950's by Sanadze and Kursunov and subsequent studies showed that these emissions were highly dependent on temperature and light (Sanadze and Kursunov, 1966; Sanadze and Kalandaze, 1966; Sandze, 1969). Rasmussen and Went independently identified isoprene emissions from plants (Rasmussen and Went, 1965) and drew attention to the importance of these emissions in the chemistry of the lower atmosphere. At the time, the notion that plants emitted these compounds was widely rejected, however Sanadze and Rasmussen both independently identified isoprene with the use of mass spectrometry (Sanadze,1969; Rasmussen, 1970). Methanol was initially identified as one of the volatile components emitted by plants by Owens and colleagues (1969), who studied emissions from alfalfa.

The sources, nature and quantities of volatile emissions from plants were subsequently investigated and it became well established that forest trees are a major contributor to tropospheric hydrocarbon totals (Rasmussen, 1972; Zimmerman, 1979; Evans et al., 1982). The major VOC species emitted by forest plants are isoprene and a small number of monoterpene compounds, which have, as such been thoroughly investigated (Zimmerman et al., 1978; Isidorov et al., 1985; Lamb et al., 1985; Khalil and Rasmussen, 1992). However, Zimmerman (1979) suggested that perhaps 30% of the total VOC's emitted from plants may be comprised of compounds other than isoprene and monoterpenes. Isidorov et

al. (1985) subsequently identified methanol emissions of evergreen cypress in the forests of Northern Europe and Asia.

Snider and Dawson (1985) carried out pioneering work investigating the concentration of atmospheric methanol in both urban and rural areas in Arizona. Mean measured concentrations were 7.9 ppb and 2.6 ppb respectively. The concentrations of methanol found in rural environments were such that they could not be accounted for purely as a result of translocation from urban environments. Following this work, several groups have carried out research in order to determine near-surface methanol concentrations in areas dedicated to various land uses (Table 1.2). Typical values for rural forested areas in Alabama vary between 11 ppb and 6 ppb during the day and night time respectively (Goldan et al., 1995a). Typical values for methanol concentrations in urban areas, for example Boston, Massachusetts and Houston, Texas, were 17.4 and 16.6 ppb respectively (Kelly et al., 1993).

Despite the fact that the majority of the research carried out on emissions from plants has concentrated on isoprene and monoterpenes, all plant species that have thus far been examined for volatile emissions have been found to emit significant quantities of methanol, especially when their leaves are rapidly expanding (Nemecek-Marshall et al., 1995). In fact, growing trees of certain species have been found to release methanol at rates that range in the same order of magnitude as isoprene and monterpenes. Typical leaf emission rates of monoterpene and isoprene have been demonstrated as 1.6 μg C h^{-1} gdw^{-1} and 35 μg C h^{-1} gdw^{-1} respectively (Guenther et al., 1994), whereas emissions of methanol were detected at rates between 0.6 and 17 μg C h^{-1} gdw^{-1} (MacDonald and Fall, 1993).

Area of Measurement/Estimate	Mean Value (ppb)	Range (ppb)	Reference
Remote Ocean (Pacific and N. Atlantic)	0.9	0.2-1.2	Singh et al., 2000, 2001
Tropical Rain Forest (Surinam)	1.1	N/A	Williams et al., 2001
Urban (Colorado)	12.0	1.0-17.0	Goldan et al., 1995
Rural Forest Day (Alabama)	11.0	1.0-20.0	Goldan et al., 1995
Rural Forest Night (Alabama)	6.0	1.0-20.0	Goldan et al., 1995
Urban (Boston, Mass.)	17.4	7.2-47	Kelly et al., 1993
Urban (Houston, Tex.)	16.6	5.6-31	Kelly et al., 1993
Rural (Arizona)	2.6	1.5-3.7	Snider and Dawson, 1985
Urban (Arizona)	7.9	5.3-10.5	Snider and Dawson, 1985
Mountain Background (Colorado)	2.0	2.0-4.0	Goldan et al., 1997

Table 1.2 – Atmospheric methanol observations (adapted from Heikes et al., 2002)

Due to the magnitude of biogenic methanol emissions and their implications on the global atmosphere (Section 1.4.6), recent attempts have been made to construct models of sources and sinks of methanol on a global scale. Heikes et al. (2002) considered global sinks of methanol, including gas and aqueous phase reactions with HO radicals, and sources, including fossil fuel combustion, biomass burning and emissions from growing terrestrial plants, in an attempt to estimate the balance of methanol in the atmosphere. From their estimates, it was concluded that the atmosphere contains approximately 4 Tg methanol and that global sources and sinks of methanol total about 340 and 270 Tg yr^{-1} respectively. Biogenic methanol emissions were estimated at over 280 Tg yr-1 based on net flux observations.

More recently, Jacob and colleagues (Jacob et al., 2005) have developed a three-dimensional model simulation of atmospheric methanol in order to consider carefully the consistency between current concentrations of atmospheric methanol and recent understanding of sources and sinks. Sources including plant growth and decay, biomass burning and anthropogenic sources were considered, together with the predominant sink being gas-phase oxidation by HO radicals. The best estimate of the global source of methanol was suggested as being about 240 Tg yr-1. However, many unknowns still exist as regards the quantities of methanol introduced to the troposphere from a variety of sources.

Despite recent efforts to construct inventories concerning sources and sinks of methanol in the global environment, many gaps still remain in our understanding of the complex processes involved. If the potential impact of methanol emissions on global tropospheric chemistry is to be fully understood, these parameters must be more accurately quantified.

1.2.4.4 The Pathway for Methanol Release from Plants

The variation between measured methanol concentrations in a forest canopy during the day compared to that at night (Goldan et al., 1995a) suggests that methanol release from growing plants is strongly correlated to photosynthetic activity, hence the day and night variation. However, despite the fact that methanol emission rates have been shown to increase with increasing light intensity, the phenomenon has been attributed to the effect of light on stomatal conductance rather than a direct effect on methanol production (Nemecek-Marshall et al., 1995). Stomatal conductance is defined as a numerical measure of the rate of passage of either water vapour, carbon dioxide or other gaseous substances through the stomata (Körner et al.,1979).

Figure 1.4 (from MacDonald and Fall, 1993) illustrates clearly the relationship between stomatal conductance and methanol emission rate from a bush bean leaf left in the dark. The changes in methanol emission rate occurred concurrently with changes in stomatal opening and closing, and independently of photosynthesis.

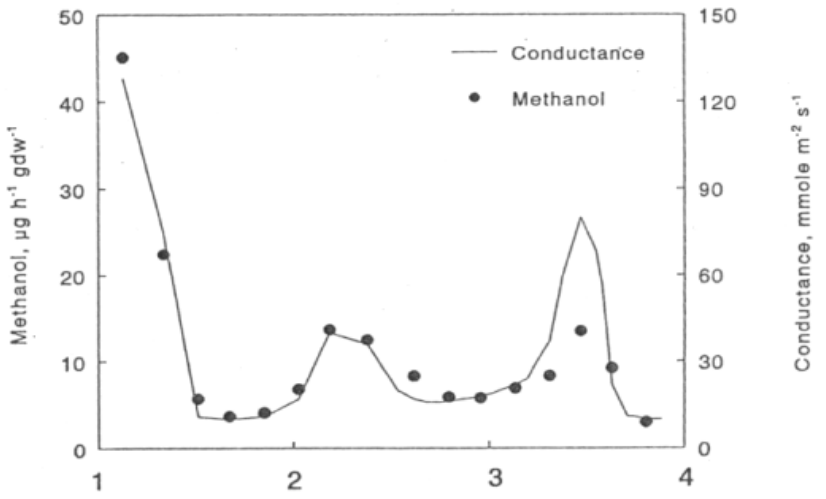


Figure 1.4 - Relationship between stomatal conductance and methanol emission rate for a bush bean leaf in the dark (MacDonald and Fall, 1993)

Similar conclusions were reached by Nemecek-Marshall et al. (1995), who found that methanol emissions were evident predominantly from leaf surfaces containing stomatal openings, demonstrating that methanol is almost exclusively emitted from stomata and not from cuticular surfaces (Nemecek-Marshall et al., 1995). Subsequent work by Hüve et al. (2007), studying the relationships between plant growth rate, stomatal conductance and methanol emission rates, likewise found that the greatest rates of methanol emission from leaves of cotton plants (*Gossypium hirsutum*) occurred first thing in the morning, coinciding with the light-dependent increase in stomatal conductance.

The mechanism involved in the production of methanol in plants is not known, but it has been suggested that the methanol is released when, during cell wall expansion, pectic polysaccharides located within the cell wall are demethylated enzymically.

The following sections will detail how, through certain developmental physiological processes which take place within the plant, methanol may be liberated and emitted from plant tissues. Previous reviews on the topic of links between emissions from plants and tropospheric methanol have failed to incorporate detailed analysis of the literature available on this topic.

1.3 The Cell Wall

The plant cell wall is a highly organised array composed of a wide variety of complex polymers, including polysaccharides, glycoproteins, polyesters and lignin, which surround the protoplast as an extracellular matrix.

The primary cell wall consists, more specifically of three structurally independent, but interacting networks. The fundamental cellulose microfibril network interlaced with xylogucan polymers is embedded in an independent network of matrix polysaccharides (Carpita and Gibeaut, 1993; McCann and Roberts, 1991). The third network consists of structural proteins in primary (growing) cell walls or a phenylproanoid network in secondary (woody) cell wall layers.

Molecules of the cell wall must provide shape, mechanical strength and extensibility, regulate porosity and control cell-cell adhesion. The plant cell wall is a source of signaling and recognition molecules that elicit the appropriate response to extracellular influences, such as pathogen invasion. The protection of the cell depends on the ability of the cell wall to allow the penetration of small molecules such as nutrients, into the cell, while simultaneously preventing predatory microorganisms from doing the same. The complexity of plant cell wall architecture provides an insight into the critical nature of the many functions, both mechanical and biological, performed by individual components of the wall.

1.3.1 Cell Wall Structure

It has been found that two distinct types of primary cell walls exist in flowering plants, known as ‘type I’ and ‘type II’ cell walls (Carpita and Gibeaut, 1993). The type II cell wall is peculiar to Poaceae and closely related monocot families, and differs from the type I walls in many ways, including the manner in which the cellulose microfibrils are interlocked within the cell wall matrix. Type II cell walls also contain considerably less pectin, and phenolic esters are present to a much greater extent than in type I walls (Carpita and Gibeaut, 1993). Type I cell walls (Figure 1.6) are by far the most abundant type, occurring in all Dicotyledonae and gymnosperms and some Monocotyledonae that have been studied.

It is worth bearing in mind that the proportions and structure of various constituents of a plant cell wall depend, to some extent, on the development, age and type of the wall. The

components are subject to many structural and chemical modifications between gene expression and cell wall assembly in the apoplast.

Many of the polysaccharide and protein constituents of cell walls have been characterised in recent years using a variety of methods including: affinity methods, for example antigen-antibody (Knox et al., 1990) and enzyme-substrate (Vian, 1985), polarised infra-red spectroscopy (Morikawa and Senda, 1978; Morikawa et al., 1978) and Fourier transform infra-red microspectroscopy (FT-IR) (McCann et al, 1992). However, initial structural characterisation of most cell wall polymers was carried out using degradative wet chemical methods, for example methylation analyses in the case of polysaccharides (Haworth and Hirst, 1933) and nitrobenzene oxidation (e.g. Leopold B. 1951) and thioacidolysis for lignin (e.g. Lapierre C. et al., 1986).

Hypotheses have since been proposed in an attempt to explain how these polymers are assembled in such a way as to enable the structure to perform its mechanical roles, such as providing extensibility to the cell. Cleland (1981) suggested that transglycosylation or hydrolysis enabled the microfibrils of the wall to 'slip', thus allowing expansion of the wall (Cleland, 1981). Albersheim and his colleagues proposed a similar structural model based upon polysaccharide and protein components, characterised by themselves and others (Keegstra et al., 1973; Talmadge et al., 1973).

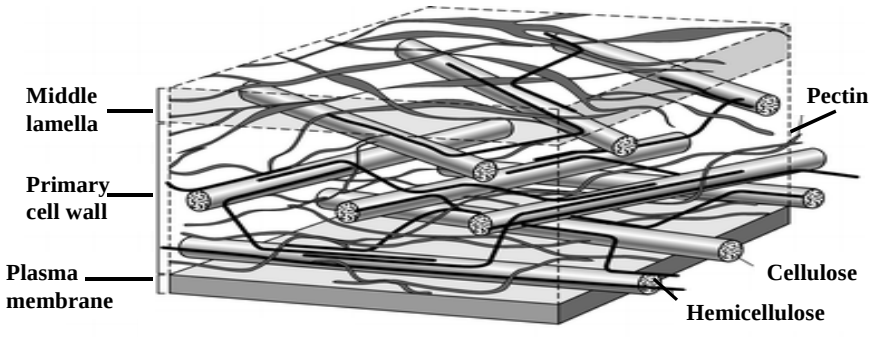


Figure 1.5 - Model of the primary cell wall of onion as propsed by McCann and Roberts (1991)

McCann and Roberts proposed a model of the primary cell wall of onion (McCann and Robert, 1991) (Figure 1.5). Subsequently, Carpita and Gibeaut (1993) proposed the model of the expanding type I primary cell wall that is shown in Figure 1.6.

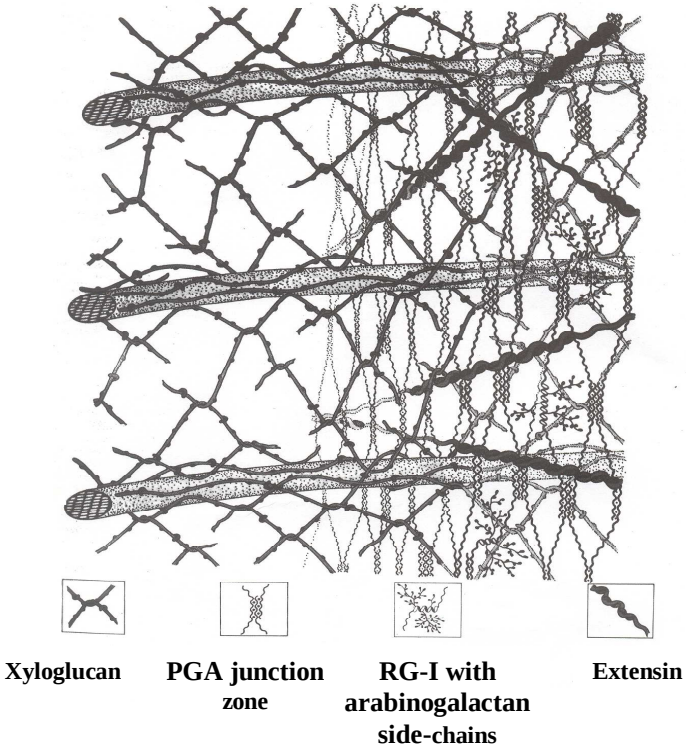


Figure 1.6 - Model of an expanding Type I plant cell wall as proposed by Carpita and Gibeaut (1993)

1.3.2 The Type-I Cell Wall

The type I cell wall is essentially a network of cellulose microfibrils, consisting of several dozen linear crystallised chains of (1→4)-β-linked D-glucose monomers (Figure 1.7). A (1→4)-β-D-glucan molecule terminated by a 'reducing end' bearing a free (unsubstituted) hemiacetal hydroxyl (on the right) and a 'nonreducing' end (on the left). The hydroxymethyl groups at C6 of alternate glucose residues are on opposite sides of the chain. Each carbon atom of the glucose ring carries an axial hydrogen atom. For clarity these are omitted.

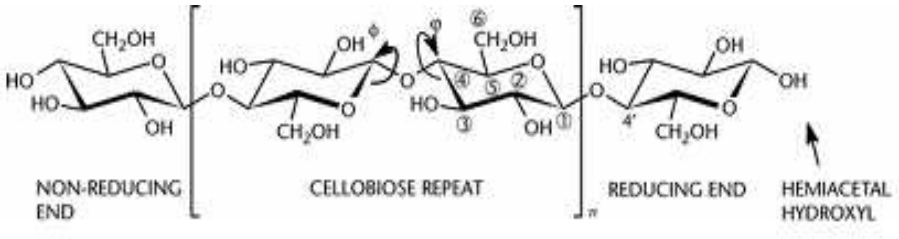


Figure 1.7 – The chemical structure of cellulose (from Stone, 2005)

Xyloglucans are the major interlocking polysaccharides that hold the microfibrils in place, although other polysaccharides including glucomannans and galactoglucomannans may also be present in some type I primary cell walls, albeit in smaller quantities. Xyloglucans consist of linear chains of (1→4)β-D-glucan. Many xylosyl units, some of them further substituted with galactosyl and other monomers, occupy the O-6 position of the glucosyl units of the chain (Stone, 2005).

Although proportions of respective cell wall fractions are highly variable, it is generally considered to be the case that the cellulose-xyloglucan framework typically makes up about 50% of the type I primary cell wall (Reid, 2000). This portion of the cell wall is embedded in matrix pectic polysaccharides, constituting approximately 30% of the total mass. The remainder of the cell wall mass is made up of structural proteins (Carpita and Gibeaut, 1993).

1.3.3 Pectin

1.3.3.1 Introduction

Pectin is one of the most structurally complex polysaccharides known. It is thought that it performs numerous complicated roles within the cell wall of a plant, including determining wall porosity, acting as recognition molecules (McNeil et al., 1984), and providing a charged surface to modulate cell wall pH and ion balance. The chemical structures of pectin molecules are highly variable, not only between plants of different species, but also between different tissues of the same plant and even between different areas of the same cell wall. The observed structural variability between pectic polysaccharides from different sources are largely as a result of numerous modification reactions which take place during the growth and development of plant tissues and during the ripening, storage and processing of fruit and vegetables.

1.3.3.2 The Chemical Structure of Pectin

The term ‘pectin’ is used to describe one member of a complex family of polysaccharides present in the cell wall and intercellular regions of plants. The major constituent of these molecules is D-galacturonic acid residues (Figure 1.8), in which ‘y’ represents the position taken by an –OH, -OCH_3 or -NH_3 group in the α-(1→4)-linked linear chain, amide groups being found only in commercial pectins. Although this structural feature is common to all

known pectic substances, the sugar residues associated with the galacturonic acid backbone vary greatly.

To date only three pectic structural units; homogalacturonan (HG), rhamnogalacturonans (RG-I and RG-II) and substituted galacturonans, have been isolated from cell walls and structurally characterised (O'Neill et al., 1989; Visser and Voragen, 1996).

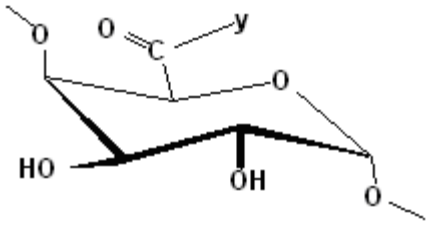


Figure 1.8 – The chemical structure of galacturonic acid, the main constituent of pectin

1.3.3.2.1 Rhamnogalacturonan

Rhamnogalacturonan is the general term given to rhamnose-rich and galacturonic acid-rich regions of a pectin molecule.

Rhamnogalacturonan-I

Rhamnogalacturonan-I (RG-I) is the name given to a group of polysaccharides comprised of repeating units of alternating α-(1→2)-linked rhamosyl and α-(1→4)-linked galacturonosyluronic acid residues, first identified by Albersheim's group (McNeil et al, 1980, 1984). These polysaccharides, isolated from suspension cultured sycamore cells, were found to be as long as 100 to 300 units (Albersheim et al. 1996; An et al., 1994) and constitute a highly diverse and complex group of structures. The fine structure of the RG-I fraction depends largely on the source of the pectin and method of isolation, although it can be said that between 20 and 80% of the rhamnosyl residues (Rha*p*) are substituted at C4 with oligosaccharide side-chains (Figure 1.9) (O'Neill et al., 2001).

The length and constituents of RG-I side-chains vary depending on the source of the pectin. They tend to predominantly contain branched and linear groups of α-L-arabinofuranosyl (Ara*f*), and β-D-galactopyranosyl (Gal*p*) residues.

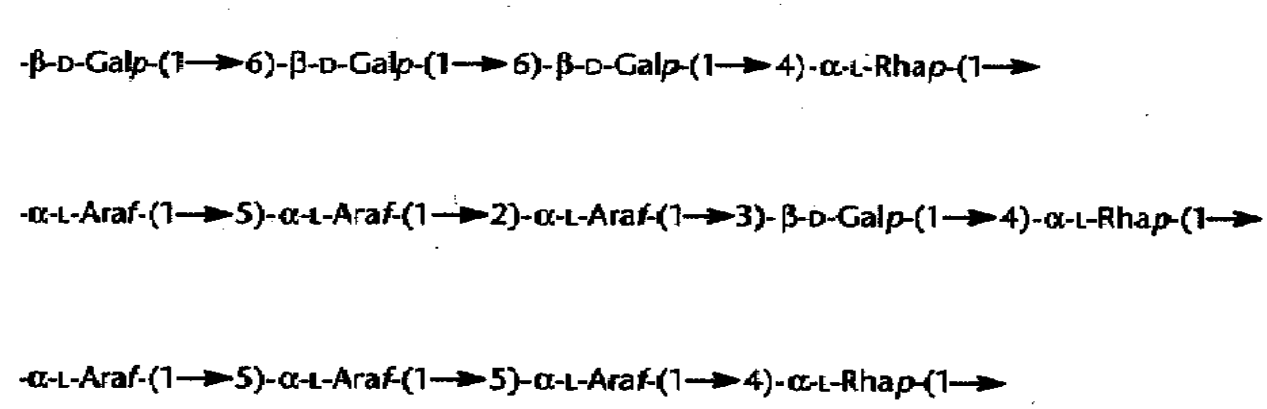


Figure 1.9 - Selected examples of structures of oligosaccharides attached to the backbone of RG-I (adapted from O'Neill et al., 2001)

Rhamnogalacturonan-II (or Highly Branched Galacturonan)

Rhamnogalacturonan-II (RG-II) accounts for around 10% of the pectin in cell walls and was first isolated and characterised by Albesheim's group (Spellman et al., 1983). RG-II was found to consist of a chain of approximately nine or ten α-(1→4)-linked galacturonosyl residues with four side chains containing numerous rare sugars such as apiose, aceric acid, DHA (3-deoxy-D-lyxo-2-heptulosaric acid) and KDO (2-keto-3-deoxy-D-mannooctulosonic acid) (Spellman et al., 1983) (Figure 1.10). The four different side chains shown in Figure 1.10 have been structurally characterised, however their exact relative locations are unknown hence the '?'.

RG-II has been found to occur in the primary cell wall, not in the middle lamella (Williams et al., 1996). Suggestions have been made for the possibility of RG-II being involved in cell wall porosity (Fleischer et al., 1999; Ishii et al., 2001).

The complex nature of the RG-II fraction of pectin has hampered efforts to fully determine the chemical sequence of the entire structure. However, due to the development of structural determining techniques such as high-field assisted laser desorption mass spectrometry (Vidal et al., 2001), the chemical structure of RG-II is being increasingly refined.

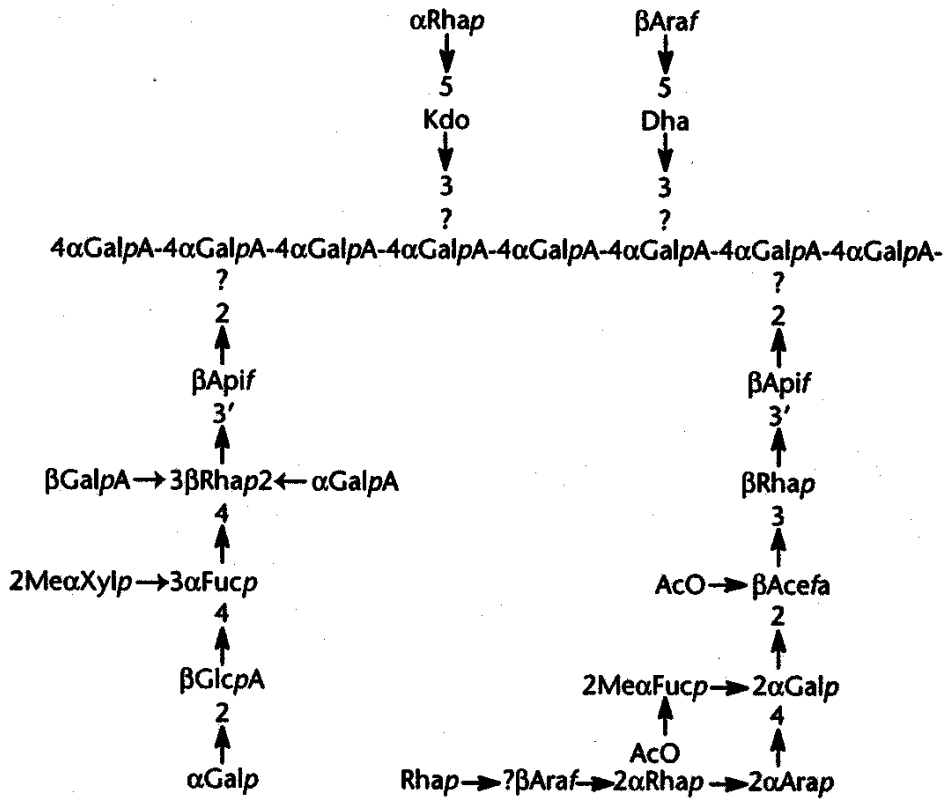


Figure 1.10 - The currently accepted glycosyl sequence of rhamnogalacturonan II (RG-II) OAc = *O*-acetyl ester, Me = *O*-methyl ether (adapted from Rodríguez-Carvajal et al., 2002)

RG-II has been found to exist in primary cell walls as a dimer, cross-linked by a 1:2 borate-diol ester (Kobayashi et al., 1996; Ishii et al., 1999). The structure of the RG-II fraction of pectin is highly homogeneous in the cell walls of higher plants, unlike that of HG and RG-I, a feature that suggests the specificity of the structural features required for these cross-links to form. Indeed, the cross-linking occurs only between the OH-2 and OH-3 of the apiosyl residues (Api*f*) on the side chains of each monomeric RG-II subunit (Figure 1.11).

The formation of this RG-II dimer is a self-assembly process, occurring in vitro without the presence of a catalytic protein. The extent and rate of dimer formation in vitro have been found to be increased in the presence of di- and trivalent cations with ionic radii >1.0Å (Ishii et al., 1999). Higher calcium and boric acid concentrations (10mM) also increase the rate of dimer formation in vitro (Matoh and Kobayashi, 1998). The role of calcium in dimer formation is not clear, but it is possible that it increases the stability of the RG-II dimer (Fleischer et al., 1999; Kobayashi et al., 1999).

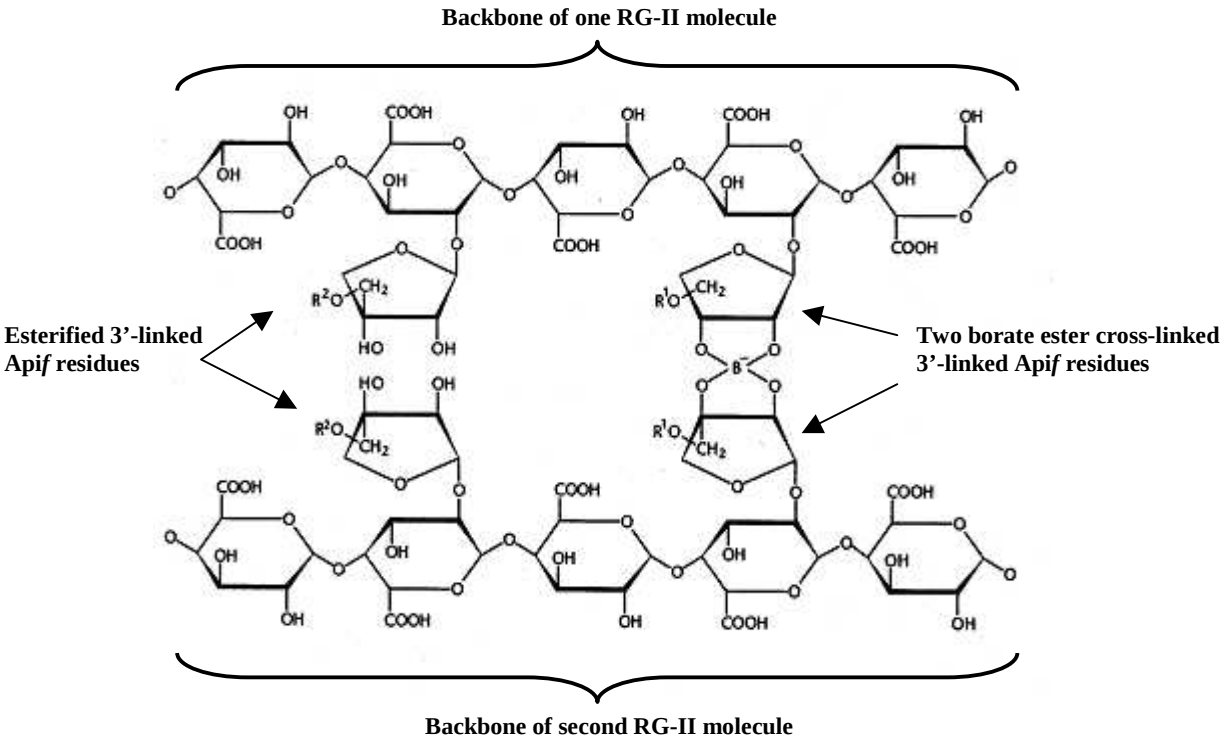


Figure 1.11 - Cross-linking of two rhamnogalacturonan II (RG-II) molecules with a borate ester (adapted from O'Neill et al., 2001)

1.3.3.2.2 Homogalacturonan

It is widely accepted that HG is the most abundant pectic polysaccharide and consists of a linear chain of α-(1→4)-D-galactopyranosyluronic acid (GalpA) residues (Figure 1.12). The main structural variation that occurs between the HG regions of pectins with different functions is the amount and distribution of carboxyl groups that are methyl esterified. HG's may also be partially O-acetylated at C-3 or C-2, depending on the plant source (Ishii, 1995).

The distribution of ester groups on an HG backbone is very complex (De Vries et al., 1983). For example, ester groups may occur in a completely random fashion, where they are distributed fairly evenly along the HG backbone. They can also occur in a more 'blockwise' pattern, where long chains of non-esterified residues are interrupted by almost completely esterified chains of galacturonic acid units.

As a consequence, the distribution of methyl esters may vary considerably between different pectin molecules, accounting for the various physical behaviours of these molecules.

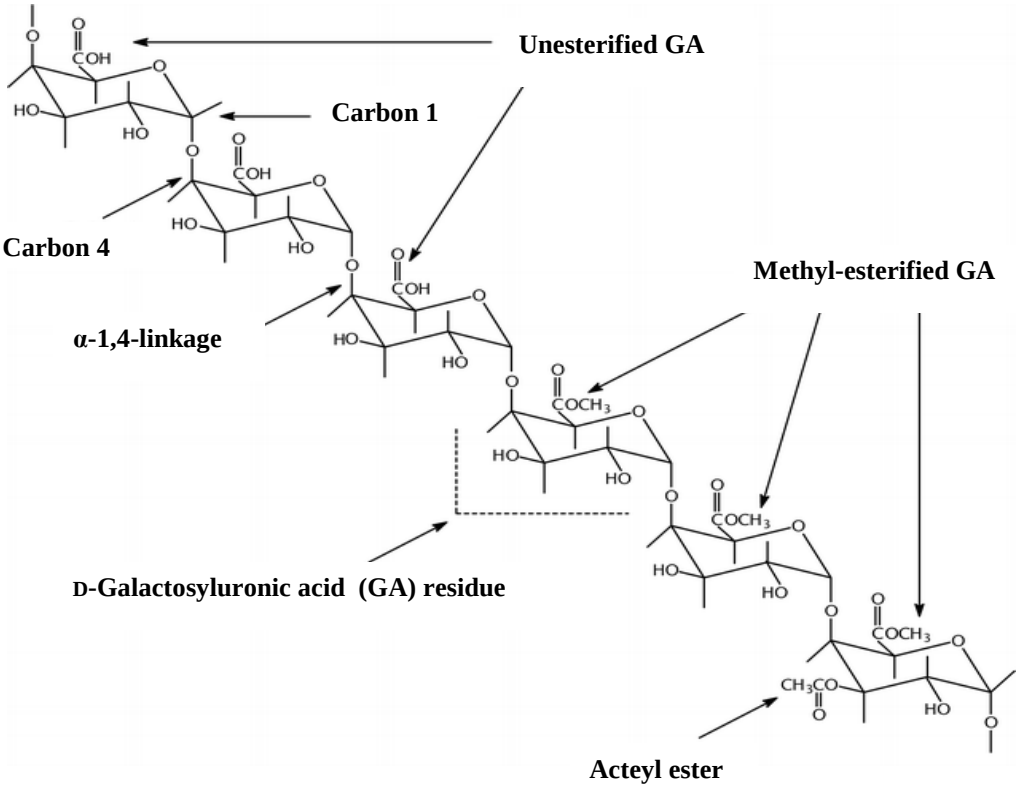
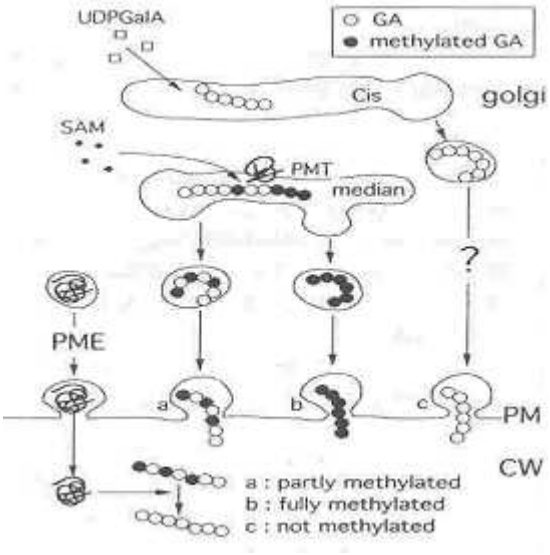


Figure 1.12 – The structure of homogalacturonan (from O'Neill et al., 2001)

1.3.3.3 Pectin Biosynthesis

The complex nature of pectin structure and function have somewhat impeded the study of pectin biosynthesis. Immunocytochemical evidence collected from research using antibodies directed against certain epitopes of cell wall carbohydrates (Hoson, 1991; Moore et al., 1991; Knox, 1992; Staehelin and Moore, 1995) suggests strongly that the synthesis of the pectic polysaccharides homogalacturonan (HG) and rhamnogalacturonan-I (RG-I) begins in the cis Golgi apparatus (Figure 1.13) (Lynch and Staehelin, 1992; Zhang and Staehelin, 1992) and continues into the medial Golgi apparatus (Moore et al, 1991; Zhang and Staehelin, 1992; Staehelin and Moore, 1995). HG esterification appears to occur in the medial and trans Golgi (Vian and Roland, 1991; Zhang and Staehelin 1992; Staehelin and Moore, 1995) and results from the action of pectin methyltransferases (PMT's) which transfer methyl groups from S-adenosylmethionine to a polygalacturonic acceptor. This process is followed by final assembly and transportation to the plasma membrane in the Golgi vesicles.



CW – cell wall
PM – plasma membrane
GA – galacturonic acid
SAM – S-adenosylmethionine
PMT – pectin methyltransferase
PME – pectin methylesterase

Figure 1.13 – Schematic representation of the hypothetical pathway for the biosynthesis of pectin (from Goldberg et al., 1996)

It is generally accepted that HG's are synthesised completely methyl-esterified and deposited into the developing cell wall with 70-80% of the galacturonic acid (GA) residues esterified with methanol. The presence of these groups prevents adjacent HG chains becoming cross-linked by divalent cations such as calcium, which are abundant in the cell wall matrix (Nari et al., 1991; Moustacas et al., 1991), and that would otherwise form a rigid, stable framework (Jarvis, 1984) (Figure 1.14).

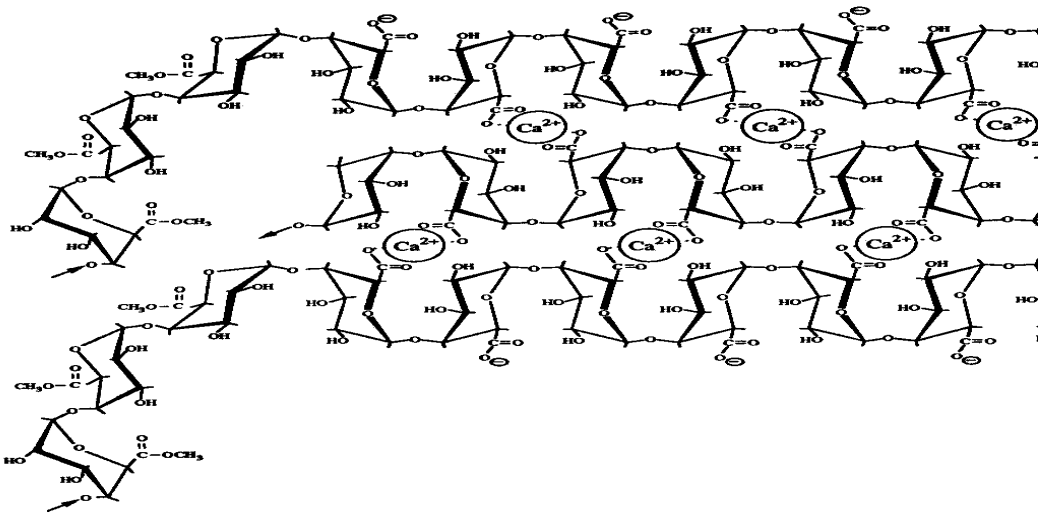


Figure 1.14 – The formation of calcium bridges leading to a rigid, stable pectic framework

Although HG is often inserted into the cell wall in a highly methyl esterified form (Carpita and Gibeau, 1993; Liners et al, 1994), a number of cell types, for example melon callus cells (Vian and Roland, 1991), have been found to secrete unesterified pectin into the cell

wall. There is also more recent evidence suggesting that the Golgi secretes pectins with low degrees of esterification (Liberman et al., 1999). It may be the case that there are randomly-esterified regions in any native HG resulting from incomplete esterification at the time of synthesis. HG regions with a blockwise pattern of esterification, with a high affinity for Ca^{2+}, may have been modified as a result of PME activity.

Once deposited in the cell wall, depending on the function to be performed by a particular polysaccharide, galacturonic acid residues may then be subjected to the action of pectin methylesterase (PME) enzymes (Figure 1.15).

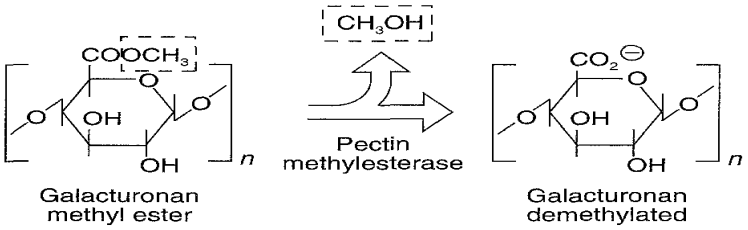


Figure 1.15 – Demethylation of an esterified GA residue by PME, the major source of methanol production in plants

Despite there being certain exceptions, it can be said that in a number of systems, the degree of esterification falls during the growth of the plant due to the action of PME. This process occurs concurrently with the stiffening of the cell wall, which can be measured mechanically (Cleland and Rayle, 1978; Ricard and Noat, 1986; reviewed by Goldberg et al., 1996). It is generally accepted that pectin is exceted into the cell wall with a high degree of esterification and that the degree of esterification reduces over time.

The demethylation of GA residues in an HG backbone is considered to be the primary source of methanol emitted from plants. Research has not only shown that methanol emissions are much greater in rapidly growing plant tissue than in mature tissue (Section 1.4.5.2.5; Nemecek-Marshall et al., 1995), but also that the demethylation of esterified GA residues by PME quantitatively liberates methanol from plant cell walls (Mangos and Haas, 1997; Frenkel et al., 1998). Insignificant quantities of methanol in plants may be associated with protein repair pathways (Mudgett and Clarke, 1993) or may be present as a result of the demethylation of DNA (Finnegan et al., 1998).

1.4 Methanol Produced by Plants

1.4.1 Introduction

The role of stomata in the control of methanol release from plants to the atmosphere is considered to be of utmost importance (Nemecek-Marshall et al., 1995), as discussed in Section 1.2.4.4. The precise pathway via which methanol is transported from the cell wall and subsequently emitted through stomata on leaf surfaces remains the subject of much speculation. A further unknown quantity is the proportion of the methanol produced in the leaf, as a result of the demethylation of pectic polysaccharides, that is released to the atmosphere. It has been shown however, that methanol within plant tissue can have several different fates (Figure 1.16). It may dissolve in water and be stored in the plant's tissue, it can be oxidised to formaldehyde (Gout et al., 2000) or a portion of the methanol released from plant tissue may also be utilised by methylotrophic microorganisms on the leaf's surface. The most likely fate of methanol produced by the tissues of plants however, is the release via diffusion to the atmosphere through stomata at the leaf's surface.

1.4.2 Enzymic Oxidation

Methanol oxidase has successfully been isolated from microorganisms, but despite extensive research on the subject, has yet to be found in higher plants. It has been found however, that plants can convert [^{14}C]methanol to $^{14}CO_2$ (Cossins, 1964) and that [^{13}C]methanol can readily be incorporated into serine and methionine (Gout et al., 2000). All the enzymic ingredients that allow the incorporation of carbon derived from formate, and possibly from methanol, into various organic compounds would appear to be present in the tissues of higher plants. However, the pathway via which methanol is initially oxidised in order that it can become incorporated into these complex compounds is, as yet, unknown. It is possible that methanol is primarily oxidised by alcohol oxidase to formaldehyde, followed by further oxidation to formate by formaldehyde dehydrogenase (Gout et al., 2000). If this enzymic pathway does exist in higher plants, a portion of the methanol released from the demethylation of pectic polysaccarides could potentially be utilised in this fashion in the production of certain amino acids.

1.4.3 Methylotrophic Microorganisms

Methanol released from leaves may also be utilised as a source of both carbon and energy by methylotrophic bacteria, which are characteristic in their ability to metabolise one-carbon compounds, such as methanol and formaldehyde. Bacterial populations on leaves occur in greatest abundance near the edges of the abaxial (lower) surface, although the abundance of these bacteria vary greatly between plant species, with the season, with leaf age and depending on hourly changes in the microclimate at the leaf's surface (Blakeman, 1982). The abundance of methylotrophic bacteria on dead and abscised leaves has been found to be much reduced compared to that on living leaves (Corpe, 1985), reinforcing the evidence for the theory that methanol emissions are indeed closely related to plant growth. The methylotrophic bacterium *Pichia methanolica* is able to grow on a medium comprised of pectic polysaccharides (Nakagawa et al., 2005). Indeed the growth yield of *P. methanolica* increases by 100% with increasing degree of methyl esterification from 0% to 90%. Although methylotrophic bacteria may consume gaseous methanol being emitted through stomata on the leaf's surface, it is likely that they do not constitute a significant sink for methanol (Galbally and Kirstine, 2002).

1.4.4 Storage Tissues

An added complication that must be taken into account when studying the emission of VOC's from plants stems from the fact that some volatiles, for example terpenes in mint and pine trees, may be stored in specially adapted tissues, depending on the volatile and the species of plant. Plants lack the ability to store isoprene in their tissues, hence a cession in the production of isoprene leads to the discontinuation of isoprene emission. The extent to which plants store certain biogenic volatile compounds seems to depend more heavily on predetermined genetic traits of the plant species than prevailing environmental conditions (Peñuelas and Estiarte, 1998).

Very limited literature is currently available describing this phenomenon occurring in the case of methanol. It has been shown however, that methyl-beta-D-glucopyranoside (MeG) is synthesised in the cytosol of cells from the leaves of the Alpine herb *Guem monatunum L.* (Aubert et al., 2004), directly from glucose and methanol. It is considered that this synthetic process may prevent the excess accumulation of methanol and its toxic derivatives in the cytoplasm. The proportion of the methanol produced as a result of demethylation of pectic polysaccharides that is ultimately utilised in this way is thought to be minimal.

1.4.5 Release of Methanol to the Atmosphere

1.4.5.1 Introduction

Although there are several possible fates for methanol and other VOC's produced by plants, it is thought that by far the greatest proportion of these volatiles is emitted directly from the plant tissue to the surrounding atmosphere. VOC emissions from plants are the result of diffusion from sub-cuticular cavities within the plant tissue along a vapour pressure gradient to the atmosphere surrounding the plant, where the concentration of these gases is relatively low (Galbally and Kirstine, 2002). Emissions of VOC's from plants will therefore ultimately be controlled by VOC volatilities and factors that alter this concentration gradient, or present a barrier to diffusion.

Figure 1.16 shows a schematic representation of the pathway that methanol produced in the plant cell wall follows as it is emitted to the atmosphere.

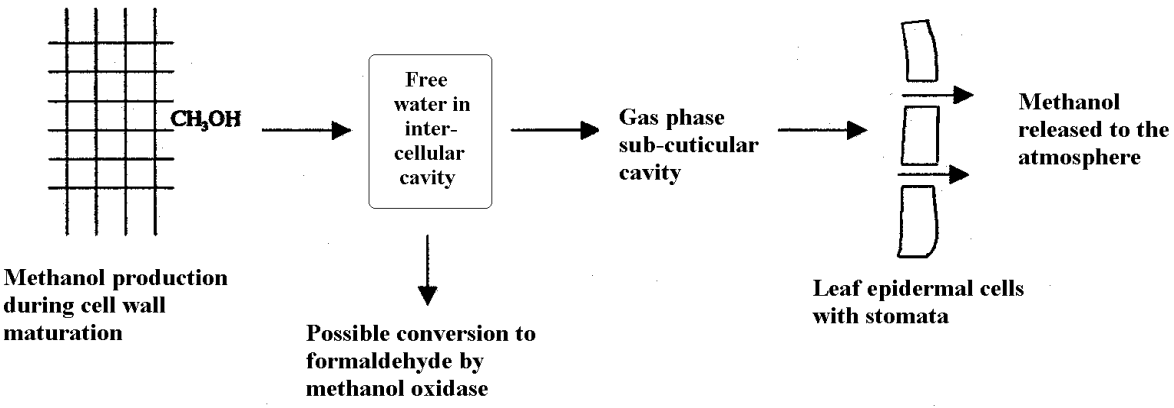


Figure 1.16 – Schematic diagram for methanol release from plant leaves

1.4.5.2 Factors Influencing the Rate of VOC Emission from Growing Plants

The rate, quantity and composition of VOC's emitted from vegetation is highly variable in both the short term (the response in emission rate up to an hour) and long term (the response after several hours, days or months), depending on both internal factors, for example genetics, and external factors, such as temperature and light intensity. The complexity of factors influencing volatile emission rates from plants is a major hindrance in the compilation of reliable data for global VOC inventories.

Unfortunately, very little work has thus far been completed regarding the effect of short-term (scale of minutes) external environmental parameters on the rate of methanol emissions from plants. In the following sections, the effects on isoprene and monoterpene emission rates of these factors will be discussed, together with data on methanol where available. It may be the case that the emission rates of all three volatiles display similar patterns, due to the fact that the emission of isoprene, terpenoids and methanol are all controlled by stomatal conductance.

1.4.5.2.1 Genetic Predetermination

Genetics plays a significant role in determining the nature of VOC emissions from plants. For example, methanol emissions from young leaves of Sassafras (*Sassafras albidum*) and Soybean (*Glycine max*) have been measured at $13.6 \pm 1.2 \mu g h^{-1} g dw^{-1}$ and $36.0 \pm 7.0 \mu g h^{-1} g dw^{-1}$ respectively (MacDonald and Fall, 1993). Assuming the methanol emitted from plants originates entirely from the demethylation of pectic polysaccharides in the primary cell wall, it is logical to assume that the varying pectin content between particular plant species will have an impact on the quantity of methanol emitted.

The predetermined genetic characteristics of vegetation species obviously have a pivotal role in determining the particular nature and quantities of VOC emissions from certain plant species. For example, certain Monocotyledonae containing type II cell walls will contain a fraction of the quantity of pectin and significantly more phenolic esters than type I cell walls, which occur in all Dicotyledonae and gymnosperms and some Monocotyledonae that have been studied (Carpita and Gibeaut, 1993). This compositional difference may lead to the emission of different quantities and possibly different volatile species depending on the prominent cell wall type. The emission of volatiles from plants is, however also highly dependent on temporal and spatial variations (Kesselmeier and Straudt, 1999).

1.4.5.2.2 Temperature

The effects of varying temperature on the emission behaviour of VOC's from plants have been investigated rather extensively. However, monoterpenes and isoprene are by far the most closely examined volatile species. Studies conducted to date have all shown that the emissions of monoterpenes and isoprene are heavily temperature-dependent, with emission rates generally increasing exponentially to an optimum at about 40 ^{o}C before decreasing rapidly (Guenther et al., 1993; Monson et al., 1995; Baldocchi et al., 1995). This phenomenon is thought to be as a result of several factors, for example elevating the

enzymic activity related to particular volatile production, increasing the vapour pressure and decreasing the resistance of emission pathways (Loreto et al., 1996).

Isoprene emissions increase rapidly from *Phragmites australis* following an increase in temperature, but this phenomenon is not sustained and after about 30 minutes, the emission rate steadily decreases to a level below the original rate of emission (Loreto et al., 2006), supporting the evidence for a lack of significant isoprene pools within the plant. This increase is not surprising given that the optimum temperature for isoprene synthase activity is 42 °C (Monson et al., 1992).

Little information is available regarding the effect of short-term temperature changes on the emission rate of methanol from plants and the effect of temperature on isoprene emission rates are likely not to be closely related to methanol emission due to the fact that isoprene emissions are related to stress events, rather than physiological changes (Sanadze, 2004). It has been shown that methanol is emitted from leaves when the stomata are open (MacDonald and Fall, 1993, Figure 1.4). Methanol emission rates from leaves therefore follow a diurnal cycle, with methanol emission rate increasing with increasing ambient temperatures (Shade and Goldstein, 2001). The rate of emission will consequently decrease significantly in times of drought or at the hottest part of the day, when stomata are closed (MacDonald and Fall, 1993).

Loreto et al. (2006) found that, on a single plant basis, methanol emissions increased in elevated temperatures over a period of about an hour and a half, but increases in emissions were observed at a much lower rate and to a much lesser extent than those of isoprene (Loreto et al., 2006). Further work has been carried out on the effects of temperature on methanol emission rates over longer timeframes and on larger scales.

The first canopy-scale measurements of methanol were recorded by Shade and Goldstein (2001), determining long-term fluxes of volatile organic compounds above a ponderosa pine (*Pinus ponderosa*) plantation. Results confirmed that methanol emission is dependent on temperature, with methanol emission rate increasing with temperature in an exponential fashion (Shade and Goldstein, 2001). However, it was also apparent that factors other than temperature were affecting the emission of methanol, particularly light which, given the pathway via which methanol is thought to be emitted (Nemecek-Marshall et al., 1995) is not unexpected. Schade and Goldstein (2006) compared seasonal quantities of methanol above a remote mountain site, the predominate plant species present being ponderosa pine. One third of the daytime variability of methanol in summer was accounted for solely by air

temperature. However, higher emissions in the springtime could not be accounted for by increases in ambient temperature, but was attributed to intensified pectin demethylation that is thought to be the source of methanol emissions from plants (Section 1.2.4.3, MacDonald and Fall, 1993; Galbally and Kirstine, 2002).

Mao et al. (2006) measured methanol concentrations in air above two sites in New Hampshire during the summer months of 2004. A close correlation between methanol concentration and air temperature was observed with methanol concentrations increasing linearly from approximately 1 to 3.5 ppbv as temperatures increased from 11 to 30 °C.

1.4.5.2.3 Light

Isoprene production in plants is closely correlated with photosynthesis, due to the fact that isoprene is formed from photosynthetic products (DMAPP) in the chloroplasts (Section 1.2.3; Loreto and Sharkey, 1990; Sharkey and Yeh, 2001). There is no large pool of isoprene within plant tissue due to a lack of suitable storage tissue. Therefore, unlike monoterpenes, synthesis and therefore emission ceases after a matter of minutes of the plant being immersed in darkness (Guenther et al., 1991; Sanadze, 1991; Sharkey et al., 1991).

The response of isoprene emission to light intensity evidently varies depending on the origin of the plant species being investigated. For example, emissions from temperate plant species tend to increase rapidly to a maximum at around 1200 μmoles m^{-2} s^{-1} and increasing light intensity beyond this has little effect on the emission rate of isoprene (Baldocchi et al., 1995). The relationship between light intensity and emission rate from typical plants in tropical deciduous forests is strictly linear up to a maximum methanol emission rate of 2000 μmoles m^{-2} s^{-1} (Lerdau and Keller, 1997).

Substantial correlation has been reported between short-term methanol emission rates and stomatal conductance from a number of plant species including cottonwood (*Populus deltoids*) and quaking aspen (*Populus tremuloides*) (Section 1.2.4.4, MacDonald and Fall, 1993). The relationship between temperature and the rate of methanol emission during the day was found to be much less convincing that that between emission rate and stomatal conductance.

Further work has been carried out on leaves of cottonwood plants whereby they were exposed to varying light intensities in constant humidity and temperature conditions (Nemecek-Marshall et al., 1995). Methanol emission rates were positively correlated with

light intensity, however following 4 hours in the dark, methanol emission had not ceased. Although methanol emissions increased with increasing light intensities, it was apparent from the results that despite the fact that methanol emissions increased with increasing light intensities, stomatal conductance, rather than a direct affect on methanol production was ultimately responsible. In the longer term, parameters more influential than light, for instance temperature and leaf age, were considered to have a greater effect on the rate of methanol production.

1.4.5.2.4 Wounding

A number of studies have been carried out investigating the effect of the process of cutting of plant material on VOC emission rates from various different plant species. For example, Kirstine et al. (1998) measured VOC's emitted from unimproved pasture in south-eastern Australia using a grab sampling technique followed by gas chromatography/mass spectrometry (GC/MS) analysis. It was found that methanol emissions constituted between approximately 15%, from grass and 33%, from clover, of the total VOC flux of approximately 3200 μg(C) m^{-2} h^{-1} emitted from the plants. The findings also showed that VOC emission rates increased significantly following the mechanical coarse cutting of the vegetation. VOC emission rates from grass increased by 180 times compared to the maximum measured emission rate from undamaged grass, with approximately 10% of this being contributed by methanol.

Studies have subsequently been carried out examining the emission of volatiles from a variety of damaged plant materials including grass (Olofsson et al., 2003) and alfalfa (Warneke et al., 2002). An immediate significant enhancement of VOC emissions was observed from all plant species studied, followed by a more prolonged period of elevated VOC emission during the drying process of cut leaves (de Gouw et al., 1999; Karl et al., 2002; Warneke et al., 2002; Olofsson et al., 2003).

Increased pectin degradation as a result of an increase in PME is known to occur in plants during episodes of mechanical and environmental stress (Kunzek et al., 1999; Fall, 2003). The rapid increase in methanol emission rate following a wounding event strongly suggests the presence of a small pool of aqueous methanol, the size of which is depleted after wounding through evaporation (Fall et al., 2003; Loreto et al., 2006). The magnitude of increase in VOC emission following a wounding event is also reported as being proportional to the size of the wound (Fall et al., 1999).

De Gouw et al. (2000) investigated further the release of VOC's from crop species, including clover and corn, following cutting and drying processes using proton-transfer chemical-ionisation mass spectrometry. It was calculated from the results of these experiments that, following a wounding event, the methanol released from clover plants was approximately 90 μg of C gdw^{-1}. Converting this figure into mg methanol per gdw plant material, we arrive at a methanol release of approximately 240 μg of methanol released per gdw clover following abscission. In the case of corn, it was found the amount of methanol released was small compared to that from clover. An interesting observation from the experiments was that there was a significant increase in methanol emission from corn plant material after the stem was cut, but this phenomenon did not occur following cutting of the leaves. This could be due to the fact that the rapidly growing part of the corn plant, where rapid cell wall expansion predominantly takes place, is at the base of the stem.

As the emission of methanol from corn was measured as being a factor of ten lower than that from clover, it may be reasonable to assume that there will indeed be an increase in methanol emission following the cutting of a grass stem. However, this increase is likely to be small in comparison to the increase in methanol emission rate during the harvest of a dicot, for example oilseed rape. Indeed, it was shown by Karl et al. (2005) that the emission rate of methanol from fully developed rice shoots and leaves did not alter significantly during drying.

Studies carried out on monoterpene emitting plants have found that, following even slight mechanical stress, there is a significant emission burst of monoterpenoid compounds (Guenther et al., 1991; Arey et al., 1995; Staudt et al., 1997), possibly a plant signalling mechanism. On the contrary, isoprene emissions have been found to be unaffected or even significantly reduced by damage to the plant (Loreto and Sharkey, 1993), although the signals regulating isoprene emissions are as yet unknown.

1.4.5.2.5 Stage of Development

Emission rates of methanol from growing leaves vary substantially during the growth and development of the plant tissue (MacDonald and Fall, 1993; Nemecek-Marshall et al., 1995). Substantial quantities of methanol were detected from the leaves of a variety of plant species by MacDonald and Fall (1993). Mature, fully expanded leaves were found to emit methanol at a rate comparable to those described as typical for isoprene emissions from leaves, about 11μg h^{-1} gdw^{-1} (Hewitt et al., 1990). However, on average, rapidly

expanding immature leaves emitted methanol at about twice the rate of mature leaves on a dry weight basis (MacDonald and Fall, 1993).

Work carried out by Nemcek-Marshall et al. (1995) found that young, rapidly expanding leaves of soybean (*Glycine max*) emitted methanol at a greatly elevated rate compared to more mature leaves. These findings support the hypothesis that methanol is released from plants as a result of the enzymic degradation of pectic polysaccharides within the plant cell wall. However, in some other plant species studied, for example bush bean (*Phaseolus vulgaris*) and cottonwood (*Populus deltoides*), methanol emission rates were greatest in very young leaves, with rates decreasing steadily as the leaves matured.

Karl et al. (2003) found that methanol emission rates over a mixed hardwood forest in the US reached a maximum in spring, with methanol emissions from young leaves apparently being in the region of three times greater than those from mature leaves, presumably as a result of the rapid expansion of leaves. A quantitative methanol emissions model, based on plant physiology, was developed by Galbally and Kirstine (2002), whereby the amount of methanol released from the plant to the atmosphere was related to the process of plant growth. The net primary productivity (NPP) was used as an estimate for the relative stage of growth of different plant species in order to take into account the variation in methanol emission rates from plant tissue at various stages of development.

The rate of methanol emissions from young, rapidly growing leaves was found to be several times greater than that from mature leaves in *Gossypium hirsutum* plants (Hüve et al., 2007). Following the removal of younger leaves, the reduction in methanol emissions suggested that approximately 75% of the total amount of methanol emitted was due to leaf growth. It was also discovered that, in concurrence with the findings of Nemecek-Marshall et al. (1995), very young leaves have a lower rate of methanol emission than those slightly older, thought to be associated with the lack of functional stomata in the former.

1.4.5.3 Methanol Emissions from Decaying Plant Material

Fluxes in methanol emission rates from a mixed deciduous woodland in the US have been reported as being 1.7 times lower in the autumn than those in the spring (Karl et al., 2003), suggesting that the majority of the methanol emitted from plant material is as a result of growth. However, observed methanol emissions in autumn were still significant and attributed predominantly to the decomposition of leaf litter on the forest floor.

The methanol contained in a plant is released to the atmosphere when it dies (Galbally and Kirstine, 2002). Pectic polysaccharides containing methyl esters may also be degraded enzymically following the death of the plant resulting in the release of methanol. Pectin methylesterase (PME) remains active under a wide range of conditions (Castaldo et al., 1997) so that, even after the death of a plant, for example following harvest, PME will continue to remove methyl groups from the HG polysaccharides in the plant material, resulting in the release of methanol. The extent to which the methyl esters are cleaved depends on the source and therefore structural characteristics of the pectin and the form of the PME. For example, the majority of PME enzymes extracted from higher plants result in the block-wise removal of methyl ester groups. As not all methyl esters present in pectic polysaccharides are arranged in block-wise configurations and often occur randomly distributed, the complete removal of methyl esters within the plant is unlikely. However, it is probable that the greatest proportion of methyl esters present is converted to methanol by PME (Rexová-Benková and Markovic, 1976; Massiot et al., 1997) and is released to the atmosphere as methanol following the death of the plant.

Significant emissions of methanol were detected from beech leaves during their degradation by Warneke et al. (1999). Emission rates of methanol were greater in magnitude than all other emitted organic compounds quantified, including acetone. The quantity of methanol emitted as a result of the decomposition of plant material was extrapolated and the annual global production of methanol from the decomposition of plant material was estimated as being between 18 and 40 Tg.

1.4.5.3.1 Fungal Degradation of Plant Material

Methanol may be emitted from decaying plant material as a result of the demethylation of lignin by the action of fungal enzymes (Ander and Eriksson, 1985; Kirk and Farrell, 1987). Lignin accounts for only about 5% of the total biomass of dead leaves (van Elsas et al., 1997), with 18% of the total mass being comprised of lignin molecules containing methoxy groups (Bourbonnais and Paice, 1992). Of this, between approximately 4 and 25% is liberated as methanol by white-rot fungus (*Phaneochaete chryososporium*) under optimum conditions (Ander and Eriksson, 1985). This process may account for a significant input of methanol from decaying plant material to the troposphere were it not for the fact that the enzymic degradation of lignin methyl ester groups is inhibited by the presence of oxygen (Ander and Eriksson, 1985). As a result, it is likely that only a small fraction, perhaps only 10% (Galbally and Kirstine, 2002), of the methanol present as methoxy groups on lignin molecules is released to the troposphere.

Several types of pectin methylesterase enzymes have been purified from the *Aspergillus* genus of fungi (reviewed by de Vries and Visser, 2001). This large group of filamentous fungi consists of a number of pathogenic fungi, but the group of black aspergilli (*A. niger* and *A. tubingensis*) are of particular importance in industrial applications (Alkorta et al., 1998). *Aspergillus* produces a wide range of enzymes that can be utilised in the degradation of plant cell wall polysaccharides, making them of major importance in the food and feed industry. Fungal pectin methylesterase (PME) enzymes cleave the ester bond between the HG pectic region and methyl residues, liberating acidic pectin and methanol (Khanh et al., 1991), in the same way as plant PME. However, the optimum pH for fungal PME activity is 3 to 4, whereas that of higher plants ranges between 7 and 8.

The ability of fungi to produce enzymes with the function of utilising plant cell walls has been well studied (e.g. Vries and Visser, 2001, St Leger et al., 1997). Saprophytic fungi, such as *Neu. crassa* and *A. nidulans*, and opportunistic pathogens such as *A. fumigatus* and *A. flavus* produce a broad spectrum of protein and polysaccharide degrading enzymes, indicative of their less specialized nutritional status (St Leger et al., 1997). It is therefore likely that a proportion of the methanol contained in abscised leaves in the form of pectic methyl esters, enters the troposphere as a result of the activity of fungal PME.

1.4.6 The Atmospheric Consequences of Methanol in the Troposphere

1.4.6.1 Introduction

Prior to 1992, literature reporting atmospheric measurements of methanol was scarce. Snider and Dawson (1985) reported mean methanol concentrations of approximately 7.9 ppbv and 2.6 ppbv in urban and rural sites respectively located in Arizona. Methanol concentrations of up to 11 ppbv have been detected during the day in a forested area in the southeastern US, compared to a concentration of about 6 ppbv for isoprene (Fehsenfeld et al., 1992).

In the continental boundary layer of the earth's atmosphere the concentration of methanol is typically in the region of between 1 and 10 ppbv and between 0.1 and 1 ppbv in the upper troposphere (Singh et al., 1995; Heikes et al., 2002). Despite the fact that methanol emission rates from plants have been quantified in some cases as being of the same order of magnitude as isoprene and monoterpenes, the ambient concentration of methanol is, after methane, the second most abundant organic gas in the earth's atmosphere.

The substantial abundance of methanol in the troposphere is due, in part to the relatively low reactivity of methanol compared to other biogenic VOC's such as isoprene. The tropospheric lifetime of methanol of approximately nine days as opposed to only a few hours for isoprene has profound consequences upon particular relationships that exist between gaseous species present in the troposphere both naturally and as a result of anthropogenic activities. The importance of methanol and indeed all VOC's present in the troposphere originating from either biogenic or anthropogenic sources, stems from their role as major precursors of hydroxyl radicals (OH), sometimes referred to as 'odd hydrogen' radicals which, in the upper troposphere, ultimately result in the formation of tropospheric ozone.

In the stratosphere, ozone is formed through the photolysis of oxygen and, in areas where ozone is depleted due to the free radical degradation of ozone by chlorinated and fluorinated organic compounds, the earth's surface is exposed to harmful solar ultraviolet radiation. Conversely, tropospheric ozone has well-documented deleterious effects on human and animal health, as well as plant ecosystems (Finlayson-Pitts and Pitts, 1993). The chemistry behind the formation of tropospheric ozone involves VOC's and is, in itself, complex, as well as being highly dependent on prevailing environmental conditions, particularly short wavelength actinic UV radiation.

1.4.6.2 Troposopheric Ozone Formation

Ozone formation in the troposphere is a highly non-linear process related to its main precursors: nitric oxide (NO), nitrogen dioxide (NO_2), collectively termed NO_X, and VOC's. In certain conditions, tropospheric ozone formation is almost entirely independent of VOC concentration and is controlled by NO_X concentrations. Certain other prevailing environmental conditions however, dictate that ozone formation is directly proportional to VOC concentration and may actually decrease with increasing NO_X concentrations.

The formation of tropospheric ozone may be categorised into two distinct series of chemical reactions, known as VOC-sensitive and NO_X-sensitive regimes. In general, a freshly-emitted plume of pollutant gases, predominantly originating from urban areas, is dominated by VOC-sensitive chemistry but develops towards NO_X-sensitive chemistry as the plume moves downwind of its source and the NO_X pollutants within the plume become less concentrated.

Tropospheric ozone formation is also heavily dependent on sunlight, particularly radiation of wavelengths 290 nm and greater, termed the actinic region, which is responsible for inducing photochemical reactions. The formation of ozone in the troposphere can be attributed directly to the presence of anthropogenic NO_2, which undergoes photolysis to form NO and O (Reaction (1.1)).

$$NO_2 + h\nu \ (\lambda < 420 \text{ nm}) \rightarrow NO + O(^3P) \qquad (1.1)$$

$$O(^3P) + O_2 (+ M) \rightarrow O_3 \qquad (1.2)$$

Reaction (1.2) shows the subsequent ozone formation, where M is a third molecule that is capable of stabilising the highly reactive reaction intermediate.

By far the greater proportion, typically more than 90%, of anthropogenic NO_X emissions, for example from fossil fuel combustion, occur in the form of nitric oxide (NO). A small amount of nitrogen dioxide (NO_2) is emitted directly to the atmosphere as a result of combustion processes (Lenner, 1987), but NO_2 is present predominantly in the troposphere as a result of the oxidation of NO after dilution in air. The formation of NO_2 from NO involves the removal of tropospheric ozone via the reaction shown in equation (1.3).

$$NO + O_3 \rightarrow NO_2 + O_2 \qquad (1.3)$$

Normally, equations (1.1) and (1.3) are counterbalanced by each other. Removal of ozone from the troposphere occurs when reaction (1.3) dominates over reaction (1.1). This occurs at night when ozone is removed via the formation of NO_2, sometimes referred to as NO_X titration, but there is no photolysis taking place. This phenomenon also occurs during the day, but only in areas close to a large NO_X emissions source as equilibrium is shifted in equation (1.3).

NO_X titration reactions (equations (1.1) to (1.3)) constitute important processes that occur in the atmosphere involving anthropogenic sources of nitrogenous combustion by-products. However, the formation of tropospheric ozone can also occur via reaction sequences involving VOC's and carbon monoxide, together with NO_X and hydroxyl radicals (OH). This series of reactions results in the conversion of NO to NO_2 through processes other than that shown in equation (1.3).

1.4.6.2.1 The Hydroxyl Radical (OH)

The hydroxyl radical plays a pivotal role in atmospheric chemistry due to its high reactivity with both organic and inorganic compounds. During daylight hours, the photolysis of existing tropospheric ozone results in OH formation via reactions (1.4), (1.5) and (1.6). It has also been suggested that another significant source of OH could be reactions of ozone with alkenes including any biogenic VOC's containing C=C bonds, for example isoprene and monoterpenes (Paulson and Orlando, 1996). These reactions may take place during the day and at night.

Ozone undergoes photolysis to form the electronically excited reaction intermediate $O(^1D)$ atom. $O(^1D)$ can either react with H_2O or become deactivated to the ground state oxygen atom $O(^3P)$ (reactions (1.5) and (1.6)).

$$O_3 + h\nu\ (\lambda < 420\ \text{nm}) \rightarrow O(^1D) + O_2 \qquad (1.4)$$

$$O(^1D) + H_2O \rightarrow 2OH \qquad (1.5)$$

$$O(^1D)\ (+\ M) \rightarrow O(^3P) \qquad (1.6)$$

It is this hydroxyl radical that plays a pivotal role in atmospheric chemistry due to the fact that it initiates the majority of organic oxidations in the troposphere (Wayne, 1985; Finlayson-Pitts and Pitts, 1986). Through the processes detailed in reactions (1.4) to (1.6), increases in tropospheric ozone itself result in increased OH formation and a subsequent decrease in the atmospheric lifetimes of organic species such as biogenic VOC's, including methanol.

The details of ozone chemistry in the troposphere are complex, involving a variety of atmospheric conditions and chemical species. Every species involved in these reactions, whether originating from anthropogenic or biogenic sources, must be fully quantified and sources understood so that tropospheric ozone formation can be better controlled. For this reason it is vital that tropospheric VOC inputs, including biogenic methanol, are better understood and quantified.

1.4.6.2.2 The Role of VOC's in Tropospheric Ozone Formation

The reaction processes that lead to ozone formation in the troposphere are almost always initiated by reactions of hydrocarbons, for example methanol, or carbon monoxide (CO)

with the hydroxyl radical (OH). Hydroxyl radicals themselves are formed from the photolysis of ozone (reactions (1.4) and (1.5)).

OH radical levels are particularly important in affecting the O_3 formation rate in the presence of NO_X because the reaction of OH with VOC's is a major, and in many cases the only process resulting in the formation of highly reactive hydrocarbon radicals, in the case of methanol CH_2OH or CH_3O (see reactions (1.7i) and (1.7ii)). The essential role played by VOC's in the formation of ozone in the troposphere is the contribution made to the atmospheric content of hydroperoxyl radicals (HO_2) which, in turn oxidise NO, present in the troposphere as a result of combustion processes, to NO_2, the immediate precursor of ozone. NO_2 then undergoes photolysis to form NO and an electronically excited oxygen atom (reaction (1.1)) which reacts with molecular oxygen to form ozone (reaction (1.2)).

Reactions (1.1) to (1.7) illustrate the role of VOC's, in this case methanol, in OH and therefore ozone formation.

$$CH_3OH + OH \begin{cases} \rightarrow CH_2OH + H_2O & (1.7i) \\ \rightarrow CH_3O + H_2O & (1.7ii) \end{cases}$$

$$CH_2OH + O_2 \rightarrow H_2CO + HO_2 \quad (1.8)$$

$$CH_3O + O_2 \rightarrow HO_2 + H_2CO \quad (1.9)$$

$$HO_2 + NO \rightarrow NO_2 + OH \quad (1.10)$$

$$OH + NO_2\,(M) \rightarrow HNO_3 \quad (1.11)$$

Initiation of the reaction sequence above involves the reaction of OH with methanol to form one of two free radicals (reactions (1.7i) and (1.7ii)). These free radicals react with molecular oxygen to form formaldehyde and the hydroperoxyl radical (HO_2) (reactions (1.8) and (1.9)). HO_2 rapidly oxidises NO to NO_2 (reaction (1.10)), which crucially results in the regeneration of OH radicals. Reaction (1.11) is the termination step of the reaction sequence and results in the formation of nitric acid, a key component of acid rain and a co pollutant of ozone (Finlayson-Pitts and Pitts, 1993). M is a third molecule, capable of stabilising the highly reactive reaction intermediate.

1.4.6.3 Current Global Methanol Inventories

Singh et al. (2000) presented the original estimate of the global budget of methanol in the environment, considering both anthropogenic and biogenic sources. Subsequent models have been developed and estimates made regarding global sources and sinks of methanol by Galbally and Kirstine (2002) and Heikes et al. (2002). These inventories were reviewed and compared collectively by Singh et al. (2004), together with further estimates made by Singh and co-workers (Singh et al., 2004). It is immediately apparent from this work that many uncertainties and inaccuracies still exist in the global estimates of methanol contributions made by a variety of sources and sinks, with the total estimated source ranging between 75 (Singh et al., 2000) and 490 Tg yr^{-1} (Heikes et al., 2002). The major inconsistency incorporated into these calculations lies in the widely differing estimates for biogenic emissions, which have been estimated at anywhere between 37 (Galbally and Kirstine, 2002) and 280 Tg yr^{-1} (Heikes et al., 2002).

More recent estimates made by Horowitz et al. (2003) and Lathière et al. (2006) have estimated mean global biogenic methanol emissions as being 107 and 106 TgC yr^{-1} respectively. This translates as approximately 285 and 283 Tg methanol yr^{-1} respectively.

Methanol emissions from plants to the troposphere are both significant and highly variable in quantity. Emission rates of methanol are dependent on several complex internal and external factors, the intricate natures of which incur an inherent degree of inaccuracy in attempts to calculate quantities contributed by various biological sources on a global scale. It is obvious from the results reviewed by Singh et al. (2004) that there remains much investigative work to be completed before an accurate quantification of the global methanol source may be achieved.

1.5 Pectin Esterification and Potato Texture

1.5.1 Introduction

Texture is a central quality characteristic of all plant-based foods. Specific cell wall components are modified during the ripening, storage, cooking and processing of these foods, resulting in spatial variations in the degree and extent of cell separation and adhesion between the walls of adjacent cells. The ripening of different fruit species results in a variety of physiological changes at a cellular level, which determine the textural changes that occur during the ripening of that particular species. For example, in fruit such

as strawberry or avocado, which develop a soft, smooth texture during ripening, swelling of the cell wall is evident. However, in fruit such as apple, which ripen to a crisp texture, cell wall swelling is not observed (Redgwell et al., 1997). Bourne (1979) separated temperate fruits into two groups according to ripening behaviour and textural properties. Group 1 consisted of fruit that softened greatly and acquired a soft, melting texture. Group 2 comprised fruit that softened moderately and were characterised by a crisp, fracturable texture.

Pectin methylesterase (PME) activity is widely implicated in the ripening of fruit, as well as in the deterioration in the texture quality of some plant-based foods during the period of storage prior to distribution.

The quality of the texture of potato tubers has been demonstrated to decline over the period following harvest, when the potatoes are stored awaiting processing and distribution.

1.5.2 Potato Texture and the Cell Wall

The degree and extent of cell adhesion and separation, which ultimately determines the texture of plant-based foods, may be strongly related to the fine structure of the pectic polysaccharides at the edge of the cell faces. More specifically, it has been suggested that the relative abundance of non-esterified and methyl-esterified homogalacturonan plays a significant role in cell adhesion and separation in plants (Jarvis et al., 2003).

In dicotyledonous plants (dicots), the adhesion between adjacent cells is provided by pectic polysaccharides located within the middle lamella and the junctions at which three cell walls meet, sometimes referred to as tricellular junctions (Figure 1.17). The regions of the cell wall at which these junctions occur are subjected to extreme stresses, as the cells' turgor pressure tends to pull the cells apart. Studies by Knox et al. (1990) in carrot root, Liners and Van-Cutsem (1992) in suspension-cultured carrot cells and Roy et al. (1994) in ripe cherry tomato, have used immunogold labelling to determine structural characteristics of the cell walls, particularly at the tricellular junctions. Results have strongly suggested the concentration of pectic polysaccharides with a low degree of esterification in these areas, allowing for extensive cross-linking by calcium ions (Figure 1.14) (Goldberg et al. 1996) and thus resulting in highly fortified adhesion between adjacent cells in these zones.

In potato cells during the cooking process, intracellular starch becomes gelatinised and forms a network, the extent of which is determined by the starch content of the raw potato.

In potatoes with a high starch content, a higher turgor-like pressure is exerted on the points in the tricellular junctions at which cell walls meet (Figure 1.17) as a result of the swelling of the starch. The cells are therefore pulled apart. In terms of the texture of cooked potatoes, a high starch content results in a substantial reduction in intercellular adhesion and a mealy consistency. Potatoes low in starch produce a waxy texture on cooking as a result of the retention of a large proportion of adhesion between cells (Van Marle et al., 1992).

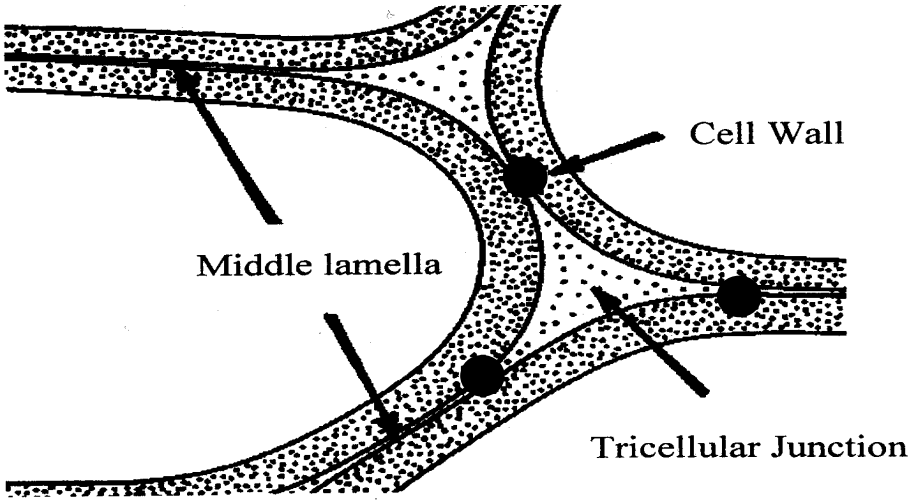


Figure 1.17 – Tricellular junction. ● denotes points of extreme stress (adapted from Jarvis and Doud, 1995)

Because the swelling of intracellular starch is the main cause of cell separation in potatoes, it could be speculated that the degree to which it occurs should correlate with starch, and consequently dry matter (DM) contents of different potato cultivars. It is also plausible to suggest that the extent of intercellular adhesion, and consequently potato texture, is influenced significantly by the nature and extent of the forces holding adjacent cells together. In other words, potato texture may be determined by the degree of esterified GA residues, whether esterified with methanol or another undetermined constituent, and consequently the extent of calcium cross-links formed in the middle lamella between the walls of each pair of cells.

During the storage of potatoes it has been found that the textural quality of tubers deteriorates (Van Marle et al., 1997). The origin of these textural changes is unknown but could be attributed to a number of structural and chemical characteristics. For example, changes in the starch content or changes in the distribution, quantity and composition of cell wall pectic polysaccharides.

One aim of this thesis is to determine whether or not the deterioration of the texture of potato tubers over the storage season can be attributed to the quantity of certain structural characteristics of pectic polysaccharides within potato tuber cell walls.

1.5.3 The History of Non-Methyl Ester Determination

Since the 1960's, researchers involved in the elucidation of the structure of pectin have concluded that chelating agents were not capable of extracting all pectins in plant cell walls (Cocking, 1960). This fact strongly suggests that forces other than the well-established Ca^{2+} cross-links (Jarvis, 1984) are involved in binding pectins within the cell wall. Since this time, researchers have been seeking to develop a repeatable, reliable, efficient method for the quantification of esters within the cell wall and subsequently to develop a method of determining the nature of covalent cross-links between pectin and another constituent of the cell wall.

A colorimetric method for the determination of pectin esterified with methanol was developed by Wood and Siddiqui (1971) and their findings related to pectin esterase metabolism. However, the methods used here were carried out on commercially available pectins rather than those extracted, in an unaltered state, from a plant cell wall. Therein lies the main hindrance in research with the aim of determining pectin structure. At this time, the main method used in pectin structural research involved a hot alkaline extraction, which resulted in depolymerisation of the pectic polysaccharides via β-elimination reactions. As a result, pectic polysaccharides extracted in this way do not necessarily reflect the structure of pectin *in situ*.

The problem of β-elimination was investigated by Keegstra et al. (1973) who attempted to extract pectin from cell walls without chemically altering the structure. Their findings strongly suggested the presence of covalent connections between xyloglucans and pectic polysaccharides in suspension-cultured sycamore cells. However, they concluded that pectin extraction required severe conditions that would inevitably result in the degradation of the polysaccharides in question.

Keijbets and Pilnik (1974) exploited the ability of pectin to reduce copper to quantify the total uronoyl content of pectins but this determination was carried out on commercially available apple pectin with a known degree of esterification. As early as the mid 1970's infrared spectroscopy was proposed as a useful tool to quantitatively determine pectic uronic acids (Bociek and Welti, 1975; Casu et al., 1978). However, the techniques

involved sample dispersal in D_2O, which interfered with sample analysis and resulted in an underestimation of total uronic acids (Casu et al., 1978).

Kim and Carpita (1992) and McCann et al. (1994) applied gas chromatographic methods to maize coleoptile cell walls and tobacco cell cultures respectively, to determine total pectic ester contents. The quantity of methyl-esterified galacturonosyl residues was determined following saponification and the presence of non-methyl esters confirmed in significant amounts in cell walls extracted from both monocots and dicots. However, the method used here was complex and relied on $NaBD_4$ to selectively reduce methyl-esterified uronic acid groups whilst leaving the free acid groups unreduced. $NaBD_4$, which has a natural pH of around 11, readily degrades to borate in non-alkaline conditions. In these conditions pectic esters may be hydrolysed rather than reduced, resulting in a lower figure for esterified galacturonosyl residues in the cell walls than the true value.

Brown and Fry (1993) isolated apparent non-methyl esters from the galacturonoyl fraction of pectin from suspension-cultured spinach plants. However, the specific nature of these esters was not determined. Hou and Chang (1996) provided further evidence for the presence of non-methyl esters and also indicated that, during the cooking of pea sprouts, changes occurred in the quantity of these esters. However, the colorimetric method employed here provided many possibilities for interference from non-galacturonoyl esters.

Diffuse reflectance Fourier transform infrared (FT-IR) spectroscopy has been utilised in the determination of the degree of esterification in commercially available pectin samples (Gnanasambandam and Proctor, 2000). This method allows the rapid analysis of a large number of samples but variations in spectral response between batches of samples introduce an element of unreliability to results.

MacKinnon et al., 2002 demonstrated that about 14% of the galacturonate units in potato pectins *in situ* carry intermolecular esters other than the established methyl or acetyl substituents. Titrimetric methods were used to quantify the total amount of anionic sites before and after saponification, hence measuring the esterified and total galacturonosyl units in the pectin in potato cell walls. A method involving the binding of copper(II) ions to non-esterified carboxyl groups on the pectic backbone was also employed. The methyl ester content was determined using a colorimetric method originally developed by Klavons and Bennett (1986). The same methods of analysis were applied to pectic polysaccharides of citrus origin and it was confirmed that all esters could be accounted for as being methyl esters.

The methods described above (MacKinnon et al., 2002) were employed in this work to determine whether the occurrence of methyl and non-methyl esters could account for the deterioration in the texture of potato tubers over the course of the storage season.

1.6 Aims and Objectives

Pectin methylesterase (PME) enzymes are responsible for the selective cleavage of methyl ester bonds on the pectic backbone, resulting in the softening of fruit and vegetables and leaf expansion. This process could result in the release of methanol to the troposphere, especially during the early, rapid stages of plant growth. Pectic non-methyl esters in the primary cell walls of plant tissue could potentially perform an intercellular bridging function, important in the separation and adhesion of adjacent cells. The initial aim of this work was to investigate the presence of these non-methyl esters in potato tuber and pea epicotyl cell walls and, in the case of potato tubers, relate the findings to the observed deterioration in tuber texture quality in the period of storage following harvest.

However, midway through my research the aims of the thesis changed. These original biochemical principles may be examined from a different angle, for example in the context of the formation of schizogenous aerenchyma in certain plant species or the potential role for waste potato material in the treatment of waste waters contaminated with heavy metal ions. However, my interests lay more towards understanding larger scale biogeochemical cycles and thus decided that, with the important introduction to the relevant biogeochemical processes, to direct the research towards a little-studied problem: the impact of plant emissions of methanol, as a result of pectin degradation, on the global troposphere. Thus, whilst the underlying principles involving the de-esterification of pectic polysaccharides and many of the methods remained, the focus of the research area shifted from biomolecular analysis to atmospheric chemistry. As a result of this, one aim of this thesis was to quantify the emissions of methanol from young, rapidly growing plants at different stages of their development.

As a result of the decomposition of leaf material during the autumn and winter months, the bonds between pectic methyl groups and the pectic backbone are cleaved, which may lead to the emission of methanol to the troposphere. Further aims of this thesis were to determine the stage of senescence at which methanol is released from leaves of species of tree native to Great Britain and to quantify the total amount of methanol contained in leaves from a variety of native tree species at the end of the summer in the form of pectic methyl esters. The ultimate aim of this work was to evaluate the approximate contribution

of UK plant-derived methanol sources, and compare these with one another and to current global totals.

Chapter Two - Methods and Materials for Pectic Ester Determinations

All glassware used in the chemical extraction and characterisation of the cell wall material (CWM) was washed in 2-5% Decon, rinsed twice with deionised water, rinsed with 0.2 M HCl and rinsed a further twice with deionised water. It was then thoroughly dried.

2.1 Methyl and Non-Methyl Ester Determination of Pea Epicotyl and Potato Tuber Primary Cell Walls

2.1.1 Growth and Harvest of Pea Seedlings

Pea (*Pisum sativum*) seeds (cv. Meteor; Dagg and Son, Bath Street, Glasgow) were soaked in deionised water overnight and grown on damp potting compost in the dark at 20 °C for 8 days. The seedlings were harvested using scissors. The main growing region, which is considered to be the top three centimetres of the epicotyl below the hook of the seedling (Martinezgarcia, 1992), was removed and discarded. Nodes were also removed, so that the plant material was as uniform as possible. The plant material was then frozen to prevent enzymic degradation of the cell wall components.

2.1.2 Harvest and Storage of Potato Tubers

Maris Piper potatoes were grown in Cambridgeshire and harvested on 24th August 2003. Estima potatoes were also grown in Cambridgeshire and harvested on 29th August 2003. Both varieties were transported to Sutton Bridge Experiment Station, Lincolnshire where Maris Piper and Estima tubers were stored at 4 ºC and 9 ºC respectively in nets. Representative samples of each variety of potato were sent to Glasgow at three different stages throughout the storage season, after 117, 215 and 299 days and 117, 218 and 299 days of storage for Estima and Maris Piper tubers respectively. A dozen potatoes from each representative sample were peeled, removing the periderm, the outer layer of dead cells, and diced into cubes with edges approximately 5 mm in length. Approximately 50 g from each sample was then sealed in a plastic bag and stored at –18 ºC until the analyses were carried out.

2.1.3 Extraction of Primary Cell Wall Material - Pea Epicotyl

This method was developed in order to isolate plant cell wall material in a form that resembles, as closely as possible, the cell wall in its natural state. Cell walls were isolated from frozen pea material. This method is based on the method used by Jardine (2002). The method was designed for various potato tissues, so some alterations were required. Starch removal was not necessary as 8-day-old pea epicotyls have a negligible starch content. This was verified by the absence of black spots in pea epicotyl material following staining with 1% KI / 0.5% I_2 solution and inspection under a light microscope.

The initial steps of the protocol were carried out in ice cold conditions to prevent extensive enzymic degradation. The entire procedure was carried out in mixed cation buffer (MCB), which mimics the conditions in the apoplast, thus retaining the natural cell wall conformation by restricting solubilisation of cell wall polymers, which may result from sudden changes in ionic conditions. The salt concentrations present in the MCB solution were derived from Goldberg et al. (1996) and are shown in Table 2.1.

Salt	Concentration (mM)
Sodium acetate	10
Potassium chloride	3
Magnesium chloride	3
Calcium chloride	1

Table 2.1 - Salt concentrations in the MCB

100 g frozen pea epicotyl tissue (cut into sections approximately 10 mm long) was blended in 300 mL chilled MCB, containing Triton-X detergent at a concentration of 2 mg L^{-1}. The detergent was necessary in order to disrupt the cell walls and solubilise cytoplasmic material and also to prevent poly-phenol-peptide interactions that may reduce the activity of enzymes utilised in future analytical methods. Approximately 5 mL octan-1-ol was added to the mixture prior to blending to prevent excess frothing. 150 g ice was also added so that enzymic degradation of the cell walls did not occur. The mixture was blended for six successive eight-second bursts.

The tissue was then transferred to and filtered through a 200 μm sieve (to collect any vascular tissue) above a 53 μm sieve. The washings have been found to contain negligible amounts of solubilised pectins (MacKinnon, 2002), not in large enough quantities to justify

retention of the washings. The cell wall material (CWM) retained in the 53 μm sieve was rinsed with cold MCB followed by methanol in the sieve.

The CWM was transferred to a soxhlet flask using 200 mL 80% methanol and boiled gently for 10 minutes to denature endogenous enzymes. The suspension was allowed to cool and then transferred to a 53 μm sieve, with several thorough washings with deionised water. It was imperative that all methanol was removed as any remaining methanol would interfere in future analyses. The CWM was transferred to a grade 4 glass sintered funnel using deionised water and rinsed several times with 100% acetone to remove all traces of moisture. The CWM was then removed from the funnel and left in a sinter to dry overnight.

2.1.4 Modifications in the Protocol for Extraction of Primary Cell Walls for Frozen Potato Tuber

100 g frozen potato tuber material was blended together with 150 g ice, 300 mL chilled MCB (with identical ionic composition as that used in the original protocol) containing 2 mg L^{-1} Triton detergent and approximately 5 mL octan-1-ol. Endogenous enzymes were denatured by boiling the plant material gently in 80% methanol for ten minutes. The primary cell wall structure was then disrupted by cryo-grinding.

Small quantities of plant material were added to a mortar containing liquid nitrogen and subsequently ground in a small coffee grinder for two successive bursts of ten seconds. Portions of the frozen, ground material were added to boiling MCB and stirred for approximately 40 seconds. Immediately following this, the potato CWM was decanted into a volume of chilled MCB, thus allowing rapid cooling of the sample to gelatinise the starch present, while causing minimal degradation of cell wall polymers.

Approximately 1 g α-amylase (containing approximately 13,400 units where 1 unit will liberate 1 mg maltose from dissolved starch in 3 minutes at 20 °C) was added to the suspension. Excess amylase was used as the exact amount of starch present in the samples was unknown. 0.03 μL pullulinase (containing 0.4 units, where one unit will liberate 1 μM maltotriose from pullulan in one minute at 25 °C) was subsequently added in order to remove the amylopectin branch points in the starch polymers.

Approximately 2 mL toluene was also added to prevent microbial degradation and the mixture was incubated overnight on orbital shaker (vortex) at 25 °C. Complete starch removal was verified following staining with 1% KI / 0.5% I_2 solution and inspection under a light microscope. Once starch removal was complete, the potato CWM was rinsed thoroughly in 100% acetone and allowed to dry overnight.

2.1.5 Chemical Characterisation of Potato Tuber and Pea Epicotyl Primary Cell Walls

The analyses undertaken in order to characterise the primary cell walls isolated from both pea epicotyl and potato tuber material were identical, with the exception of the determination of residual starch, which was carried out on samples isolated from potato tuber only.

2.1.5.1 Determination of Residual Starch in MCB-insoluble Material Isolated from Potato Tuber

This procedure was based on the enzymic methods developed by Batey (1982) and Karkalas (1985). Residual starch was simultaneously gelatinised and hydrolysed by heating in suspension with thermostable α-amylase at 94 ºC. Amyloglucosidase and o-dianisidine were then added sequentially in order to firstly hydrolyse the maltose to glucose and secondly oxidise the liberated glucose, which forms a coloured complex upon reaction with o-dianisidine. The procedure was carried out in order to quantify starch not removed by treatment with α-amylase/pullulinase (Jardine, 1998). This quantification ensures that subsequent results are calculated accurately; taking into account the fact that a certain proportion of the cell wall mass is comprised of starch.

To a screw-cap Pyrex tube, 25 mg potato tuber CWM, 5 mL 0.05 M TRIS buffer (where the buffer pH had been reduced from approximately pH 11 to pH 8.2 using about 25 mL 0.1 M HCl) and 0.1 mL thermostable α-amylase were added and boiled for one hour. After the suspensions were cooled, they were centrifuged. 1 mL of the supernatant was removed from each sample and to each 0.2 mL amyloglucosidase from *Aspergillus niger* (diluted to 45 units per mL where 1 unit liberates 1 mg of glucose from starch in 3 minutes at pH 4.5 and 55 ºC) was added. The solutions were then incubated for 2.5 hours to produce glucose.

Glucose standards of concentrations between 10 and 80 mg L^{-1} were made up in distilled water. 3.5 mL of a mixed enzymic solution were added to both samples and standards. Solutions were then incubated at 25 ºC in the dark for fifty minutes.

The mixed enzymic solution contained the following:

1. 65 mg glucose oxidase type II (containing 15000 units) from *Aspergillus niger*, Sigma (where 1 unit liberates 1 mg of glucose from starch in 1 minute at pH 5.1 at 35 ºC),

2. 3.5 mg peroxidase type I (containing 300 units) from horseradish, Sigma (where 1 unit liberates 1 mg purpurogallin from pyrogallol in 20 seconds at 20 ºC),

3. 50 mg o-dianisidine

As the glucose resulting from the depolymerisation of starch reacts with this enzymic mixture, glucose is oxidised to gluconic acid, liberating hydrogen peroxide. A red colour is produced as a result of the oxidation of o-dianisidine by hydrogen peroxide in the presence of peroxidase. Absorbances of samples, standards (glucose solutions ranging from 0.1 to 1.0 mg mL^{-1}) and blanks were measured at 455 nm, in triplicate, in 4.5 mL disposable plastic cuvettes.

2.1.5.2 Determination of Non-esterified Carboxylic Acid Content

Two different methods were used to measure the extent of carboxyl groups present in the cell wall that were not esterified.

2.1.5.2.1 Titration with NaOH

This method was developed by MacKinnon et al. (2002). The cell walls were acidified with dilute acid in aqueous ethanol solution to remove ester groups present on the homogalacturonan (HG) backbone. The resulting acidified cell walls were then titrated against dilute NaOH. 70% ethanol was used in this procedure so that if any hydrolysis of the polysaccharides occurred, they were not released into solution. It was imperative that analytical grade ethanol was used in order to eliminate the possibility of contamination.

0.125 g isolated CWM was acidified overnight with 10 mM HCl in 70% ethanol at 4 °C. A second batch of CWM was acidified for two nights to ensure that, following

acidification, all cell wall polysaccharides were present in the free acid form. The acid was completely removed in a grade three sintered glass funnel with several thorough washings using 70% ethanol. To ensure that all the acid was removed, the pH of each rinse was monitored. The acid was judged to be completely removed when the filtrate had a pH greater than 4. The CWM was then washed thoroughly with acetone and left to dry.

0.03 g CWM was transferred to a three-necked round-bottomed flask using 50 mL degassed 0.1 M NaCl. Degassed 0.01 M NaOH was then titrated against this under nitrogen gas. Degassing of the solutions was achieved by submerging each in an ultrasonic bath for 10 minutes immediately prior to the analysis. It was important to remove carbon dioxide from all solutions in this procedure as carboxyl ions may artificially lower the pH of the solutions thus increasing the volume of NaOH required to reach the end point. The pH of the CWM suspension was monitored closely throughout and the titration was halted at pH 7. When the titration neared the end point, NaOH was added gradually and the pH reading was allowed to stabilise, which took about two minutes. Deesterification would take place if the pH of the solution was allowed to rise above pH 7 for any length of time, resulting in a higher than actual value for non-esterified carboxylic acid content.

2.1.5.2.2 Copper Binding Method

Divalent copper cations bind chemically to non-esterified galacturonic acid residues in pectin molecules. These ions can subsequently be removed using excess acid. The copper (II) content in the resulting filtrate gives a value for the amount of galacturonic acid residues that are not esterified.

The pH of the copper solution is of utmost importance in this procedure. The pH must be maintained at approximately 6.1. If the pH is greater than 6.1, $CuOH^+$ will bind to the non-esterified sites, resulting in a higher value for non-esterified carboxyl acid content than is actually present in the pectin molecule (i.e. the binding of the copper to non-esterified carboxyl groups will exceed the stoichiometric level). If the pH is significantly less than 6, a proportion of the carboxyl groups will become protonated, reducing the proportion of galacturonic acid residues carrying a negative charge and thus resulting in a smaller measurement for bound copper (Jardine et al., 2002; Keijbets and Pilnik, 1974).

0.075 g CWM were saturated in 30 mL 0.2% copper acetate for 30 minutes. Copper acetate was chosen as the pH is approximately 5.9, very similar to the natural pH of CWM. This procedure was also carried out using 2.0% copper acetate, 2.0 % copper sulphate and

0.2% copper sulphate, in order to find out how the values for chemically bound copper varied with the concentration and pH of the copper solutions. The pH and conductivity of the solutions were monitored throughout the procedure.

The copper not chemically bound to the pectin was removed using deionised water in a grade four glass sintered funnel. All unbound copper was considered to have been removed when the conductivity of the filtrate was less than 10 μS. Bound copper was removed using approximately 40 mL 1 M HNO_3 in 60% ethanol. Each replicate was made up to 50 mL, diluted to 1 in 10 and analysed by atomic absorption.

2.1.5.3 Determination of Total Galacturonic Acid

This method uses the same principles as the titration method for determination of the non-esterified carboxyl acid content (Section 2.1.5.2.1). However, CWM was saponified prior to acidification to remove ester groups present on the HG backbone of the pectic polysaccharides. Figures obtained from these analyses can then be subtracted from each other in order to determine the ester content of the pectin present.

0.125 g isolated CWM was saponified overnight with 0.1M NaOH in 70% ethanol at 4 °C to remove ester groups from the HG backbone of pectic polysaccharides present. CWM was then acidified overnight with 10mM HCl in 70% ethanol at 4 °C in order to ensure that the resultant free carboxyl groups were completely acidified. Following acidification, the CWM was washed thoroughly in a grade 3 sintered glass funnel using 70% ethanol. Several washings were required in order to remove all acid not associated with the pectin. The pH of the filtrate was monitored and it was judged that all non-bound acid was removed when the pH of the filtrate was greater than 4.

0.03 g CWM was transferred to a three-necked round-bottomed flask using 50 mL degassed 0.1 M NaCl. Degassed 0.01 M NaOH was then titrated against this under nitrogen gas. The pH of the CWM suspension was monitored closely throughout and the titration was halted at pH 7. When the titration neared the end point, NaOH was added gradually and the pH reading was allowed to stabilise, which took about two minutes. De-esterification would take place if the pH of the solution was allowed to rise above pH 7 for any length of time, resulting in a higher than actual value for non-esterified carboxylic acid content.

2.1.5.4 Methyl Ester Determination

The extent of methylation of esters in cell walls was measured using a method based on that used by Klavons and Bennet (1986). This involves the cleavage of the methyl ester groups from the pectic molecules, resulting in the release of methanol. The methanol is then oxidised to formaldehyde using alcohol oxidase. The formaldehyde concentration can subsequently be measured colorimetrically on addition of pentanedione, which forms a coloured complex.

12 mg isolated CWM was weighed accurately in a plastic centrifuge tube and 6.25 mL distilled water followed by 1 mL 1 M KOH was added. The resulting suspensions were left at room temperature for one hour, after which time they were neutralised with 1 mL 0.49 M H_3PO_4. Controls consisting of CWM with 6.25 mL distilled water and 1 mL acid followed by the addition of 1 mL KOH were simultaneously made up. These suspensions had an identical ionic composition as the samples, however the ester groups were not cleaved from the HG. Samples and controls were centrifuged for 5 minutes at 3000 rps, releasing 1 mL of supernatant.

Methanol standards ranging between 2 and 16 μMoles in 50 mL volumetric flasks were made up in phosphate buffer (0.2 M monobasic and 0.2M dibasic sodium phosphate) from a 10 mM methanol stock solution in phosphate buffer. Samples, standards and controls were incubated for 15 minutes at 25 ºC with 1 mL alcohol oxidase (1 unit mL^{-1}) from *Pichia pastoris* (Sigma, where 1 unit oxidises 1 μmole of methanol to formaldehyde at pH 7.5 at 25 ºC). 2 mL 0.02 M 2,4-pentanedione in 2 M ammonium acetate and 0.05 M acetic acid was added to each sample, standard and control and solutions were subsequently incubated at 60 ºC for 15 minutes. The solutions were cooled and the absorbance measured at 412 nm in 4.5 mL plastic cuvettes using a Hitachi U-1500 spectrophotometer.

If sample dilutions were required, they were made up prior to enzymic incubation, so all suspensions containing CWM were maintained until satisfactory results were obtained.

2.2 Pectic Methyl Ester Content of Mature Leaves from Native Tree Species

2.2.1 Collection of Mature Leaf Material

Approximately 100 g of fully matured leaves (prior to colour change and abscission) were collected from trees of twelve indigenous species at the end of August and beginning of September 2005 from three sites in Scotland. The tree species and sites from which they were collected, together with the dates on which they were collected, are listed in Table 2.2.

Tree Species	Location of Trees	Date of Leaf Harvest
Oak (*Quercus robur*)	Carstramon Wood, Gatehouse-of-Fleet	29th August
Wych Elm (*Ulmus glabra*)	Carstramon Wood, Gatehouse-of-Fleet	30th August
Ash (*Fraxinus excelsior*)	Carstramon Wood, Gatehouse-of-Fleet	31st August
Beech (*Fagus sylvatica*)	Carstramon Wood, Gatehouse-of-Fleet	32nd August
Sycamore (*Acer pseudoplatanus*)	Carstramon Wood, Gatehouse-of-Fleet	33rd August
Horse Chestnut (*Aesculus hippocastanum*)	Glasgow University Campus	30th August
Silver Birch (*Betula Pendula*)	Glasgow University Campus	30th August
Lime (*Tilia x europea*)	Botanic Gardens, Glasgow	5th August
Alder (*Alnus glutinosa*)	Botanic Gardens, Glasgow	5th August
Scots Pine (*Pinus sylvestris*)	Botanic Gardens, Glasgow	5th August
Yew (*Taxus baccata*)	Botanic Gardens, Glasgow	6th August
Hazel (*Corylus avellana*)	Botanic Gardens, Glasgow	6th August
Oilseed Rape (*Brassica napus*) Leaves	Lochwinnoch	2nd September
Oilseed Rape (*Brassica napus*) Flowers	Lochwinnoch	2nd September

Table 2.2 - Tree species, the names of sites from which leaves were collected and dates of collection

Leaves were collected from at least three typical healthy trees of each species from the locations listed in Table 2.2. Discoloured leaves (for example due to attack by micro organisms) were not included in the sampling process. Due to the possibility of losses of methyl esters from the pectic polysaccharides within the cell walls of sampled leaves as a result of the action of indogenous PME immediately after removal from the tree, leaves were frozen at -18°C within three hours of sampling until analyses were carried out.

2.2.2 Extraction of Primary Cell Wall Material – Mature Leaves

This method was adapted from those protocols utilised in the extraction of pea epicotyl cell wall material (Section 2.1.3), based on the method used by Jardine (2002).

20 g frozen leaf material, together with 150 g ice, approximately 5 mL octan-1-ol and 300 mL chilled MCB containing Triton-X detergent at a concentration of 2 mg L^{-1}, was blended for ten successive ten second bursts.

The blended plant tissue was then quantitatively transferred to and filtered through a 200 μm sieve (to collect any vascular tissue) above a 53 μm sieve. The CWM was rinsed several times with cold MCB followed by acetone in each of the two sieves and ice was added as required. The fine plant material and subsequently the vascular tissue, was then transferred quantitatively to a grade 3 sintered glass funnel and rinsed thoroughly with acetone, which was completely removed under suction. The fine CWM was transferred to a pill jar and placed in an oven at 100 ºC overnight. The vascular tissue was treated in the same way. The CWM was not boiled in 80% methanol in this procedure because of the risk of residual methanol, which would contribute greatly to the determination of the methyl ester content of the pectic cell wall ploysaccarides.

2.2.3 Determination of Methyl Ester Content in Mature Leaf Primary Cell Wall Material

The total amount of homogalacturonan (HG) monomers carrying a methyl ester group in the cell walls of mature leaves was determined using a method based on that developed by Klavons and Bennet (1986), adapted from the method described in Section 2.1.5.4. This involves the cleavage of the methyl ester groups from the pectic molecules, resulting in the release of methanol. The methanol can then be converted to formaldehyde using alcohol oxidase. The formaldehyde concentration can then be measured colorimetrically on addition of pentandione, which forms a coloured complex with formaldehyde.

The percentage yield of CWM from the original 20 g frozen leaf material used in the cell wall isolation method was determined. The total weights of both fine material and vascular tissue obtained from the cell wall isolation method were also obtained. 12 mg of isolated CWM, comprised of the same proportion of fine and vascular tissues as was present in the original frozen leaf material of each of the sample species, was weighed into a plastic centrifuge tube. 6.25 mL deionised water and 1 mL 1 M KOH were added and the suspensions were left for 1 hour. This solution was neutralised with 1 mL 0.49 M H_3PO_4. Controls were made up using 12 mg CWM comprised of fine and vascular CWM in the same proportions as in the sample analyses. 6.25 mL water and 1 mL H_3PO_4 were added to each. Controls were left for 1 hour before neutralisation with 1 mL 1 M KOH.

The following concentrations of methanol standard solutions were made up from a 1 M methanol stock solution in deionised water using a phosphate buffer (0.2 M monobasic, 0.2 M dibasic sodium phosphate solution): 0, 1, 2, 3, 4, 5, 6, 8 and 10 μg mL^{-1} methanol.

1 mL of each sample, control and standard solution was incubated at 25 °C with 1 mL alcohol oxidase (1 unit mL^{-1} activity) for 15 minutes. 2 mL 0.02 M 2,4-pentandione (acetyl acetone) in 2 M ammonium acetate and 0.05 M acetic acid were added.

The solutions were incubated at 60 °C for 15 minutes and the absorbance at 412 nm was measured in plastic cuvettes using a Hitachi U-1500 spectrophotometer.

2.3 Determination of the Stage of Senescence at which Methanol is Lost from Leaves

2.3.1 Sampling of Leaf Material From Mugdock Country Park

Oak (*Quercus petraea*), alder (*Alnus glutinosa*) and beech (*Fagus sylvatica*) trees were selected as the three sample species to be studied as they exhibit very different trends in relation to the senescence and abscission of their leaves. For example, premature abscission is a characteristic of alder trees, resulting in the majority of senescence of alder leaves occurring on the ground. Conversely, beech leaves appear to remain on the trees for a relatively long period of time following senescence.

Approximately 50 g of leaves from oak, beech and alder trees were collected at three different dates throughout October, November and December 2005, at approximately monthly intervals. Three sample trees of each of the three sample species (oak, alder and beech) were selected from three different sites within the same area located within Mugdock Country Park (Figure 2.1). All three sites are located within an area occupied by deciduous woodland. The National Grid References for the centre of Sites A, B and C are NS 54617590, NS 54797595 and NS 54877592 respectively.

Leaves classed as completely green, intermediate and completely brown were collected, where available, off the branches of selected sample trees and also from the ground immediately surrounding the sample trees.

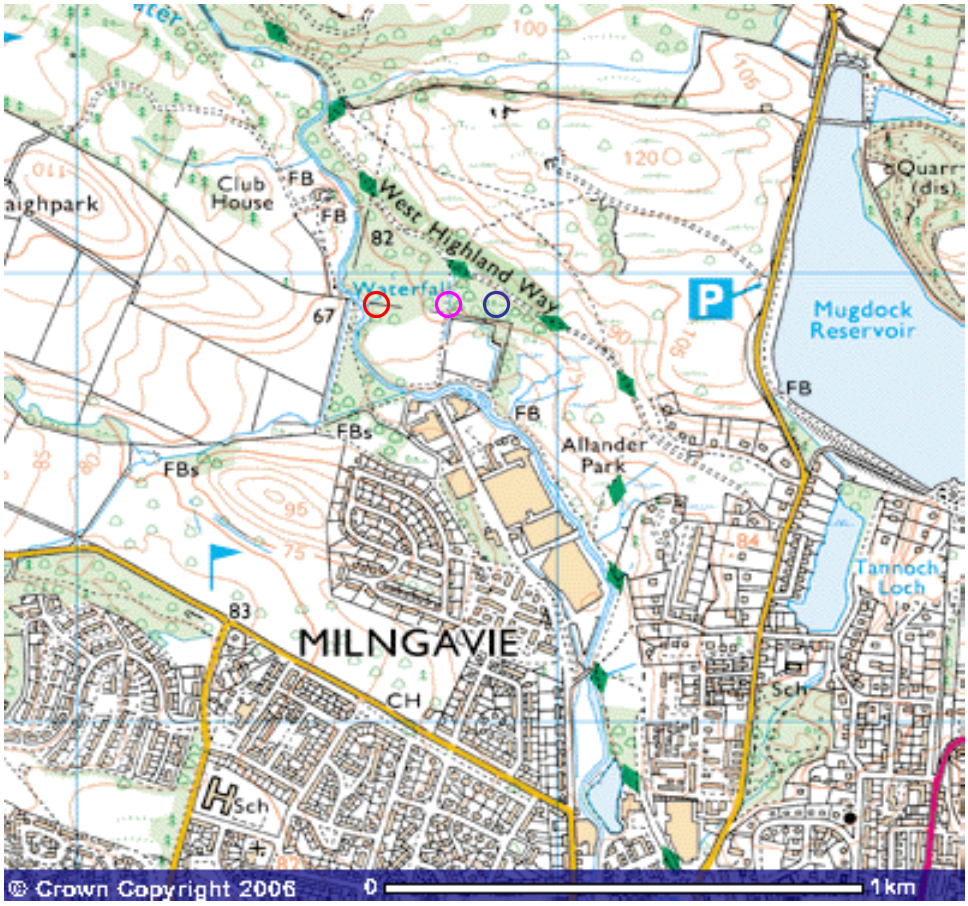


Figure 2.1 - Map showing positions of the three sampling sites. Red, pink and blue circles denote Sites A, B and C respectively

Estimates were recorded of the proportion, in percentage terms, of leaves that were green, intermediate and brown (the colour immediately preceding abscission) (see Figures 2.4-2.6), both for the leaves on the branches of selected sample trees and for those leaves that had apparently been abscised by each of the individual trees. Leaves at different stages of senescence were collected from sample trees in approximately the same proportions as they were present on the trees. The apparent proportions of leaves that had abscised previous to the sampling dates were also estimated in percentage terms for each of the nine sample trees on each site.

1 metre-square quadrats were placed in precise, random positions around the base of sample trees and the proportion of the quadrat area comprised of green, intermediate and brown leaves apparently originating from that specific sample tree was estimated and recorded in percentage terms. On each of the three sampling dates, the quadrats were placed in their original, specific positions, which had been accurately recorded on the original sampling date. Representative samples were collected from the ground by sampling leaves at different stages of senescence from around the trunk of each of the

sample trees in similar proportions to the estimates made from the quadrats. Those leaves sampled from the ground were collected from areas surrounding the base of the particular sample tree out-with the quadrat positions so that leaf coverage was not underestimated during subsequent sampling visits. The moisture content and subsequently the methyl ester content of leaf samples collected from each of the three sample trees of each of the three sample species from each of the three sample sites, for leaves sampled from both the tree and the ground surrounding the tree were determined.

Figure 2.2 - Examples of a green (leaf on the left), intermediate (two central leaves) and brown (leaf on the right) oak leaves as collected from Mugdock Country Park in order to determine the stage of decomposition at which methanol is lost from leaves

Figure 2.3 - Examples of a green (leaf on the extreme left), intermediate (two central leaves) and brown (leaf on the extreme right) alder leaves as collected from Mugdock Country Park in order to determine the stage of decomposition at which methanol is lost from leaves

Figure 2.4 - Examples of a green (leaf on the left), intermediate (four central leaves) and brown (leaf on the right) beech leaves as collected from Mugdock Country Park in order to determine the stage of decomposition at which methanol is lost from leaves

2.3.2 Extraction of Cell Wall Material - Leaf Samples from Mugdock Country Park

This cell wall isolation method is based on the method for the extraction of cell wall material from mature leaves (Section 2.2.3) and based on the method used by Jardine (2002).

20 g frozen leaf material, in the proportions of green, intermediate and brown (colour immediately preceding abscission) leaf that were present in the sample, was blended for ten successive ten second bursts together with 150 g ice, approximately 5 mL octan-1-ol and 300 mL chilled MCB containing Triton-X detergent at a concentration of 2mg L^{-1}.

The blended plant tissue was then transferred quantitatively to and filtered through a 53 μm sieve. The cell wall material (CWM) was rinsed several times with cold MCB followed by acetone and ice was added as required. The CWM (including both the fine material and vascular tissue) was transferred quantitatively to a grade 3 sintered glass funnel and rinsed thoroughly with acetone, which was completely removed under suction. The CWM was transferred to a pill jar and placed in an oven at 100 ºC overnight.

2.3.3 Determination of Methyl Ester Content in Primary Cell Wall Material of Leaves Sampled from Mugdock Country Park

The determination of methyl ester content in the cell walls of leaves collected from different sites in Mugdock Country Park was based on the method used by Klavons and Bennet (1986) and adapted from the method described in Section 2.2.3.

A representative sample of 50 mg CWM, comprised of a representative proportion of both fine and vascular CWM, was weighed out into a plastic centrifuge tube. 8 mL deionised water and 1 mL 1 M KOH were added to the samples. Controls were made up simultaneously by adding 8 mL water followed by 1 mL 0.49 M H_3PO_4 to 50 mg CWM. The suspensions were left for 1 hour at 25 °C, after which time samples and controls were neutralised with 1 mL 0.49 M H_3PO_4 and 1 M KOH respectively.

Methanol standards of the following concentrations were made up from a 1 M methanol stock solution in deionised water using a phosphate buffer (0.2M monobasic, 0.2M dibasic sodium phosphate solution): 0, 1, 2, 3, 4, 5, 6, 8 and 10 μg mL^{-1} methanol.

1 ml of supernatant was removed from each of the samples, controls and standards and incubated at 25 °C with 1 mL alcohol oxidase (1 unit/mL activity) for 15 minutes. 2 mL 0.02 M 2,4-pentandione (acetyl acetone) in 2 M ammonium acetate and 0.05 M acetic acid were added.

The solutions were incubated at 60 °C for 15 minutes. The absorbance of the resulting coloured compound was measured at 412 nm in plastic cuvettes using a Hitachi U-1500 spectrophotometer.

2.4 Methyl Ester Determination of Barley Seedling Primary Cell Walls

2.4.1 Introduction

The total amount of methanol present in barley seedlings at various stags of development was determined. The purpose of these analyses was several-fold. The methyl ester content of very young barley seedlings could be quantified, together with how this characteristic altered over time as the seedlings developed. These data could also be combined with the

results of the quantification of methanol emitted from barley seedlings using GC-FID (Section 3.4) and extrapolated in order to determine the proportion of the total methyl ester content of seedlings at early stages of development that is emitted to the troposphere as methanol as the plants grow. Using the results from this calculation, the residual methyl ester content of the barley plants following a typical growing season could be determined, thus determining the amount of methanol that could be released to the troposphere as a result of the decomposition of barley straw.

2.4.2 Extraction of Primary Cell Wall Material – Barley Seedlings

Barley seedlings were harvested at three different stages of their development and the cell wall material was extracted from the plant material. The method used in this procedure was based on the method developed by Jardine et al. (2002) and developed from that utilised in Section 2.2.3.

Six seedlings were harvested after 6, 9,11 and 13 days of growth. The plant material was blended for ten successive ten second bursts together with 100 g ice, approximately 5 mL octan-1-ol and 200 mL chilled MCB containing Triton-X detergent at a concentration of 2 mg L^{-1}.

The blended plant tissue was then transferred quantitatively to and filtered through a 53 μm sieve. The CWM was rinsed several times with cold MCB followed by acetone and ice was added as required. The CWM (including both the fine material and vascular tissue) was transferred quantitatively to a grade 3 sintered glass funnel and rinsed thoroughly with acetone, which was completely removed under suction. The CWM was transferred to a small glass vial and placed in an oven at 100 ºC overnight.

2.4.3 Determination of Methyl Ester Content in Primary Cell Wall Material of Barley Seedlings

The method used by Klavons and Bennet (1986) and described in detail in Section 2.2.3 was used in order to quantify the methyl ester content of primary cell wall material extracted from barley seedlings at different stages of growth.

12 mg of isolated CWM was weighed out into a plastic centrifuge tube. 6.25 mL deionised water and 1 mL 1M KOH were added to the samples. Controls were made up at the same time. The suspensions were left for 1hour at 25 °C, after which time samples and controls

were neutralised with 1 mL 0.49 M H_3PO_4 and 1 M KOH respectively. Methanol standards of concentrations between 1 and 10 μg mL^{-1} methanol were made using a phosphate buffer (0.2 M monobasic, 0.2 M dibasic sodium phosphate solution).

1 mL of supernatant was removed from each of the samples, controls and standards and incubated at 25 °C with 1mL alcohol oxidase (1unit/mL activity) for 15 minutes. 2 mL 0.02 M 2,4-pentandione (acetyl acetone) in 2 M ammonium acetate and 0.05 M acetic acid were then added. The solutions were incubated at 60 °C for 15 minutes and the absorbance at 412 nm was measured in plastic cuvettes using a Hitachi U-1500 spectrophotometer.

Chapter Three -
Method Development for the Quantification of Methanol in Plant VOC Emissions

3.1 Introduction – Methods Previously Used in the Quantification of Methanol in the Atmosphere

Determining the quantity of methanol emitted from plants has proven to be a complex, arduous challenge for a variety of reasons. The highly volatile, reactive nature of methanol demands a reliable capturing technique followed by rapid transfer to the researcher's chosen instrumentation for quantification. In the case of quantifying methanol emitted from growing plant material, the plant or part of plant being studied must be enclosed in such a way as to minimise the impact on the typical physiological functioning of the plant.

The particular methods implemented in the quantification of methanol emitted from plants vary between groups of researchers, with no particular method having been proven more reliable or accurate than another. Novel analytical techniques, designed with the aim of improving the ease of the procedure and accuracy of results in the quantification of methanol emitted from plants, are consequently continually being developed and implemented.

The most commonly applied techniques used to quantify methanol and indeed VOC's in general emitted from plant material, incorporate gas chromatography (GC) in one form or another. GC may be coupled with a number of detector systems, the role of which is to identify individual volatile species within a sample. For example, GC-FID (GC coupled with a flame ionisation detector) is a well-established method for the quantification and identification of volatile hydrocarbons. This technique may additionally be coupled with mass spectrometry (MS) in order to determine the identity of unknown compounds or confirm the identity of species within the sample, present in concentrations in the order of parts per million or parts per trillion per volume. The highly unstable chemical nature of methanol compared to other common VOC's emitted from plants, such as isoprene and terpenoid compounds, means that some analytical techniques employed in the quantification of these VOC's are unsuitable for methanol quantification.

In work which originally confirmed the emission of methanol from plants in quantities comparable to those of isoprene, MacDonald and Fall (1993) devised a method using gas chromatography coupled with a flame ionisation detector (GC-FID) to quantify methanol

emitted from a variety of plant species. Nemecek-Marshall et al. (1995) utilised a water-jacketed, clamp-on cuvette, originally devised by Fall and Monson (1992) to determine isoprene emission rates from certain tree species, to quantify methanol emitted from the abaxial and adaxial surfaces of leaves. Methanol emitted from each of the leaf's surfaces was quantified using either the GC-FID method developed by MacDonald and Fall (1993) or a colorimetric method involving methanol oxidase.

Jordan et al. (1992) quantified an array of volatile organic compounds such as alcohols, although not including methanol, aldehydes, and aromatic compounds emitted from *Ceratiola ericoides*, an evergreen shrub native to southern USA. Leaves and stems were placed in a 'purge vessel' and the volatiles adsorbed onto Tenax TA prior to thermal desorption and quantification using GC-MS. Hakola et al. (1998) enclosed a branch of a willow enclosed in a Teflon chamber to quantify the emission rates of terpenes and isoprene. The collected volatiles were pumped through a canister containing Tenax TA which was subsequently thermally desorbed and analysed using GC-FID.

Other techniques such as Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) (Fall et al., 1999), vapour phase-Fourier transform infrared spectrometry (FT-IR) (Garrigues et al., 1997) and Headspace Solid Phase Microextraction (HS-SPME) (Alcaraz Zini et al., 2001) have also been used in the determination of VOC's emitted from plants or other media. PTR-MS allows the quantification of VOC's without a pre-concentration step which may bias the detection of compounds more efficiently being trapped on adsorbent material (Fall et al., 1999). The utilisation of FT-IR allows quantitative monitoring of methanol in the vapour phase following volatilisation in a heated glass chamber and transportation into an FT-IR gas cell (Garrigues et al., 1997). SPME is a simple, sensitive pre-concentration and extraction technique which, when coupled with GC and a sensitive detection system, may semi-quantitatively determine VOC emission rates from plants (Alcaraz Zini et al., 2001).

3.2 Development of Method

3.2.1 Introduction

The method of plant volatile quantification developed by Nemecek-Marshall et al. (1995) from initial work carried out by MacDonald and Fall (1993) was used as a starting point in developing a headspace capture and purge technique for the quantification of methanol emitted from growing plants. A headspace capture and purge sample collection system was considered to be more reliable than a continuous-flow adsorption technique, as it

significantly reduces the risk of analytes being removed from the adsorbent material by the continuous through-flow of air. This method also minimises disturbance to the atmosphere within the chamber during the sampling procedure as only a small proportion of the air within the chamber was actually removed for a single analysis. A method involving the collection of a headspace sample over a period of time, followed by sample concentration on an adsorbent material, could also easily be adapted to sample many different volatiles from a wide variety of plant materials, growing or decomposing in different environmental conditions. The development of a much larger chamber than was utilised by MacDonald and Fall (1993) allowed the incubation of larger or whole plant samples, which would contribute to the accuracy of the scaling-up of emissions data collected using this method.

Due to economic constraints, GC-FID was considered to be the most appropriate method of methanol quantification, as the necessary instrumentation was readily available. Multiple GC columns were tested to determine the most appropriate for the analysis. The column decided upon as being most useful in this application was different to that used by researchers in previous studies of this nature (for example Nemecek-Marshall et al. (1995)). This demonstrates that there is some flexibility in the choice of column for the quantification of methanol in headspace samples. Whereas Nemecek-Marshall et al. (1995) contained a single leaf and quantified methanol from abaxial and adaxial leaf surfaces, in order to speculate on the potential amount of methanol emitted by certain plant species on a global scale, it was deemed more logical to quantify methanol emissions from whole plants growing hydroponically. Volatiles emitted by the plant material studied by this group were concentrated onto the GC column using a liquid nitrogen cryotrap, a technique also employed in this work.

Solid-phase micro extraction (SPME) was also explored as a possible method for the quantification of methanol emitted from plant material. However, although this technique is a useful semi-quantitative method for the detection of low-molecular weight volatiles, it was considered to be lacking the level of accuracy required for the analyses in this study.

3.2.2 Sample Analysis

Gas chromatography (GC) with flame ionisation detector (FID) was considered to be a suitable method for the analysis of methanol in a headspace sample as, by incorporating an appropriate column, trace volatiles can be isolated and quantified from a complex gaseous mixture. GC-MS was also considered to be a viable option in the quantification of

methanol although this method is complicated by the fact that O_2, which is present in reagent gases, has the same molecular ion mass as methanol (32).

A Hewlett Packard 5890 GC-FID split injection system coupled with a Varian 4270 integrator was considered to be a suitable instrument for the quantification of methanol in headspace samples. The temperature was initially set at 50 °C although this was altered throughout the development of the method as needed to alter retention times on chromatograms. The injector and detectors were 220 and 200 °C respectively. The He carrier flow rate was initially 45 mL s^{-1} but this was also altered as necessary throughout the method development.

3.2.3 Sample Collection

The initial concept, as shown in Figure 3.1, involved encapsulating a piece of growing plant material in an airtight, transparent container, and was based on a system developed by Nemecek-Marshall et al. (1995). The gases evolved by the plant material would then be circulated continuously through an adsorbent material such as Tenax using a peristaltic pump. Suitable flow rates are considered to be between 20 and 40 mL min^{-1}.

Tenax-TA (80/100 mesh (Supelco, Bellefonte, PA)) was initially considered to be a suitable adsorbent material for this method of methanol analysis due to its physical properties. These properties include very high thermal stability (Sakodynskii et al., 1974), which allows the material to be conditioned and desorbed at very high temperatures with no detrimental effects. Tenax-TA has no affinity for water (Russell, 1975) which, if present on the adsorbent material, would result in the rapid saturation of the adsorbent resin. Tenax-TA is a porous polymer [poly(p-2,6 diphenyleneoxide)] which has been specifically designed for the trapping of volatiles and semi-volatiles from matrices including air. Tenax-TA can be conditioned as many as fifteen times without affecting its trapping efficiency. It can also be stored for up to three weeks without significant loss of adsorbed compounds (Pellizzari et al., 1976).

An alternative adsorbent resin, also suitable for the collection of small volatile compounds is Carbosieve SIII (60/80 mesh (Supelco, Bellefonte, PA)). This particular carbon molecular sieve has a higher affinity for water than most other adsorbent resins, but the small pore size provided by this resin is ideal for trapping the smallest volatile compounds, such as methanol. Carbosieve SIII can also be used up to a temperature of 400 °C.

Experiments were conducted using both adsorbent resins to determine the impact of resin type on the quantification of methanol emissions from growing plants.

Prior to being packed with adsorbent material, glass tubes must be silanised to prevent the occurrence of reactions between the active sites on the glass and analytes in the sample.

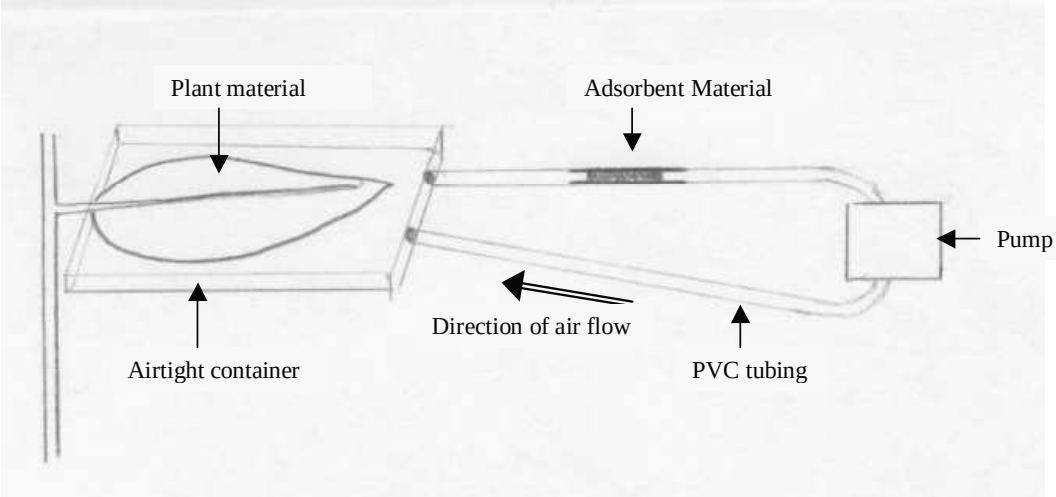


Figure 3.1 - Initial idea for collection of VOC's emitted from plant material

3.2.4 Sample Desorption

Desorption of the VOC's could be achieved in one of two ways. The first would involve dissolving the methanol contained on the adsorbent material in a suitable solvent, for example 2-methoxyethanol, acetonitrile, hexane or pentane. The greatest disadvantage with this method was considered to be the incorporation of impurities from the solvent into samples. 2-methoxyethanol may contain small amounts of methanol, which would affect results of methanol determinations in experiments.

The second possible method for desorbing VOC's off the adsorbent material and onto the GC column would involve thermally desorbing the analytes directly onto the column without the use of a solvent. This method was considered to be preferable, as it would eliminate the possibility of the incorporation of impurities from the solvent into samples.

3.2.5 Silanisation of Glass Tubes for Methanol Adsorption

A number of glass tubes with lengths and internal diameters of 80 mm and 4 mm respectively were obtained. The glass tubes were submerged in acetone followed by toluene for 5 minutes each. Subsequently they were submerged in a 5%

hexamethyldisilazane (HMDS) solution in toluene for 15 minutes. Through the silanisation process bonding sites on the surface of the glass are deactivated and thus possible reactions between polar compounds in a sample and the glass are eliminated. Following removal from the HMDS solution, the tubes were rinsed in toluene followed by acetone and dried in an oven at 110 °C for at least one hour. Once dry, the glass tubes were removed from the oven and stored in a desiccator.

3.2.6 Packing of Silanised Glass Tubes with Adsorbent Material

The adsorbent material (Tenax or Carbosieve) was packed into each tube between two silanised glass wool plugs at a depth of 30 mm (85 mg and 110 mg respectively).

3.2.7 Conditioning of Adsorbent Tubes

Before use, the adsorbent tubes were conditioned in order to remove any contaminants that might have been held on the adsorbent material originating from the ambient air. Each packed tube was inserted into a specially constructed aluminium heating block, ensuring that the adsorbent material was sitting fully within the heating block, at approximately 200°C with nitrogen gas passing through each tube at a rate of approximately 30 mL min^{-1}. The tubes were conditioned for 3 hours, after which they were removed from the heating block and left to cool with nitrogen gas continuously flowing through each. Once cooled, the ends of each tube were sealed with PTFE tape and individually wrapped in aluminium foil. The conditioning process was repeated each time the tubes were used to collect samples. Boyd (1984) showed that conditioned precolumns could be stored for up to 7 days at 20 °C prior to use with no significant accumulation of background volatiles.

In the methods used here, precolumns were stored in the fridge (3-4 °C) after conditioning and used within four days. Samples were generally desorbed immediately following sample collection, thus minimising the loss of analytes and accumulation of contaminants for the air.

3.2.8 Determination of Most Suitable Column for GC Quantification of Methanol

Having decided on a method for desorbing the analytes off the adsorbent material (Tenax-TA initially), a GC system was required that was capable of quantifying methanol accurately at trace levels. In order to determine which columns were capable of achieving this, a series of methanol standards were made up in hexane, ranging between 0% and 1%. Standards were initially injected directly onto a PLOT (porous layer open tubular) fused silica capillary column, 50 m x 0.53 mm, film thickness 10 μm (Varian BV, Middleburg, The Netherlands). Following several direct injections of methanol standards onto this column under various conditions, it was concluded that it was unsuitable for this particular analysis due to the instability of the baseline following injections, possibly as a result of column bleed or general poor condition of the column.

The PLOT fused silica column was replaced by a DB-5 column 50 m x 0.2 mm, film thickness 0.33 μm (J & W Scientific, Folsom, CA). Initially small volumes (0.3 μL) of HPLC grade methanol were injected directly onto the column at 130 °C in order to determine whether it was capable of qualitatively analysing methanol. Resulting chromatograms showed two adjacent peaks of roughly the same size with retention times of approximately 8 minutes. To determine whether the column would be capable of quantitatively measuring methanol, a series of injections of HPLC grade methanol were made, of volumes varying between 0.1 and 0.8 μL.

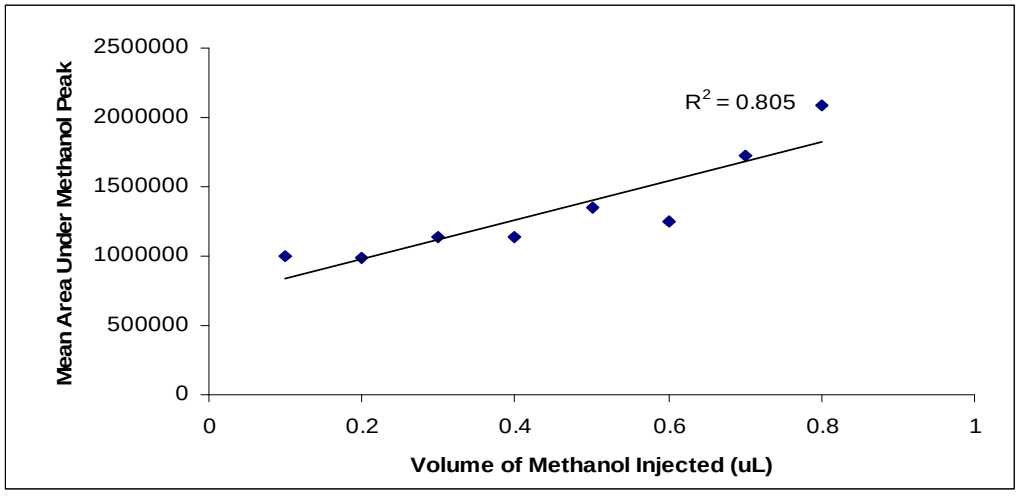


Figure 3.2 – Calibration attempt using DB-5 column with varying volumes of methanol injected directly onto column

Figure 3.2 shows that, although there was a degree of correlation between the volume of methanol injected directly onto the DB-5 column and the area under the methanol peak at retention times of approximately 5.5 minutes, the relationship was not considered to be strong enough to accurately measure methanol using this calibration method.

An alternative calibration method was attempted with the DB-5 column whereby methanol standards ranging between 0% and 10% were made up to determine whether or not this column was capable of quantifying methanol in smaller amounts. Water was considered a suitable solvent but the chromatogram resulting from a direct injection of water consisted of a sloping baseline with a large amount of noise. Hexane was thought to be an appropriate alternative to water as a solvent. Direct injections of HPLC grade hexane resulted in chromatograms with a single, clear peak at approximately 9 minutes. A series of methanol standards from 0% to 10% in hexane was made up and directly injected onto the DB-5 column at 130 °C.

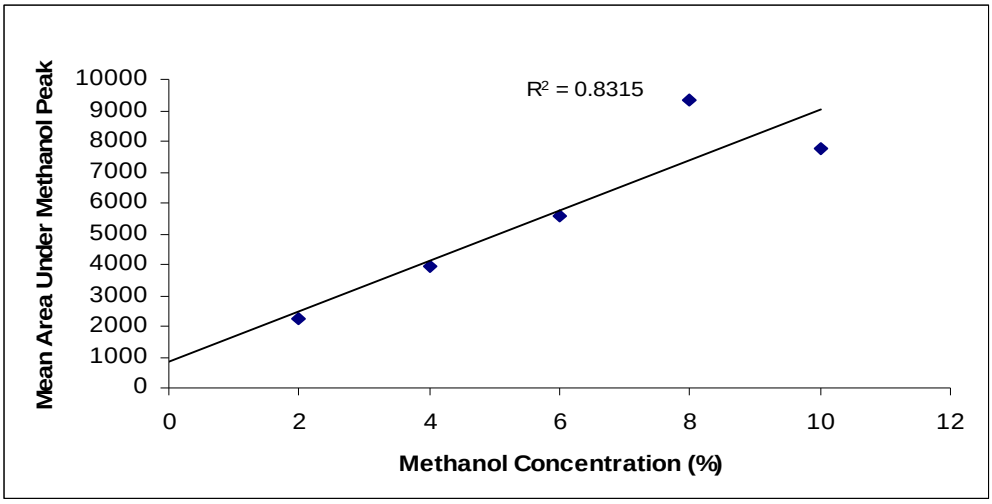


Figure 3.3 – Calibration attempt using DB-5 column with varying concentrations of methanol in hexane

Figure 3.3 shows that again there was a degree of correlation between the size of the methanol peak and the concentration of the methanol solution injected directly into the GC. However, although the relationship between the size of the methanol peak and the concentration of the methanol solution was relatively strong, especially with the exclusion of the higher concentrations of methanol, the hexane and methanol peaks on the chromatograms were overlapping to a large extent (Figure 3.4), thus giving an inaccurate value when the peaks were integrated. The temperature of the oven and the carrier gas flow rate were altered accordingly but the retention times of the methanol and hexane remained too similar for the individual peaks to be fully separated. It was therefore concluded that the results of the calibration of DB-5 for methanol were inconclusive and

there was no obvious way in which to determine whether or not this column was capable of quantifying methanol at trace levels.

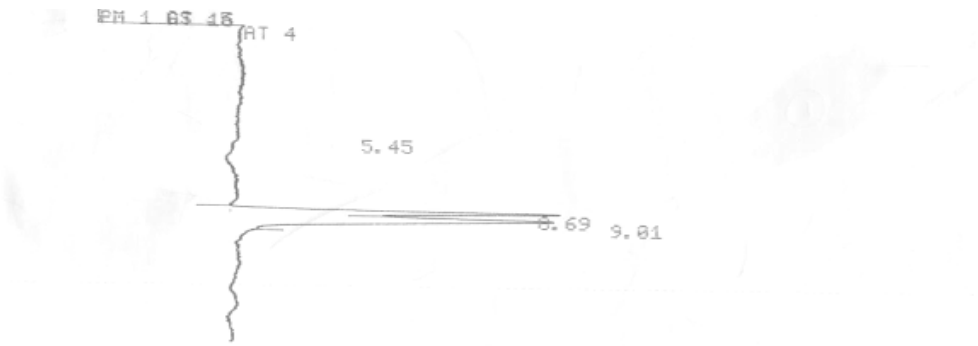


Figure 3.4 – An example of the chromatograms obtained when methanol standards in hexane were injected directly onto the DB-5 column

A calibration was then attempted using a Carbowax column (15 m x 0.54 mm, film thickness 1.2 μm; Alltech, Deeerfield, IL) and a series of methanol standards in HPLC grade hexane. Figure 3.5 shows that there was reasonably good correlation between the size of the methanol peak, with retention time of approximately 1.5 and the concentration of methanol in the standard injected directly onto the column.

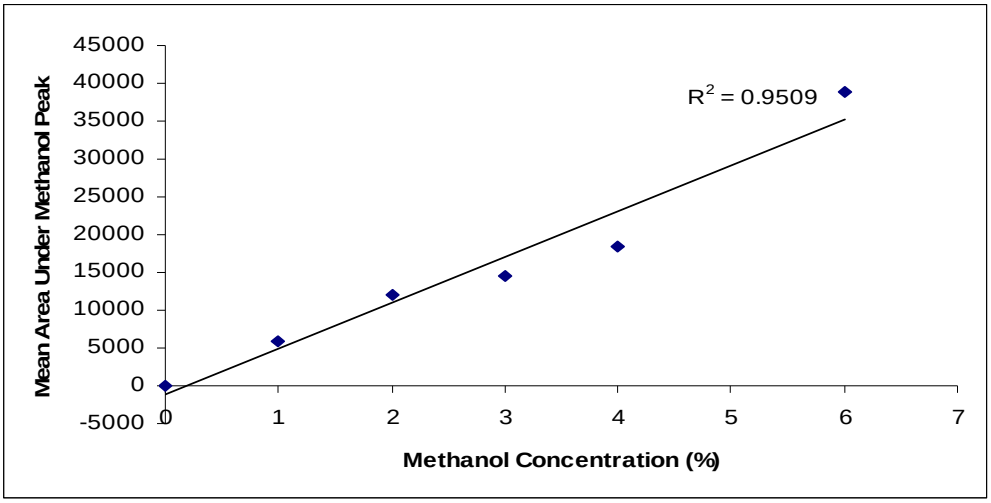


Figure 3.5 - Calibration attempt using Carbowax column with varying concentrations methanol in hexane (0-6%)

Despite this however, the concentrations of methanol expected within a system such as that shown in Figure 3.1, containing a sample of growing plant material, were predicted to be in the ppb range. It was therefore considered advisable to ascertain whether the Carbowax GC column was capable of quantifying methanol at these low concentrations.

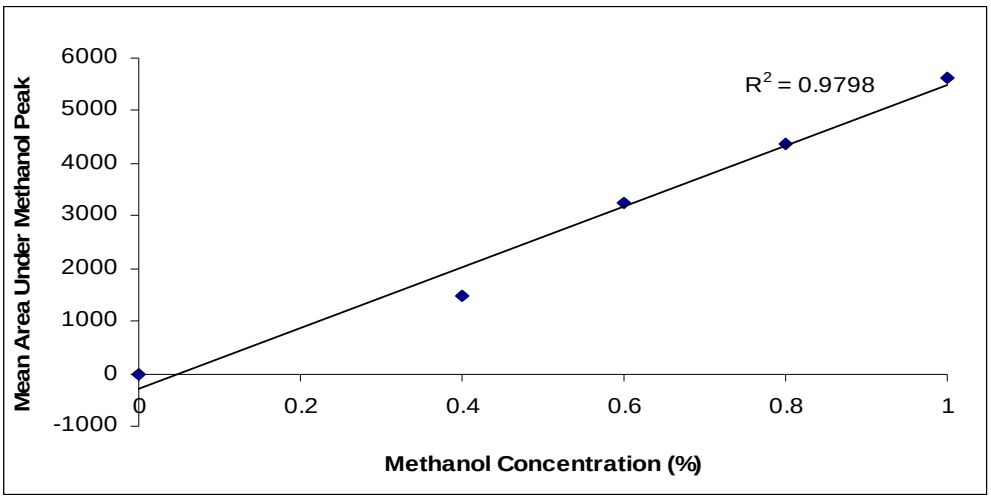


Figure 3.6 - Calibration attempt using Carbowax column with varying concentrations methanol in hexane (0-1%)

A series of methanol standards of concentrations less than 1% were subsequently directly injected onto the Carbowax column to determine whether or not it was capable of quantifying methanol at much lower concentrations. Figure 3.6 illustrates the close correlation between the mean areas under the methanol peaks on the chromatograms and the concentration of the methanol solution injected directly onto the column. On the basis of this calibration and the fact that the retention times for hexane and methanol were approximately 0.7 and 1.5 minutes respectively (Figure 3.7), it was concluded that a Carbowax column would be suitable for the quantification of methanol emitted from plant material.

Figure 3.7 - An example of the chromatograms obtained when methanol standards in hexane were injected directly onto the Carbowax column

3.2.9 Sample Desorption Technique

Having concluded that the most suitable method for methanol quantification involved the use of a Carbowax column, a method was required that would allow the sample to be thermally desorbed from the adsorbent material (initially Tenax-TA) directly onto a Carbowax column.

A mechanism was devised whereby a glass precolumn containing the adsorbent material, onto which the sample VOC's were adsorbed, was inserted into a heated block. A carrier

gas flow was connected to the top of the glass tube and the base of the tube was coupled directly to the GC injection port. The precolumn was flash heated to a temperature of 220°C to volatilise VOC's trapped on the adsorbent material, which were then flushed onto the GC column with the flow of He carrier gas (Figure 3.8).

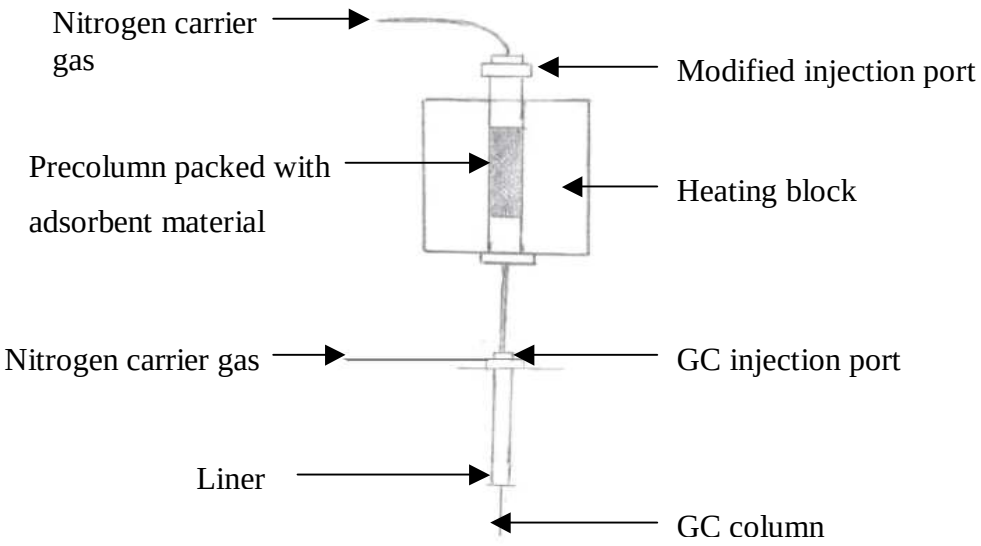


Figure 3.8 – Schematic representation of the heating block system coupled with the conventional injector system for gas chromatography

This system was considered to be a suitable method for thermally desorbing the VOC's off the adsorbent material and onto the GC column, as the block allowed sample Tenax tubes to be analysed without interrupting the carrier gas flow to the column. It also allowed the direct injection of standard solutions onto the column via the modified injection port above the precolumn. Sample tubes could simply replace the tube already inserted in the heating block. It also negated the need for a solvent extraction step, which might have introduced contaminants to the procedure.

Figures 3.9 and 3.10 show responses obtained when 0.3 µL HPLC grade hexane and 0.3 µL HPLC grade methanol respectively were injected into the Tenax tube which was held within the heating block at a temperature of 220 °C.

Figure 3.9 – An example of the chromatograms obtained when hexane was injected into a Tenax tube within the heating block

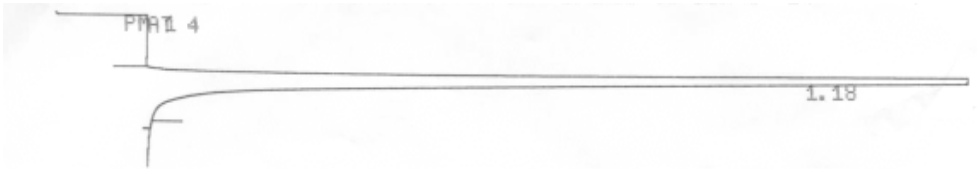


Figure 3.10 - An example of the chromatograms obtained when methanol was injected into a Tenax tube within the heating block

Figures 3.9 and 3.10 illustrate the fact that the thermal desorption of hexane and methanol from the Tenax material within the heating block resulted in relatively sharp peaks. However, due to the additional distance that the desorbed gases had to travel before reaching the GC column and the subsequent decrease in temperature of the system, and therefore the volatiles, there was a significant increase in the amount of tailing observed in the responses for each of the VOC's.

Figure 3.11 - An example of the chromatograms obtained when methanol standard in hexane was injected into a Tenax tube within the heating block

Figure 3.11 shows a typical chromatogram resulting from the injection of 0.3 μL 1% methanol standard in hexane into the Tenax tube within the heating block. Not only has the hexane peak become split, but due to tailing of the hexane peak, there is also a significant amount of overlapping. The temperature of the heating block was increased to 240 °C and the length of tubing between the block and the GC was reduced in order to try to reduce the extent to which the peaks were overlapping. Neither of these alterations resulted in the peaks becoming completely separated. The temperature of the oven was reduced from 45 °C to 35 °C and although this change lessened the overlapping, it was still considered to be significant. The overlapping meant that the integration of the methanol peak might have been inaccurate resulting in an inaccurate methanol calibration. Therefore the accurate quantification of methanol would not be possible using this method. It also transpired that the process of replacing the precolumn tube was time consuming and

awkward, possibly resulting in the loss of volatiles from a sample precolumn as it was introduced to the heating block.

An alternative thermal desorption method was devised whereby the injection liner at the head of the GC column was replaced by a Tenax-packed tube onto which the sample VOC's were adsorbed. This method involved interrupting the carrier gas flow between sample introductions, which might have damaged the column had the oven been run at a high temperature. In this case however, the oven was run at temperatures no greater than 50 °C and consequently interruption of the carrier gas flow would have no detrimental effect on the column.

The initial tests using this thermal desorption method involved the injection of methanol standards in hexane directly into the liner at the head of the GC, which had been replaced by a glass tube with the same dimensions, packed with Tenax. Figure 3.12 is an example of the typical response following the injection of 0.3 μL 1% methanol standard in hexane.

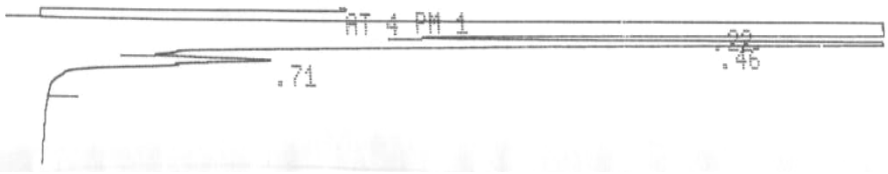


Figure 3.12 – An example of the chromatograms obtained following direct injection of a methanol standard in hexane into a 'Tenax liner'

The methanol and hexane peaks are overlapping to a much greater extent than those obtained from the same standard being thermally desorbed within the heating block. It was concluded that if a thermal desorption method was going to be employed for the methanol determinations in the future, a new GC column would have to be purchased.

An Equity^{TM}-1 fused silica capillary column (30 m x 0.53 mm, film thickness 3.0 μm) was purchased from Supelco, Bellefonte, PA. This particular column was chosen as the retention times for methanol and hexane on this column at a temperature of 40 °C with a carrier gas flow rate of 40 mL s^{-1}, differed by approximately 8 minutes. This meant that the respective peaks would be completely independent of each other on a chromatogram, thus the calibration of methanol would be accurate.

Initially a series of methanol standards in hexane were injected directly into the liner, which had been replaced with a precolumn tube containing approximately 85 μg Tenax-TA. The responses showed clear, but rather wide peaks. It was concluded that the

methanol and hexane in the standards were being adsorbed onto the Tenax and being held for a small but significant time resulting in peaks that were not as sharp as they would be following direct injection of the same solutions into the system containing a liner rather than the Tenax precolumn.

In order to rectify this problem a cryotrap was introduced to the system (Figure 3.13). A portion of the GC column was submerged in liquid nitrogen for a period of time prior to the introduction of a sample or standard to the system in order to focus VOC's that were trapped on the adsorbent resin.

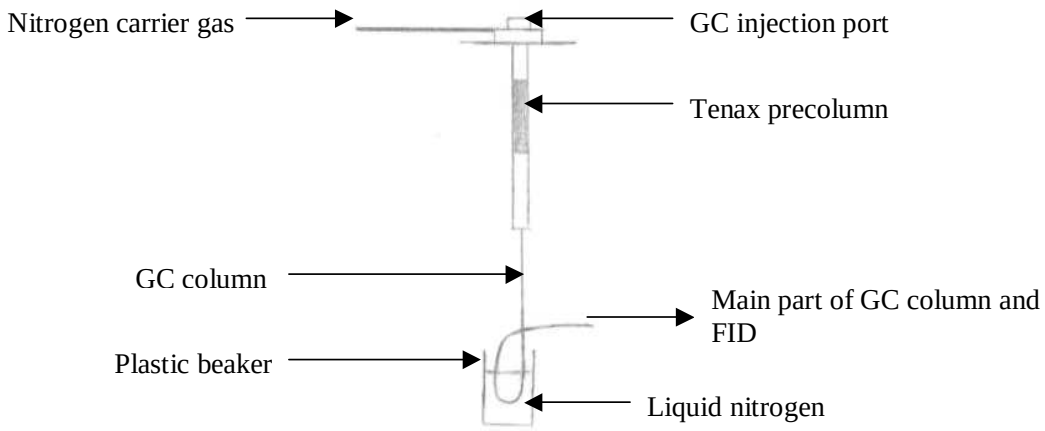


Figure 3.13 – Schematic representation of the cryogenic trapping system with a Tenax precolumn replacing the liner and a portion of the Equity™-1 column submerged in liquid nitrogen

A standard protocol was devised that allowed the Equity™-1 GC column to be accurately calibrated for methanol using methanol in hexane standards. Initially, methanol standards in hexane were introduced by direct injection into the system via the GC injection port. A portion of the column was submerged in liquid nitrogen for ten minutes prior to and five minutes following the introduction of a standard solution to the system.

Figure 3.14 is an example of the typical response obtained following direct injection of 0.3 μL 1% methanol in hexane. The retention time for methanol was approximately 1.4 minutes.

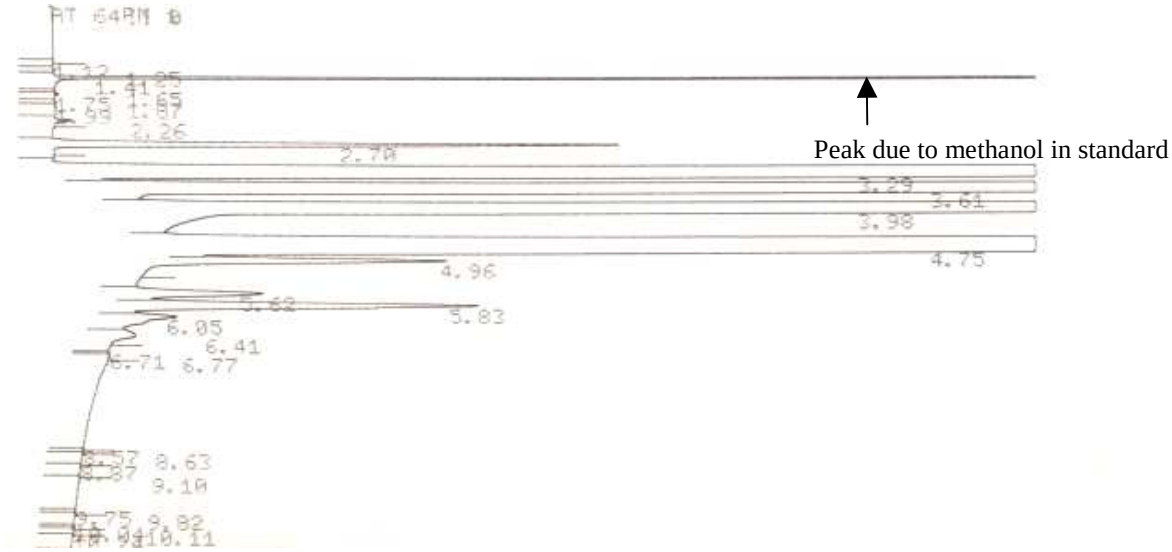


Figure 3.14 - An example of the chromatograms obtained following direct injection of a methanol standard in hexane into the cryogenic trapping system

The response from the direct injection of hexane into the cryogenic trapping system was a large number of peaks with retention times ranging between approximately 2.5 to 5.5 minutes, the majority of which being as a result of impurities present in the hexane. This did not prevent the column from being calibrated accurately for methanol however, because the retention times for methanol and hexane were significantly different from each other.

A series of methanol standards in hexane ranging between 0 and 1% were subsequently injected directly into the cryogenic trapping system. Figure 3.15 illustrates the very close correlation between the concentration of the methanol standard injected into the system and the mean area under each of the respective response peaks.

The relationship between methanol concentration and area under the response peaks is such that this method was considered to be suitable for the accurate quantification of methanol in VOC's emitted from plant material.

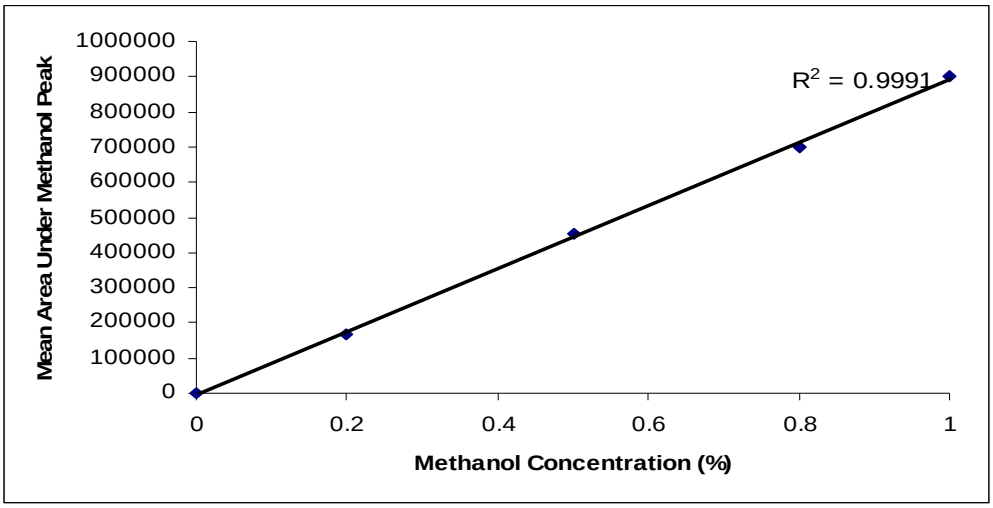


Figure 3.15 - Calibration attempt using Equity™-1 column and cryogenic trapping system with varying concentrations methanol in hexane

3.2.10 Standard Protocol for the Introduction of Samples to the Cryogenic Trapping System

The GC oven was switched off and allowed to cool before a portion of the GC column was submerged in liquid nitrogen for ten minutes. The carrier gas flow was halted using the column head pressure gauge and the existing liner was replaced with a precolumn Tenax tube containing the sample VOC's. The flow of carrier gas was resumed at 40 mL s^{-1} and the column remained in liquid nitrogen for a further 5 minutes. The column was removed from beaker containing the liquid nitrogen, the oven door closed and the oven switched on at 40 ºC. The integrator was immediately switched on.

Figure 3.16 is an example of the typical response given when the GC liner was replaced with a clean, dry, empty glass tube of the same dimensions of the liner, following the protocol above. There is only one significant peak at a retention time of 1.63 minutes.

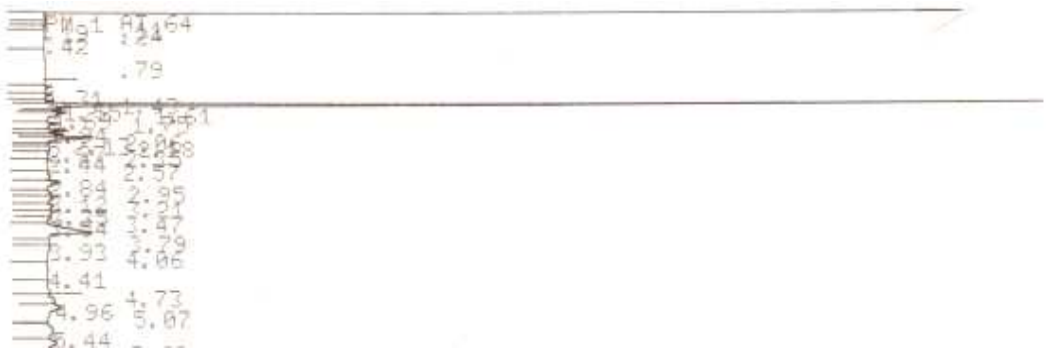


Figure 3.16 – A typical chromatogram resulting from the replacement of the liner with an empty glass tube of the same dimensions following the standard protocol

Figure 3.17 shows the same obvious peak at a retention time of 1.61 minutes that is present in Figure 3.16. All subsequent chromatograms, from standards or samples, as a result of following the standard protocol for the introduction of samples to the cryogenic trapping system, contained this peak. It was speculated that this peak is a result of the carrier gas being switched back on following the introduction of the Tenax precolumn to the system. It could possibly be due to the presence of an unknown contaminant in the Tenax adsorbent resin.

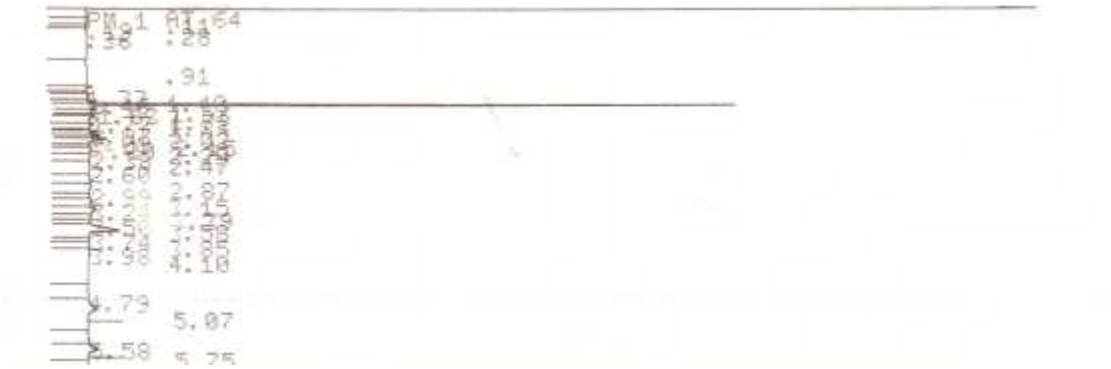


Figure 3.17 – A typical chromatogram resulting from the replacement of the liner with a freshly-conditioned Tenax precolumn glass tube following the standard protocol

3.2.11 Sample VOC Collection

3.2.11.1 TLC Tank Experiments

Having decided on a suitable method for the quantification of methanol in VOC emissions from plants, an appropriate method for sample VOC collection was required. The initial sample collection concept detailed in Figure 3.1 was scaled up in order to accommodate a large quantity of leaves that were in a state of decay (Figure 3.18).

The lid of a 5 L TLC tank was adapted in such a way that it contained two circular holes of diameter 6 mm. Onto the outer edge of each hole a small length of threaded 6 mm external diameter glass tubing was attached using adhesive. The lid was attached to the tank using adhesive tape so that an airtight seal was achieved. The holes in the tank's lid were sealed using screw caps into which septa had been fitted.

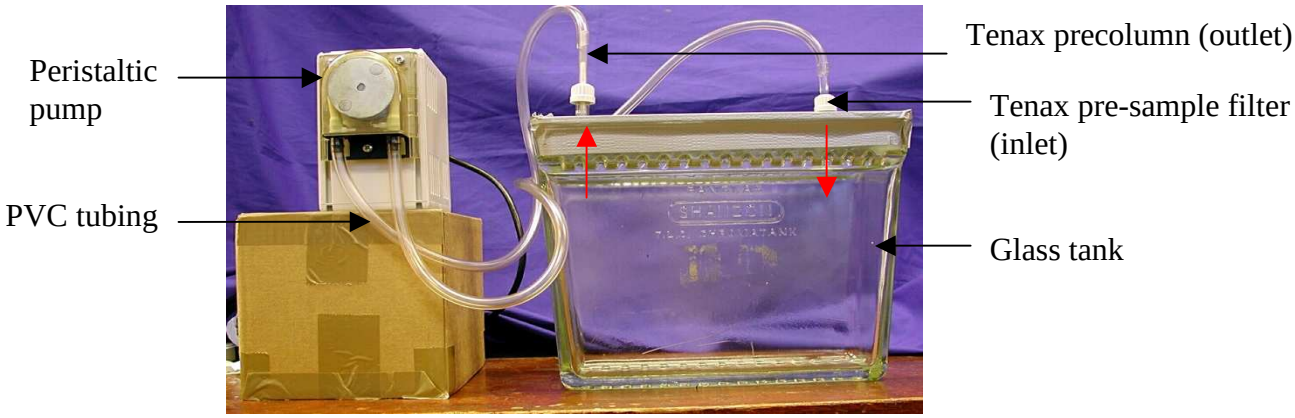


Figure 3.18 – Initial set-up for the collection of VOC's emitted from a large quantity of decomposing leaves. The direction of air flow is denoted by red arrows

During sample collection a Tenax tube was inserted into the tank at the inlet end of the system in the hope that it would filter out any contaminants present in the circulating air. A further Tenax tube, the sample collection tube, was inserted in the outlet end of the tank to pick up VOC's from the sample. During the sampling process, it was proposed that the sample itself would be located within the tank. An airtight seal was achieved between the inlet and outlet Tenax tubes and the threaded glass tubing on the tank lid by sliding a Teflon collar onto each of the Tenax glass tubes and tightening the screw cap onto it.

Tenax-TA has a low breakthrough volume for methanol. The term breakthrough volume is defined as the volume of carrier gas that will purge an analyte through 1 g of adsorbent resin in a desorption tube at a specific temperature, given in litres or millilitres per gram of resin. It is vital to be aware of the breakthrough volume of a specific analyte for a specific adsorbent material in order to ensure that these analytes are not purged off the resin during a dynamic headspace sampling process. The breakthrough volume for methanol on Tenax-TA is quoted as being in the region of 40 mL per 100 mg of resin at 20 °C. It has been shown that very little methanol is removed from the resin following purging with 20 mL carrier gas. For this reason, the pump was set to pump at a rate of 4 mL min^{-1} and for each sample or standard and the collection time was 5 minutes.

Figure 3.19 illustrates a typical chromatogram obtained after the empty tank was incubated for 3 hours at a temperature of 25 °C, followed by 5 minutes of pumping through a pre-sample filter and a precolumn Tenax tube at a rate of 4 mL min^{-1}.

Considering the tank was completely empty and had been rinsed thoroughly with deionised water and dried at a temperature of 100 °C, there were many peaks on the chromatogram, the origins of which were unknown. One possible source of the apparent contamination was the PVC tubing connecting the pump to both the Tenax filter and precolumn. The tubing was significantly reduced in length and then rinsed with deionised water and dried at a temperature of 100 °C to attempt to remove any residual chemical contaminants. However, following this process there were apparently still a number of contaminants adsorbed on the Tenax after the empty tank had been incubated for 3 hours and the gas contained in it purged through a Tenax precolumn at a rate of 4 mL min^{-1} for 5 minutes.

These contaminants could potentially have been plasticizers or antioxidants originating from the PVC tubing in the sampling system. The PVC tubing was replaced with Teflon tubing in an attempt to alleviate the risk of contaminant introduction to the system from the PVC. The alternative tubing did nothing to reduce the number or extent of the contaminant peaks in the resulting chromatograms.

Although the tank itself was rinsed and dried thoroughly between each analysis, there continued to be contaminants present in the system. Several analyses were carried out on the outlet Tenax precolumn following thorough flushing of the tank with air prior to any incubation. Again, this failed to eliminate the source of the volatile contamination.

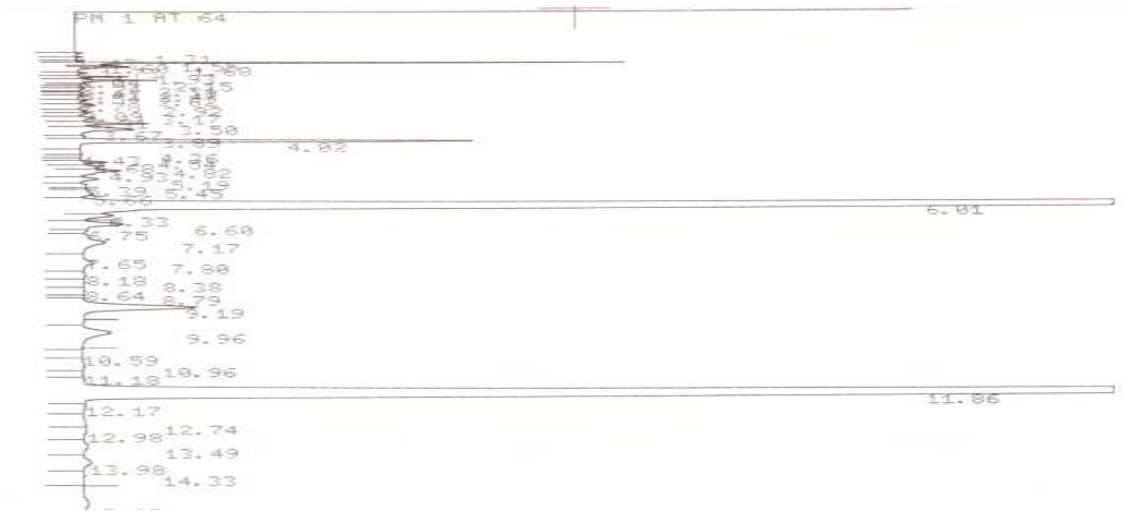


Figure 3.19 – A typical chromatogram obtained following 3 hours of incubating the empty airtight tank at 25 °C and circulating air through the Tenax precolumn for 5 minutes at a rate of 4mL/min

Araldite was used as an adhesive in attaching the threaded glass tubing to the tank lid in the experimental set-up shown in Figure 3.18. This may have been a source of volatile contamination that could lead to the responses seen in Figure 3.19. The Araldite surrounding the glass tubing in the tank lid was therefore covered in Teflon tape, which contains no adhesive and therefore will not introduce volatile compounds to the system. The resulting chromatograms however, continued to display several significant peaks.

The peristaltic pump could potentially have been introducing contaminants to the system. In order to determine whether or not this was the case, a series of experiments were set up whereby a Tenax precolumn was directly attached at either end to the pump via small lengths of polyethylene tubing. The pump was also dismantled and the Santoprene tubing within the pump was cleaned and dried. The resulting chromatograms displayed much the same peaks as were shown in the earlier experiments. It was deemed likely therefore, that the main source of the contamination seen in the chromatograms was the pump.

A system was devised that eliminated the need for the peristaltic pump and relied on the purging of the system using an air cylinder coupled to a regulator. The flow was monitored using an SKC low range flotometer. However, due to the large volume of gas contained within the tank, the air flow rate was difficult to control and fluctuated significantly during sampling. It was vital to be certain of the volume of air that had passed through the Tenax precolumn, not only to be sure that the breakthrough volume had not been reached, but also in order to accurately calculate methanol emission rates from samples of plant material. The TLC tank set up was therefore abandoned due to difficulties in accurately regulating the air flow rate and also due to the fact that contamination originating within the system continued to be detected.

3.2.11.2 Modified Glass Bell Jar Experiments

An airtight glass jar was designed (Figure 3.20), which did not require adhesive or sealant materials that could potentially be a source of volatile contamination within the system.

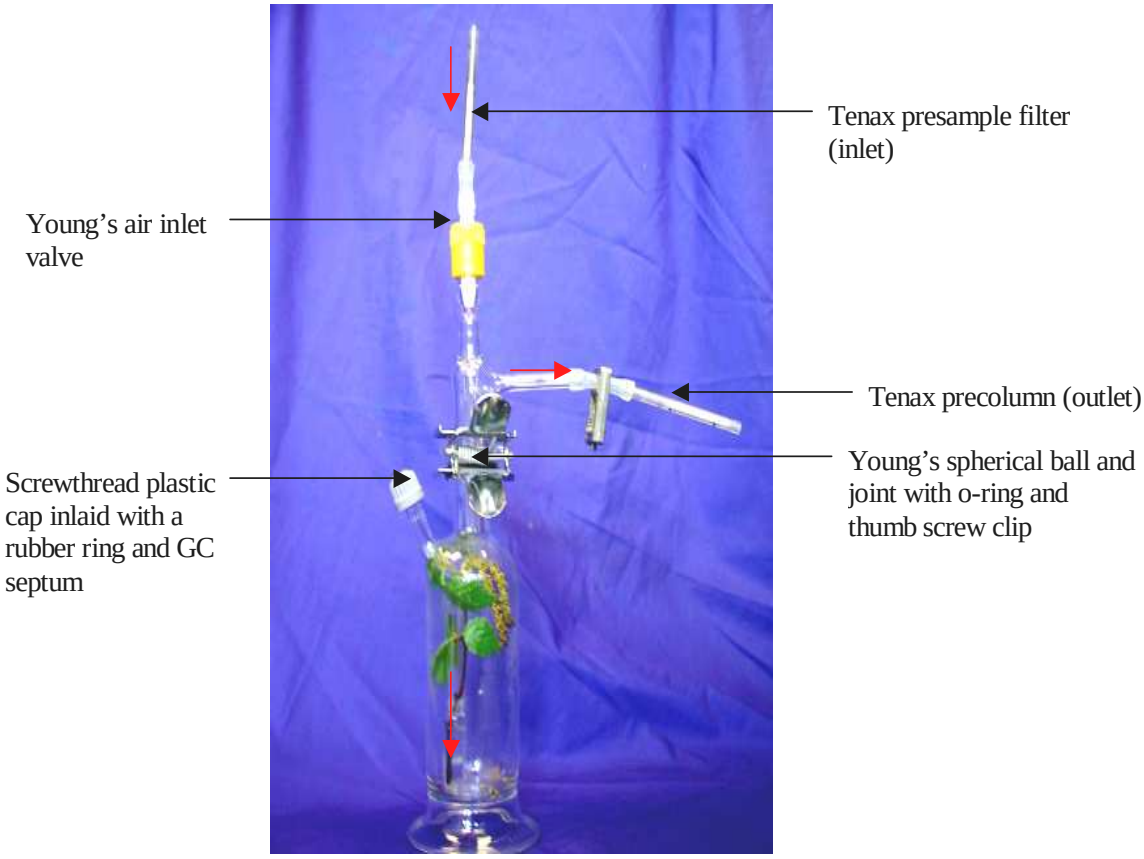


Figure 3.20 – The bell jar system utilised in the trap and purge method for the determination of methanol emitted from growing plant material. The direction of air flow is denoted by red arrows

The jar lid comprised of an inlet attached to a length of glass tubing that reached to approximately 10 mm above the base of the bell jar. The outlet was also located in the lid of the jar. The additional inlet in the body of the jar, sealed with a screwthread plastic cap inlaid with a rubber ring and GC septum, allowed for the introduction of a known volume of methanol into the system, in order to calibrate the adsorbent resin for methanol. An airtight seal was achieved between the lid and body of the jar by inserting a rubber o-ring and clamping them together tightly with a thumbscrew clip. A length of PTFE tubing was attached to the Tenax presample filter, connecting the glass tube packed with adsorbent resin to an air cylinder via a gas flow regulator. The Tenax precolumn attached to the outlet of the bell jar lid was, in turn connected to an SKC low range flotometer via a small length of PVC tubing.

3.2.11.2.1 Calibration of Equity-1 GC Column for Methanol Using the Modified Bell Jar System

The adsorbent resin, initially Tenax, was calibrated for methanol using the bell jar system in order to ultimately quantify the volume of methanol emitted from a known amount of plant material.

Volumes of methanol varying between 0 µL and 2 µL were introduced to the modified bell jar, after it had been thoroughly rinsed with water and dried at a temperature of 100 ºC. The methanol was allowed to equilibrate with the air inside the jar for 1 hour at 21 °C, following which time air was introduced to the system at a rate of 4mL min^{-1} for 5 minutes.

5 minutes prior to the end of the hour equilibrium time, a portion of the Equity-1 GC column was submerged in liquid nitrogen as described in Section 3.2.8 and shown in Figure 3.13. Once the column had been submerged for 5 minutes, the air flow through the bell jar system was commenced, so that by the time 20 mL of air had passed through the precolumn at a rate of 4 mL min^{-1}, the GC column had been submerged in liquid nitrogen for 10 minutes, as originally described. The precolumn was removed from the system following 5 minutes of sample collection and sealed at both ends using PTFE tape. The precolumn was then inserted directly into the injection port of the GC chromatograph as described in Section 3.2.8 and shown in Figure 3.13. The GC column was left submerged in liquid nitrogen for a further 5 minutes following the introduction of the standard precolumn into the injection port. The oven door was then closed and the integrator started.

Prior to the calibration process, the exact retention time of methanol under these conditions had to be determined. Figure 3.21 shows a typical chromatogram resulting from the introduction of 0.2 µL methanol into the jar, followed by the procedure described above for the trapping and introduction of the sample into the GC. Comparing this to the typical chromatogram following the introduction of a blank Tenax precolumn into the GC injection port, it was apparent that there were a number of additional peaks in the standard precolumn chromatogram. By varying the volume of methanol within the jar it was possible to determine that the response for which methanol was responsible was that with a retention time of 1.63 minutes.

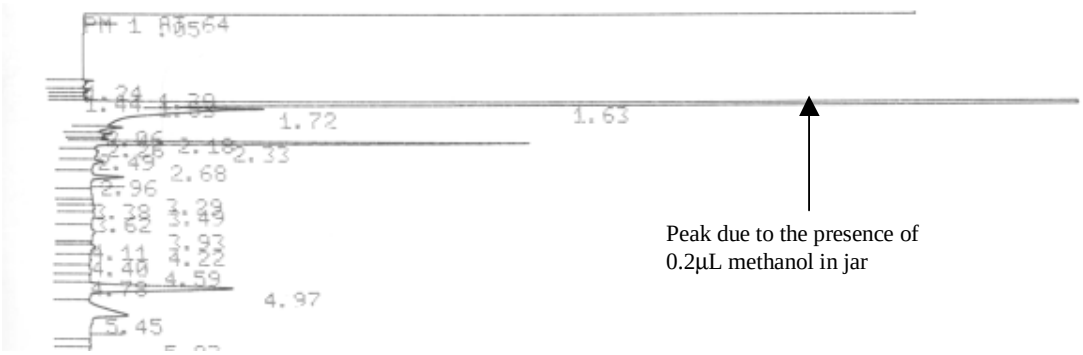


Figure 3.21 – A typical chromatogram obtained following the introduction of 0.2µL methanol into the bell jar system and collection on a Tenax precolumn as described above

In order to verify the assertion that the breakthrough volume for methanol on Tenax was greater than 20 mL per 100 mg of resin, two Tenax precolumns were set up in sequence at the outflow end of the bell jar system containing 0.2 µL methanol. The chromatogram obtained from the second Tenax precolumn, following the purging of the system with 20 mL air, displayed no response at the retention time typical for methanol, demonstrating that no methanol had broken through the first Tenax precolumn.

3.2.11.3 Results of the Calibration of Tenax for Methanol

Figure 3.22 illustrates the close correlation between the mean area underneath the peak due to methanol, with retention times of between 1.55 and 1.74, and the volume of methanol introduced to the bell jar. This method was therefore considered to be suitable for the quantification of methanol emitted from plant tissue.

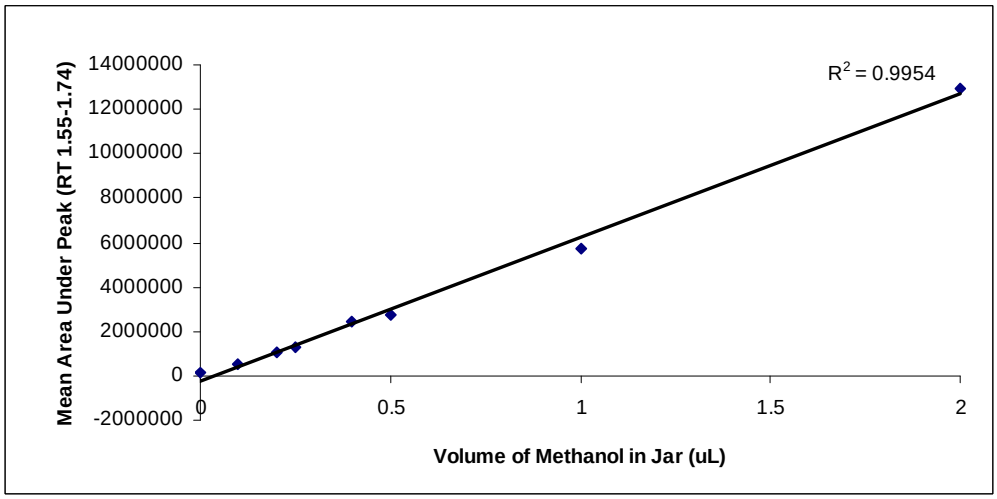


Figure 3.22 – Calibration graph for Tenax with methanol using the modified bell jar system and following the protocol described in Section 3.4

3.2.11.3.1 Determination of the Recovery Factor for Methanol using Tenax-TA in Conjunction with the Modified Bell Jar System

The volume of the bell jar, together with the lid, was determined to be 340 mL. A volume of 0.2 μL methanol was introduced to the sealed jar using a syringe through the septum-sealed inlet. If a representative sample of 20 mL of the air inside the bell jar was purged through the Tenax precolumn, then the equivalent volume of methanol that would be subsequently adsorbed onto the precolumn would be approximately 0.012 μL. Following the introduction of methanol, the sealed jar was incubated at 21 °C for 1 hour. 20 mL of air was then purged through the system and the sample precolumn, and introduced to the GC as described in Section 3.2.9. This process was repeated several times and the methanol responses shown in the resultant chromatograms recorded. The averaged result is shown in Table 3.1.

In order to determine the recovery factor of methanol on the adsorbent resins, a freshly reconditioned adsorbent resin precolumn was introduced to the injection port of the GC after a portion of the GC column had been submerged in liquid nitrogen for 10 minutes. Approximately 0.012 μL methanol was injected directly onto the precolumn and the GC column was subsequently left in liquid nitrogen for a further 5 minutes, after which time the liquid nitrogen was removed, the oven switched on at 40 °C and the integrator started. The responses shown on the resulting chromatograms were then recorded and compared to those obtained from the purge and trap protocol described above. This procedure was repeated five times and the results obtained for the recovery of methanol from Tenax using the methods developed in this chapter are shown in Table 3.1.

Mean Area Under Methanol Peak Following Injection of 0.012μL Methanol Directly into Tenax Precolumn	Mean Area Under Methanol Peak Following Trap and Purge Method with 0.2μL Methanol Injected into Jar (Tenax precolumn)	Recovery Factor (%)
10201853	1093576	10.7 (9.23-13.2)

Table 3.1 – Comparison of methanol responses obtained during recovery experiments for methanol with Tenax-TA (error range in brackets)

The calculated recovery factor of 10.7% is low, but because it was relatively consistent for the series of recovery experiments that were carried out using this system, it was deemed a suitable figure for the calculation of methanol emissions from plants. The observed losses of methanol were proved not to be a result of breakthrough. It was therefore concluded that losses were incurred during the transfer of the adsorbent tube from the bell jar system to the injection port of the GC.

The alternative adsorbent resin, Carbosieve SIII, was subsequently investigated to determine its suitability for the determination of methanol emitted from plant material using the modified bell jar system (Section 3.2.10.4).

3.2.11.3.2 Determination of Methanol Released from Birch Twigs using Tenax-TA as the Adsorbent Resin

Although it had been established that the modified bell jar could be used in conjunction with Tenax precolumns to quantify the amount of pure methanol present in the jar, it was unclear whether or not the method could be used to quantify methanol being emitted from a growing plant system. There was a possibility that the plant material could emit a VOC with a similar retention time on the Equity-1 GC column to that of methanol. If this was the case, the peaks of the respective compounds could overlap on sample chromatograms, resulting in inaccurate integration measurements for the methanol peak. Subsequent calculations for methanol concentration in the jar would therefore be inaccurate.

Initially, a series of experiments were carried out to determine how the amount of methanol detected within the modified bell jar system varied depending on the length of time for which a birch twig was sealed within the jar. This experiment was designed to confirm that the incubation process had minimal effect on the sample plants' natural physiological processes, therefore the rate of methanol release from the plant material, whilst ensuring that a measurable quantity of methanol would be released. From the results of this series of experiments, the optimum incubation time for subsequent experiments, involving different types of plant material, could be determined.

A number of twigs were removed from a healthy, rapidly growing silver birch tree in May 2006 and sealed in the bell jar for lengths of time varying between 3 and 24 hours, at 15-17 ºC and 20-21 ºC night and daytime temperatures respectively. The relative humidity was between 60 and 70% and light intensity was approximately 350 μmol m^{-2} s^{-1}. After this time the sample VOC's were adsorbed onto a Tenax precolumn by purging air through the precolumn tube at a rate of 4 mL min^{-1} for 5 minutes as described in Section 3.2.10.2.1. The sample VOC's were then thermally desorbed directly onto the Equity-1 GC column following the procedure described in Section 3.2.9.

In order to determine whether or not any sample VOC's emitted from sample plant material interfered with the peak obtained methanol, a series of experiments were carried out involving the incubation of silver birch twigs within the jar and the purging of trapped VOC's through a Tenax precolumn. Twigs were removed from a silver birch tree using

secateurs and placed in a modified glass bell jar, with the severed end of the twig submerged in water in a small vial, as shown in Figure 3.20. The jar was incubated for 24 hours in the light/dark regime described above, after which time the VOC's were purged through the system with air and adsorbed onto the Tenax precolumn at a rate of 4 mL min^{-1} for 5 minutes, as described in Section 3.2.10.2.1. The sample precolumn tubes were introduced to the GC injection port following the procedure described in Section 3.2.8.

A jar containing the glass vial holding 5 mL water together with an injection of 0.3 µL methanol was set up along with a jar containing only 0.3 µL methanol to determine whether or not the water absorbed any methanol from the atmosphere within the jar, resulting in a measured rate of methanol emission lower than the actual figure. The resulting chromatograms showed that the methanol was not dissolved in the water in significant quantities.

3.2.11.3.3 Results from the Determination of Methanol Released from Birch Twigs Using the Modified Bell Jar System

Figure 3.23 shows that the emission of methanol from birch twigs is a progressive process and that, as far as can be ascertained from this set of experiments, headspace collection up to a time of 24 hours does not significantly affect physiological processes taking place in the plant tissue. Therefore a headspace collection time of 24 hours was determined to be suitable for subsequent experiments using various plant materials.

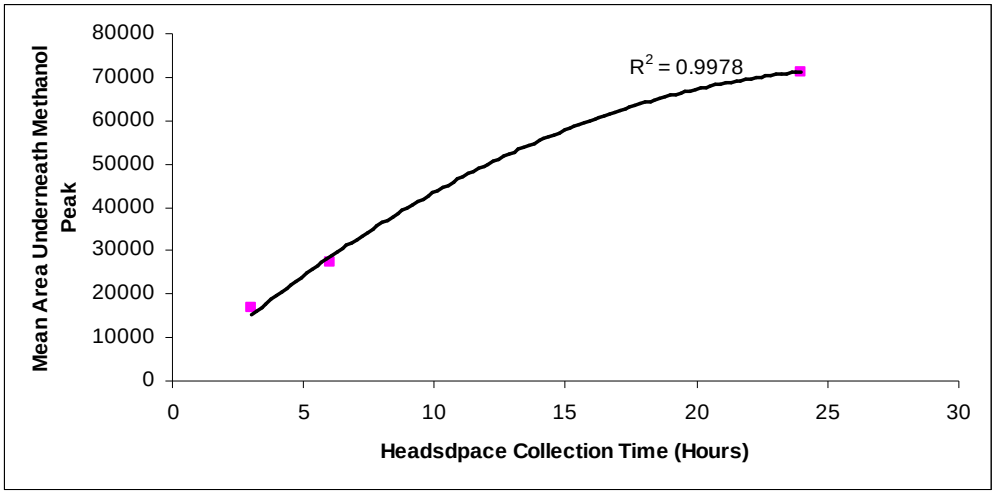


Figure 3.23 – Graph to show the area under the methanol peak following headspace collection above birch twigs for various lengths of time

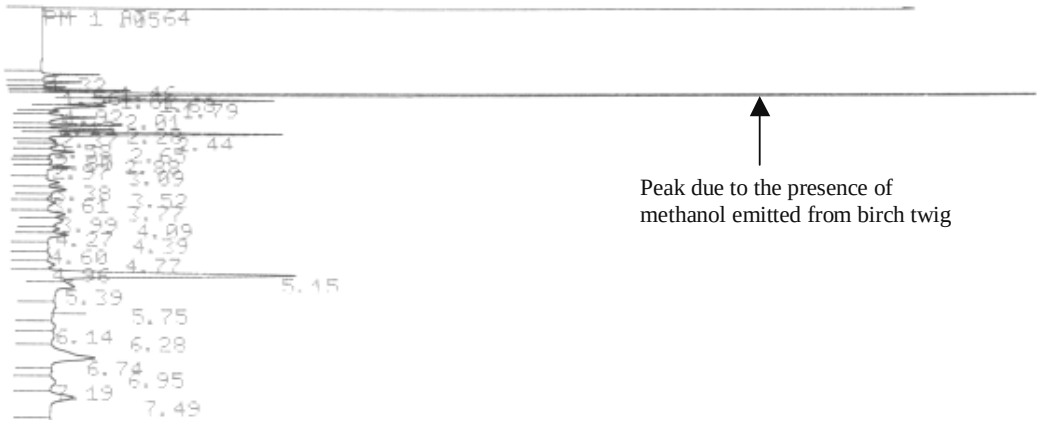


Figure 3.24 - A typical chromatogram resulting from headspace collection above a birch twig incubated for 24 hours

Figure 3.24 illustrates the fact that the response that is due to the presence of methanol (at approximate RT of 1.70) is not significantly overlapped by any of the other peaks present on the chromatogram, possibly resulting from volatile compounds with similar physical and chemical properties to methanol emitted from the plant material.

The chromatogram shown in Figure 3.24 supports the theory that the sample collection, trapping and analysis methods developed in this chapter can be implemented in the quantification of methanol emitted from a range of different plant materials.

3.2.11.4 Carbosieve SIII as an Adsorbent Resin in the Determination of Methanol Emitted from Plants

Carbosieve SIII was considered to be a suitable adsorbent resin for the quantification of methanol emitted from plant material due to its physical properties and its affinity for methanol. Carbosieve SIII (60/80 mesh (Supelco, Bellefonte, PA)) is a high surface area molecular sieve and, although it has a high affinity for water relative to other adsorbent resins, its small pore size enables it to effectively trap volatile organic compounds and straight chain alcohols. Like Tenax TA it is also thermally stable, up to a temperature of 400 °C. In addition, Carbosieve SIII was considered to be a suitable adsorbent material for this analytical method as it has a relatively high breakthrough volume for methanol. The breakthrough volume of methanol for Carbosieve SIII is quoted as being 7.5L for 1.0 g of resin at 20 °C. This means that even following purging with approximately 3.75L of air in the modified bell jar system, methanol would not be removed from the resin.

Identical glass tubes to those used to make Tenax precolumns were packed with Carobsieve SIII to a depth of approximately 3 cm (roughly 0.3 g) as described for Tenax in

Section 3.2.5. Carbosieve precolumns were conditioned and stored as described for Tenax precolumns in Section 3.2.6. The precolumn tubes were then used to determine whether Carbosieve SIII was a suitable resin for the quantification of methanol released from plant material in the purge and trap method described for Tenax TA. Figure 3.25 is an example of the chromatograms obtained following the incubation of an empty modified bell jar for 1 hour at 21 °C. The air inside the jar was subsequently purged and the VOC's present were trapped on the resin in exactly the same way as was carried out using Tenax TA, as described in Section 3.2.10.

The chromatogram in Figure 3.25 shows several small peaks across the region of the chromatogram between retention times of approximately 1 minute and 2.5 minutes. These peaks were present on all subsequent chromatograms, whether resulting from the introduction of a blank, standard or sample Carbosieve precolumn to the injection port of the GC. In order to determine whether these peaks would interfere with the methanol peak on standard and sample chromatograms, a series of experiments were undertaken whereby 0.2 μL methanol was introduced to the modified bell jar through the septum-sealed inlet of the jar (Figure 3.20).

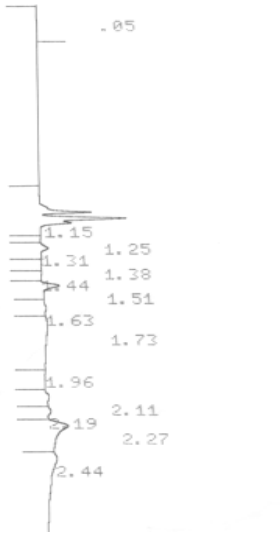


Figure 3.25 – A typical chromatogram obtained from a blank Carbosieve SIII precolumn; one-hour incubation of an empty bell jar with subsequent purging with 20 mL air

The jar was then incubated for an hour at 21 °C before a portion of the air was passed through the system and trapped on the Carbosieve resin. The procedure that was carried out with Carbosieve was identical to that used with Tenax as the adsorbent resin in Section 3.2.10.2.1. Figure 3.26 is an example of a chromatogram obtained following this procedure.

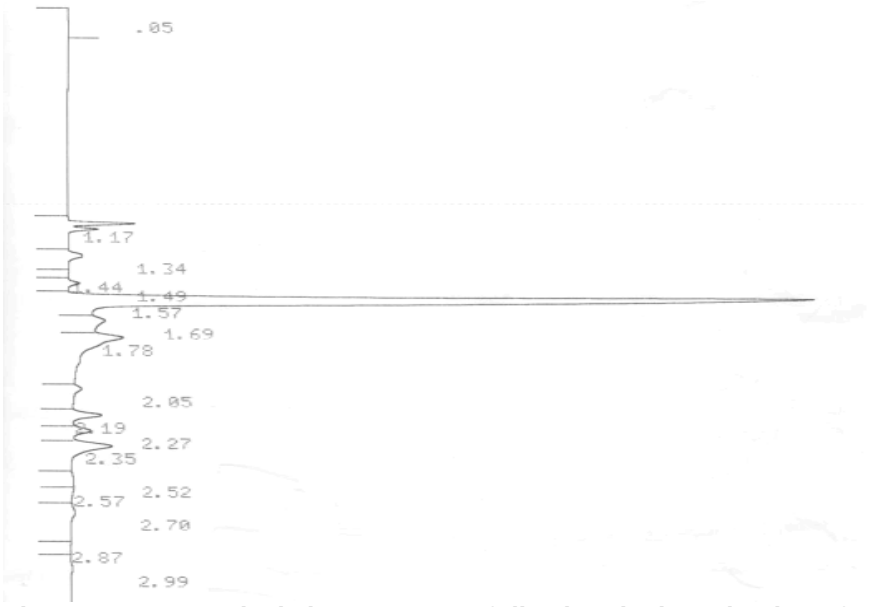


Figure 3.26 – A typical chromatogram following the introduction of 0.2µL methanol into the bell jar system and collection on a Carbosieve precolumn

The chromatogram shown in Figure 3.26 shows clearly that the retention time for methanol under the conditions used in the experiment was 1.57 minutes. To verify that this peak was indeed due to the presence of methanol, a series of experiments was conducted whereby volumes of methanol ranging between 0 and 2 µL were introduced to the jar. The procedure carried out for the calibration of Tenax for methanol using the modified bell jar system, detailed in Section 3.2.10.2.1, was followed in order to calibrate the system for methanol using Carbosieve. The results of the calibration experiments are shown in Figure 3.27.

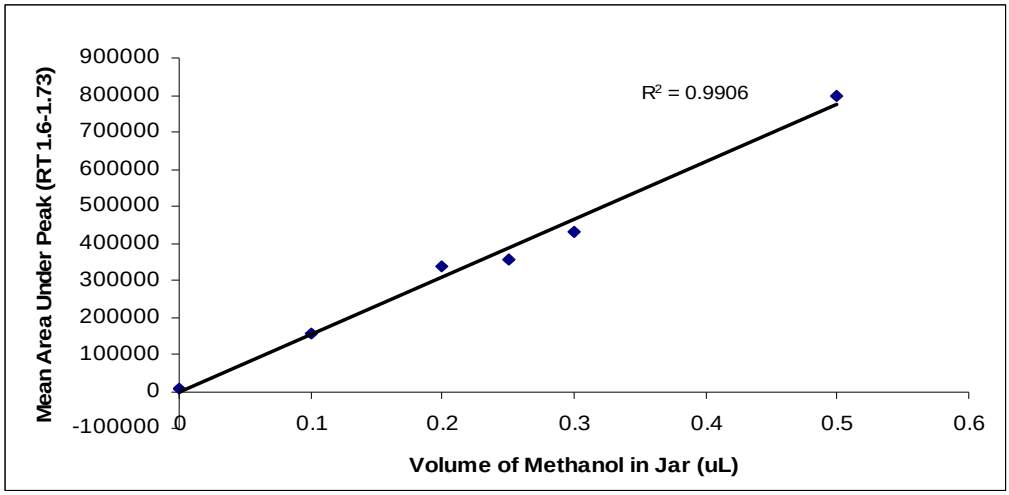


Figure 3.27 - Calibration graph for Carbosieve with methanol using the modified bell jar system

Figure 3.27 illustrates the close correlation between the mean area underneath the peak due to methanol, with retention times of between 1.55 and 1.74, and the volume of methanol introduced to the bell jar. This method was therefore considered to be suitable for the quantification of methanol emitted from plant tissue.

3.2.11.4.1 Determination of the Recovery Factor for Methanol using Carbosieve SIII as the Adsorbent Resin in the Modified Bell Jar System

The recovery factor for methanol on a Carbosieve precolumn was determined in exactly the same way as was achieved for that on a Tenax precolumn, as described in Section 3.2.10.3.1. A volume of 0.2 μL methanol was introduced to the sealed jar using a syringe through the septum-sealed inlet of the modified bell jar system. Following incubation at 21 °C for 1 hour, the system was purged with 20 mL of air from an air cylinder and the VOC's adsorbed onto the Carbosieve precolumn. The precolumn was then immediately introduced into the injection port of the GC as described in Section 3.2.9. This process was repeated several times and the areas underneath the methanol peaks on the resultant chromatograms were recorded. The mean peak area resulting from this procedure is shown in Table 3.2.

From the initial 0.2 μL methanol in the jar, it was calculated that the equivalent volume of methanol that should theoretically have been adsorbed onto the Carbosieve precolumn was approximately 0.012 μL following the introduction of 20 mL air to the system.

To determine the recovery factor for methanol during this experiment, a freshly conditioned precolumn was inserted into the GC injection port and approximately 0.012 μL methanol injected directly into it, following exactly the same procedure as was followed for a Tenax precolumn in Section 3.2.10.3.1. This experiment was repeated five times. The results obtained from the experiments undertaken to determine the recovery of methanol from Carbosieve using the methods developed in this chapter are shown in Table 3.2.

Mean Area Under Methanol Peak Following Injection of 0.012μL Methanol Directly into Carbosieve Precolumn	Mean Area Under Methanol Peak Following Trap and Purge Method with 0.2μL Methanol Injected into Jar (Carbosieve precolumn)	Recovery Factor (%)
9991024	162424	1.63 (1.39-2.32)

Table 3.2 - Comparison of methanol responses obtained during recovery experiments for methanol with Carbosieve SIII (error range in brackets)

The recovery factor for methanol on a Carbosieve precolumn is significantly lower than that for Tenax. However, as with the Tenax precolumn, the reproducibility of the results were such that it was considered suitable for the method developed in this chapter to be used in conjunction with a Carbosieve precolumn to quantify methanol. Consequently, both adsorbent resins were employed in separate sets of experiments quantifying the rate of methanol emission from growing plants, using the modified bell jar.

3.3 Final Experimental Procedure for the Determination of Methanol Using the Bell Jar System

The quantification of methanol emitted from growing plant material was undertaken using the modified bell jar system shown in Figure 3.20 and both adsorbent resins used in the method development process. Plant material was introduced to the jar through the neck of the jar and sealed in such a way that any disruption to the growth of the plant was kept to a minimum. The plant material was incubated for 24 hours in a growth cabinet with a temperature program of 15-17 ºC and 20-21 ºC night and daytime temperatures respectively. The relative humidity was between 60 and 70% and light intensity was approximately 350 μmol m^{-2} s^{-1}.

Five minutes prior to the end of the 24-hour incubation period, a portion of the Equity-1 GC column was submerged in liquid nitrogen, as shown in Figure 3.13. The aim of this process was to ensure that VOC's thermally desorbed off the adsorbent resin were subsequently trapped and focused before being introduced to the body of the GC column. At the end of the 24-hour incubation period the jar containing plant material was attached, via a pre-sample Tenax filter, to an air cylinder. A precolumn containing either Tenax TA or Carbosieve adsorbent resin was then attached to the outlet tube of the jar using small lengths of PVC tubing. A low range flowmeter was attached to the outflow end of the sample precolumn using a further length of PVC tubing.

The flow of air passing through the system was set at 4 mL min^{-1} and regulated using the air inlet valve at the top of the lid of the jar. Air was allowed to pass through the jar containing the sample for 5 minutes, after which time the sample precolumn was inserted directly into the injection port of the GC. The GC column was immersed in liquid nitrogen for a further 5 minutes before being removed and the oven and integrator switched on.

3.4 Quantification of Methanol Emitted from Plants Using GC-FID

3.4.1 Introduction

Barley, ryegrass and oilseed rape were chosen as suitable plant species for the determination of methanol emitted from leaves of growing plants as they are all grown commercially in large quantities across the UK. By quantifying the amount of methanol emitted from individual, or near-individual plants on a dry weight basis, the amount of methanol introduced to the UK troposphere could be estimated by extrapolating these findings to account for the total amount of each of these plant species grown in the UK. Two graminaceous plant species were chosen (barley and ryegrass) together with oilseed rape, a non-graminaceous species, in order to evaluate any differences in the amount of methanol emitted by plants of different classes.

3.4.1.1 Barley

With a UK acreage totalling 881 thousand hectares in June 2006 (Anon, 2007a), barley is the second most abundant arable crop grown in the UK, second only to wheat. Barley is the most widespread arable crop in Scotland and in 2005 some 298 thousand hectares of barley were grown north of the border. It is reasonable to assume therefore, that any volatiles emitted from the barley plants as they grow will have a considerable impact on the overall composition of the UK stratosphere. Chariot is a high yielding malting variety of spring barley and is therefore grown predominantly for the beer-making industry. A total of 14752 tonnes of spring barley of the variety Chariot was produced in England and Scotland in 2003.

3.4.1.2 Ryegrass

Perennial ryegrass is a tufted, fibrous rooted perennial grass that is common throughout the UK and regarded as a native plant. The wide distribution may be due to the fact that perennial ryegrass was once used exclusively in the sowing of leys. It has been in cultivation as a forage grass since the 17th century. Ryegrass became established along roads probably as a consequence of the spreading of seeds during the transportation of hay from fields. Perennial ryegrass prefers fertile soils and occurs as a weed in cereals and other arable crops.

3.4.1.3 Oilseed Rape

Oilseed rape was barely known as a crop in the UK prior to the 1970's. At this time an explosion in commodity prices encouraged farmers to grow the crop. The total area used to grow oilseed rape in the UK is growing year on year as farmers appreciate the increasing value of the crop in relation to the production of cattle feed and oil, which may be processed for use as biodiesel.

Essex Broadleaved is a spring-sown, fast growing forage rape. Rape pastures have been used to fatten lambs and feed ewe flocks for well over 600 years. Forage varieties of rape are commonly employed today in many UK, Northern European, Australian and New Zealand farming systems. Spring rape may also be used as a break crop in cereal rotations so that, for example, weeds may be treated with a selective herbicide. Spring oilseed rape is predominantly a crop of northern parts of the UK due to its ability to grow in cool and damp weather conditions. Between 12 and 24% of the total oilseed rape production in the UK between 1993 and 1998 consisted of spring oilseed rape and one third of that total amount was grown in Scotland (Anon, 2007a). Although a large number of varieties of oilseed rape are grown in the UK, it is assumed that, certainly at the early stages of growth, plants of different varieties will not emit significantly different amounts of VOC's from their leaves.

3.4.2 Growth of Barley Seedlings

20-30 spring barley (*Hordeum vulgare* cv. Chariot) seeds (Nickersons Seeds Ltd., Lincolnshire) were rinsed by swirling in approximately 100 mL deionised water for 20-30 seconds. The excess water was decanted off the seeds, which were then placed in a 600 mL beaker together with 100 mL deionised water. The beaker containing the water and seeds was placed in a growth chamber at 15-17 ºC and 20-21 ºC night and daytime temperatures respectively and the water was bubbled gently overnight using a small pump. The relative humidity was between 60 and 70% and light intensity was approximately 350 μmol m^{-2} s^{-1}.

Following 24 hours of bubbling in the growth chamber, 5 l 0.5 mM $CaSO_4$ solution was made up in a 5 L volumetric flask and transferred to a 10 L opaque plastic tray. The water was decanted off the imbibed seeds, which were rinsed using approximately 100 mL deionised water for 10-20 seconds. The seeds were poured evenly onto a wire mesh platform suspended above the $CaSO_4$ solution. Deionised water was added to the $CaSO_4$

solution up to the level of the wire mesh that held the barley seeds. The tray was placed in a growth chamber under the conditions detailed above, covered with aluminium foil and the solution was bubbled gently using an electric pump connected to several lengths of PVC tubing. The seeds remained covered in the growth cabinet for 72 hours. The level of the $CaSO_4$ solution was topped up with deionised water intermittently to prevent the seed coat and endosperm drying out.

After the seeds had been growing under darkness for 72 hours, the aluminium foil was removed and the seedlings exposed to the light. The seedlings were left on the wire mesh above the aerated $CaSO_4$ solution for a further 72 hours in the light/dark regime detailed above.

A 10 L stock solution of modified Hoagland nutrient solution was made up in two 5 L volumetric flasks. The first volumetric flask contained a solution comprised of macronutrients, while the second contained a solution of micronutrients. The concentrations of the various constituents of the macronutrient and micronutrient solutions are listed in Tables 3.3 and 3.4.

Constituent of Macronutrient Solution	Concentration of Constituent in Modified Hoagland Solution (mM)
$NH_4H_2PO_4$	0.5
$(NH_4)_2HPO_4$	0.5
KNO_3	2
$MgSO_4$ (x $7H_2O$)	0.5
NaCl	0.5
$Ca(NO_3)_2$ (x $4H_2O$)	2

Table 3.3 - Concentrations of constituents used in the macronutrient fraction of the modified Hoagland solution

Constituent of Micronutrient Solution	Concentration of Constituent in Modified Hoagland Solution (mM)
H_3BO_3	6.25
$CuSO_4$ (x $7H_2O$)	0.125
$MnSO_4$ (x H_2O)	0.5
Na_2MoO_4 (x $2HO_2$)	0.19
$ZnCl_2$	0.5
FeIII NaEDTA	27

Table 3.4 - Concentrations of constituents used in the micronutrient fraction of the modified Hoagland solution

The 6-day-old barley seedlings were transferred to Hoagland solution as shown in Figure 3.28. Approximately 450 mL of each of the macronutrient and micronutrient solutions detailed above were poured into a 1 L beaker which had been wrapped in aluminium foil. The seedlings were placed within a sponge bung and inserted into a polystyrene lid so that the root tips were submerged in nutrient solution. The seedlings were placed in a growth cabinet under the same light and temperature regime described above. The nutrient solution was bubbled using a small pump.

Seedlings were removed from the Hoagland solution two at a time at different stages of development, between 6 and 15 days old, and placed in a modified glass bell jar, as shown in Figure 3.30. A cotton wool disk of approximate diameter 40 mm was placed in the base of the bell jar, together with 30 mL modified Hoagland solution. The barley seedlings were introduced carefully to the bell jar to prevent damage to the plants, especially the roots which are particularly fragile. It has been shown that mechanical damage to plant tissue can result in the increased emission of certain VOC's, possibly including methanol (Karl et al., 2005). The jar was sealed and placed in the growth cabinet for 24 hours under the light and temperature regime detailed above. The emitted methanol was then adsorbed onto an adsorbent resin and quantified using GC-FID as described in Section 3.4.

Figure 3.28 - 10-day-old barley seedlings growing in modified Hoagland solution

3.4.3 Growth of Ryegrass Seedlings

Perennial ryegrass (*Lolium perenne*) seeds were obtained from Cotswold Seeds, Gloucestershire. Cotton wool disks of approximate diameter 35 mm were placed in a seed tray. 20 ryegrass seeds were placed on top of each disk. Each disk was saturated with 5 mL modified Hoagland solution (see Section 3.4.2) and a further 150 mL modified Hoagland solution was carefully added to the base of the tray to prevent the seed coat and endosperm drying out. The tray was placed in a growth cabinet at 15-17 ºC and 20-21 ºC night and daytime temperatures respectively. The relative humidity was between 60 and 70% and light intensity was approximately 350 μmol m^{-2} s^{-1}. The tray was covered with tin foil and left for 72 hours, by which time the vast majority of the seeds had germinated. The foil was removed and a further 150 mL modified Hoagland solution added to the tray. Further volumes of modified Hoagland solution were added to the tray to prevent the seedlings from drying out. Ryegrass seedlings are shown in Figure 3.29 following 9 days of growth on cotton wool disks saturated with modified Hoagland solution.

Figure 3.29 - 9-day-old ryegrass seedlings

Ryegrass seedlings were removed from the tray at different stages of development and the intact cotton wool disk placed into a modified glass bell jar. 30 mL modified Hoagland solution were added to the base of the bell jar to prevent the cotton wool from drying out and the sealed jar placed in the growth cabinet under the light and temperature regime detailed above for 24 hours.

3.4.4 Growth of Oilseed Rape Seedlings

Oilseed rape (*Brassica napus*, cv. Essex Broadleaved) seeds were obtained from Cotswold Seeds, Gloucestershire. Cotton wool disks of approximate diameter 35 mm were placed in a seed tray. 20 oilseed rape seeds were placed on each cotton wool disk, which were then saturated with 5 mL modified Hoagland solution (see Section 3.4.2). The tray was covered with tin foil and placed in a growth cabinet. The growth cabinet was set on a temperature program of 15-17 ºC and 20-21 ºC night and daytime temperatures respectively, with relative humidity of between 60 and 70% and light intensity of approximately 350 μmol m^{-2} s^{-1}. The foil was removed after 72 hours, by which time the majority of the seeds had germinated.

The intact disks were removed at 24-hour intervals and the seedlings placed in a modified bell jar, as shown in Figure 3.30. The seedlings were incubated for 24 hours in the growth cabinet, after which time the methanol emitted by the seedlings was quantified using GC-FID.

Figure 3.30 - 11-day-old oilseed rape seedlings in modified bell jar

3.4.5 Quantification of Methanol Emitted from Plants

GC was performed using a Hewlett Packard 5890 GC split injection system with an Equity^{TM}-1 fused silica capillary column (30 m x 0.53 mm, film thickness 3.0 μm; Supelco, Bellefonte, PA) coupled with a Varian 4270 integrator. He carrier gas flow was set at 4 ml min^{-1} and the oven temperature was 40 °C. The injector and detector temperatures were 220 and 200 °C respectively.

Ten minutes prior to the end of the 24-hour incubation period, the modified bell jar containing the sample plants was removed from the growth cabinet. The GC was switched off and a portion of the GC column was submerged in liquid nitrogen, as illustrated in Figure 3.13. A glass tube containing adsorbent resin was attached to a small length of PVC tubing at both the inlet (to filter impurities from the air) and outlet end of the bell jar, as shown in Figure 3.20. An air cylinder and low-range flotometer were coupled to the inlet and outlet end of the jar respectively, so that the rate of air flow purging the system could be accurately monitored throughout the sampling period. After the column had been submerged in liquid nitrogen for 5 minutes, the flow of air through the system was commenced and maintained at 4 mL min^{-1} using the air inlet valve. Following a sampling time of 5 minutes, the sample adsorbent tube, occupying the outlet end of the system, was removed and transferred immediately to the injection port of the GC, as illustrated in Figure 3.13. The column was left in the liquid nitrogen for a further 5 minutes with the GC oven switched off to allow the complete thermal desorption of the volatile compounds adsorbed on the resin. The column was subsequently removed from the liquid nitrogen and the oven door closed. The oven switched on and the integrator started.

Chapter Four –
Methyl and Non-Methyl Ester Determination on Pea and Potato Material

4.1 Methyl and Non-Methyl Ester Determination of Pea Epicotyls

4.1.1 Introduction

Pectic polysaccharides are required for intercellular adhesion in dicotyledonous plants (Jarvis et al., 2003). To fulfil this function, they need to be cross-linked, covalently or non-covalently. Partial cell separation by chelating agents (Cocking et al., 1960) implied that both covalent and non-covalent cross-links were involved.

There is accumulating evidence to suggest that, as well as calcium cross-linking, covalent linkages are responsible for cell to cell adhesion. For example, treatment with EDTA has been shown to result in minimal cell separation, whereas additional treatment with PG caused separation of both transverse and longitudinal walls (Briggs, 2004). It has been suggested that these covalent linkages are formed by glycosidic bonds between xyloglucan and acidic pectins (Thompson and Fry, 2000) or, alternatively, that ester bonds could be fortifying the calcium cross-links (Hou and Chang, 1997).

It has also been suggested that alcohols other than methanol can form galacturonoyl esters in a similar manner to methanol. It has already been established that acetic acid forms ethanoyl, rather than galacturonyl, esters with the O-2 and O-3 hydroxyl groups of galacturonosyl residues.

Here, methods described in Section 2.1 were utilised in order to quantify non-esterified galaturonyl residues, total uronic acid content, and methyl esters content of pea epicotyl cell wall material and ultimately detect the presence of and quantify any non-methyl esters in these cell walls.

4.1.2 Calculations

4.1.2.1 Non-Esterified Carboxylic Acid Determination

4.1.2.1.1 Titration with NaOH

The method detailed in Section 2.1.5.2.1 was carried out in order to quantify the carboxylic acid groups present in the cell wall that were not esterified in any way. Cell walls were acidified to ensure galacturonyl residues were present in their free acid form and titrated with NaOH under nitrogen gas.

The calculation used to determine the number of μmoles of non-esterified carboxyl groups per gram of cell wall material (CWM) was as follows:

(0.01 * (Titre (mL) / 1000) * 1000) * ((1 / weight of CWM (g)) * 1000)

4.1.2.1.2 Copper Binding Method

The method described in Section 2.1.5.2.2 was used as an alternative method to the titration with NaOH to quantify non-esterified carboxylic acid groups in the cell wall. Cell walls were saturated with Cu(II) ions, which bind to carboxyl groups not containing an ester group, and are subsequently removed using excess acid.

Using this method, the calculations required to determine the number of μmoles of non-esterified carboxyl groups per gram of cell wall material (CWM) was as follows:

AA copper concentration (μg mL^{-1}) / 63.55 = copper concentration (mM)

Concentration (mM) * 50 (mL) * 2 = μMoles carboxyl

μMoles carboxyl / weight of CWM (mg) = μMoles carboxyl mg CWM^{-1}

4.1.2.2 Total Galacturonic Acid Determination

This method is described fully in Section 2.1.5.3 and involves the saponification of the CWM, which cleaves the ester bond. The cell walls are then acidified so that all carboxyl groups are in the acidified form and titrated with NaOH.

The calculation used to determine the total number of μmoles of carboxyl groups per gram of cell wall material (CWM) was as follows:

(0.01 * (Titre (mL) / 1000) * 1000) * ((1 / weight of CWM (g)) * 1000)

4.1.2.3 Methyl Ester Determination

Methyl esters were quantified using the method described in Section 2.1.5.4, which involves the colorimetric determination of formaldehyde produced from the oxidation of methanol. The methanol is produced following the cleavage of methyl ester bonds by saponification with KOH.

The following calculations were used to determine the number of μmoles of methanol contained in 1 g pea epicotyl CWM:

Colorimetric values obtained from spectrophotometer from samples, standards and controls

Methanol concentrations of samples in μg mL^{-1} determined from calibration graph

Concentration (μg mL^{-1}) * Dilution Factor = Actual Methanol Concentration (μg mL^{-1})

Methanol Concentration (μg mL^{-1}) / 32 = μmoles MeOH mL^{-1}

μmoles MeOH mL^{-1} * 8.25 = μmoles MeOH in 8.25 mL (total solution volume in analysis)

μmoles MeOH / weight of CWM (g) = μmoles MeOH g^{-1}

Averages taken of samples (a) and controls (b) and (b) subtracted from (a) to give methanol concentration in μmoles g^{-1}

4.1.2.4 Overall Calculations

The non-methyl esterified galacturonic acid content of the cell walls of pea epicotyls was determined using the four calculations detailed in Sections 4.1.2.1, 4.1.2.2 and 4.1.2.3 above. The total ester content of the galacturonic acid residues (GalUA) in the cell walls and the non-methyl ester content of the cell walls were determined using equations 4.1 and 4.2 respectively.

$$\text{Total Esterified GalUA} = \text{Total GalUA} - \text{Non-Esterified GalUA} \quad (4.1)$$

$$\text{Non-Methyl Esterified GalUA} = \text{Total Esterified GalUA} - \text{Methyl-Esterified GalUA} \quad (4.2)$$

4.1.3 Results

Table 4.1 shows the figures resulting from the calculations described in Section 4.1.2. There is good agreement between the results determined from the titration and copper binding methods for non-esterified GalUA residues.

	Content in Cell Walls (umol g CWM-1)					
Plant Material	Total Carboxyl	Non-Esterified Carboxyl (Titration)	Non-Esterified Carboxyl (Copper Binding)	Mean Non-Esterified Carboxyl	Total Ester	Methyl Ester
	a	b	c	(b+c)/2	d	e
Pea Epicotyl	630.5	399.5	418.3	408.9	221.6	180.8

Table 4.1 – Results from the chemical characterisation of pea epicotyl cell walls

Table 4.2 shows the methyl and non-methyl ester contents of GA in terms of percentages of the total GA content of pea epicotyl cell walls. The calculations relate to figures displayed in Table 4.1.

	Percent Esterification		
Plant Material	Total Ester Content	Methyl Ester Content	Non-Methyl Ester Content
	100d/a	100e/a	100(d-e)/a
Pea Epicotyl	35.1	28.7	6.47

Table 4.2 – Percentage methyl and non-methyl ester contents

Statistical analysis of the results was hampered by the fact that different numbers of replicates were used in determinations for total and methyl esters. However, it was determined that there was a significant quantity of non-methyl esters in cell walls extracted from pea epicotyl tissue.

4.1.4 Discussion

The results for the methyl and non-methyl ester content of pea epicotyl cell walls displayed in Table 4.2 are shown in terms of percent of total carboxyl content. They confirm the presence of pectic non-methyl esters in pea epicotyl cell walls. This suggests the presence of a cross-link between certain galacturonoyl groups and some other constituent of the cell

wall. However, from this set of experiments, the exact nature of this cross-link cannot be determined.

4.2 Methyl and Non-Methyl Ester Determination of Potato Tuber Primary Cell Walls

4.2.1 Introduction

The texture of potatoes is strongly correlated with the starch content of the potato (Jarvis and Duncan, 1992). As different varieties of potato have varying specific gravities and subsequently starch contents, texture is also specific to particular varieties of potato and to some extent determines their main uses; for example, in the food industry different potato varieties are used in different products depending on their texture.

The texture of plant-based foods is affected by modifications to specific cell wall components during growth, ripening, storage, cooking and processing, which result in spatial variations in cell separation and adhesion between the walls of adjacent cells. Research suggests that the formation of non-covalent calcium cross-links following enzymic de-esterification of galacturonic acid (GA) residues results in the softening of some plant based foods, for example tomato pericarp (Orfila et al., 2001). However, in other systems heat treatment or treatment with pectinases is required for cell separation, indicating that the bonds linking adjacent cells are not calcium-mediated. It has been demonstrated that about 10% of the galacturonate units in potato pectins *in situ* carry intermolecular esters other than the established methyl or acetyl substituents (MacKinnon et al., 2002).

The quantities of methyl and non-methyl esters in the pectic polysaccharides of cell walls from two varieties of potato tuber over the course of a storage season were determined and related to observed changes in tissue texture as the storage season progresses.

4.2.2 Calculations

The starch remaining in the MCB-insoluble cell wall material following treatment with α-amylase and pullulinase was quantified (Section 2.1.5.1) so that subsequent calculations involved an accurate figure for the weight of cell wall material. Figures for percentage dry matter were calculated (Equation 4.3 from Burton, 1989) in order to ultimately obtain a

conversion factor for the weight of cell wall material used in methyl ester quantification experiments. The starch contents of fresh material from each of the two potato tuber cultivars were also calculated using equations devised by Burton (1989) (Equation 4.4).

$$\text{Dry Matter (\%)} = (196.58 * \text{Specific Gravity}) - 189.41 \quad (4.3)$$

$$\text{Starch (\%)} = 17.55 + 199 * (\text{Specific Gravity} - 1.098) \quad (4.4)$$

Table 4.3 shows the figures for specific gravity and starch contents of the two potato cultivars studied here. Specific gravity values were taken from Wilson (unpublished).

Cultivar	Specific Gravity	Dry Matter (%)	Starch (% Fresh Weight)	Starch (% Dry Weight)
Estima	1.07	21.68	12.73	58.74
Maris Piper	1.09	24.00	15.08	62.85

Table 4.3 – Specific gravities and starch content of potato tubers

A conversion factor was calculated for each of the two potato cultivars to take into account the residual starch in subsequent calculations using Equation 4.4. The resulting figures are shown in Table 4.4.

$$\text{Conversion Factor} = (100 - (\text{Dry Matter (\%)} + \text{Residual Starch (\%)})) / 100 \quad (4.5)$$

Cultivar	Residual Starch (%)	Dry Matter (%)	Conversion Factor
Estima	11.96	6.39	0.82
Maris Piper	9.90	6.21	0.84

Table 4.4 – Residual starch and dry matter of MCB-insoluble material

In subsequent calculations used to determine the methyl and non-methyl ester content of potato tuber cell walls, the weight of MCB-insoluble material used was multiplied by the conversion factor so that results could be expressed accurately on a cell wall weight basis.

Calculations detailed in Section 4.1.2 were used to determine the non-esterified carboxylic acid content, the total galacturonic acid content, the methyl ester content and subsequently the non-methyl ester content of the cell walls of Estima and Maris Piper potato tubers at various stages over the storage season. In each calculation involving a weight of CWM,

this figure was multiplied by the conversion factor to take into account the presence of residual starch.

4.2.3 Results

Table 4.5 shows the results from the calculations described in Section 4.1.2. Again, there is good agreement between the results determined from the titration and copper binding methods for non-esterified GalUA residues. Table 4.6 displays the results of the methyl and non-methyl ester determination experiments in terms of a percentage of the total carboxyl groups in the cell walls. Standard deviations are also displayed. Due to variations in the number of replicates involved in the analyses involved in the chemical characterisation of potato tuber cell walls, analyses of variance could not be performed on the raw data. The standard deviation figures for data obtained from the non-methyl ester determination was calculated using the following formula:

$$\text{Standard Deviation of Non-Methyl Ester Content} = \sqrt{(\text{Variance of Total Ester Content} + \text{Variance of Methyl Ester Content})}$$

By using this formula, errors incurred from the two figures used in the calculation used to determine non-methyl ester content are taken into account.

From the figures shown in Table 4.6, it can be seen that there is no significant variation in methyl ester content of the potato tubers studied over the storage season. The methyl ester content of Estima tubers decreases over the storage season whereas in Maris Piper tubers, the quantity of methyl esterified GA residues remains relatively constant. One may expect the quantity of methyl esterified GA residues to increase over the storage season as the texture of the tubers deteriorates. The fact that potato tubers become softer over the storage season alone suggests that the methyl ester content of tubers increases and as the occurrence of methyl esters increases, the extent of calcium cross-linking decreases.

Non-methyl esters appear to increase in occurrence in Estima as the storage season progresses, while in the case of Maris Piper, the proportion of the GA residues esterified with something other than methanol appears to decrease over time.

There is obviously a large difference between the non-methyl ester content of the two cultivars at all times throughout the storage season of the potatoes. It is clear that the quantity of non-methyl esters in Maris Piper cell walls is much greater than that in Estima.

In fact, at this early stage in the storage season, there is a greater proportion of non-methyl esters than that of methyl esters in the cell walls of Maris Piper tubers. The methyl ester contents of the cultivars are relatively similar to each other, but the proportion of the total GA residues that are not carrying an ester of any kind is much greater in Estima cell walls than in Maris Piper. Just over 50% of the carboxyl groups in Maris Piper cell walls are not esterified, compared to about 70% in Estima cell walls.

		Content in Cell Walls (umol g CWM -1)					
	Time Between Harvest and Analyses (Days)	Total Carboxyl	Non-Esterified Carboxyl (Titration)	Non-Esterified Carboxyl (Copper Binding)	Mean Non-Esterified Carboxyl	Total Ester	Methyl Ester
Potato Sample		a	b	c	d = (b+c)/2	e = a - d	f
Estima 1	117	701	460	447	454	248	159
Estima 2	215	548	381	376	378	170	93
Estima 3	299	543	365	366	365	178	92
Maris Piper 1	117	826	456	457	457	370	144
Maris Piper 2	218	783	423	420	421	362	187
Maris Piper 3	299	729	430	424	427	301	146

Table 4.5 - Results from the chemical characterisation of potato tuber cell walls at different stages during the storage season

		Percent Esterification					
Potato Sample	Time Between Harvest and Analyses (Days)	Total Ester Content	Standard Deviation of Total Ester Content	Methyl Ester Content	Standard Deviation of Methyl Ester Content	Non-Methyl Ester Content	Standard Deviation of Non-Methyl Ester Content
		100e/a	100SD/a	100f/a	100SD/a	100(e-f)/a	100SD/a
Estima 1	117	35.3	2.20	22.7	1.46	12.6	4.35
Estima 2	215	31.0	5.50	17.0	0.84	14.0	3.38
Estima 3	299	32.7	0.98	16.9	2.66	15.9	2.83
Maris Piper 1	117	44.7	0.42	17.4	0.82	27.3	0.92
Maris Piper 2	218	46.2	4.43	23.8	2.26	22.4	4.98
Maris Piper 3	299	41.4	1.38	20.0	3.31	21.4	3.59

Table 4.6 - Percentage methyl and non-methyl ester contents and standard deviations of potato tuber cell walls at different stages during the storage season

4.2.4 Discussion

There is, in both potato cultivars studied here, a significant quantity of non-methyl esters present in the pectic fraction of the cell walls. Although the methods employed are not highly specific for esters of a specific origin, the quantities are large enough to discount the possibility that results are significantly contributed to by anionic constituents of the cell wall other than the pectic polysaccharides.

Despite the fact that there is no obvious correlation between duration of storage following harvest and degree of GA methyl and non-methyl esterification, from the data in Tables 4.5 and 4.6, it is evident that there are significant varietal differences in overall pectin composition.

Typical methyl ester contents of potato cell walls range between 37% in cv. King Edward (Binner et al., 2000) and 53% in cv. Bintje (Ng and Waldron, 1997). It is clear that the methyl ester contents of both potato cultivars studied here are below these figures, with Estima and Maris Piper containing approximately 23 and 17% methyl esterified GA residues respectively. These figures refer to the composition of pectic polysaccharides within the cell wall following 117 days of storage. It may be possible that the methyl ester content decreases in the time following harvest and the non-methyl ester content increases, thus de-stabilising the cell walls by interrupting the calcium network that would otherwise create a rigid framework (Figure 1.14).

However, as there is no obvious trend between the methyl and non-methyl ester contents over the storage period, the observed deterioration of potato texture may be solely due to the decrease in starch content and therefore specific gravity of the potato tubers.

4.2.5 Conclusions

These experiments have confirmed the presence of non-methyl esters and it has been demonstrated that variations in their quantities occur in different varieties of potato tuber. While there was no obvious correlation between duration of storage following harvest and degree of GA methyl-esterification, significant varietal differences in overall pectin composition were observed. The modifications observed in potato texture over the period

of storage can evidently be directly attributed to the general decrease in specific gravity of potato tissues, leading to reduced turgor pressure within the cell.

Chapter Five -
Pectic Methyl Ester Content of Mature Leaves of Native Tree Species

5.1 Introduction

The pectic methyl ester content of mature leaves of certain tree species found growing extensively throughout Great Britain was determined (Section 2.2). This series of experiments were designed with the ultimate aim of determining the amount of methanol that is potentially released to the troposphere during the autumn and winter months as the leaves senesce.

5.2 Results

Leaf material was sampled from several species of tree growing commonly throughout Great Britain, located at the various locations in Scotland during August and September, 2005, as listed in Table 2.2. The mean methanol content of three replicates of each species is displayed graphically in Figure 5.1.

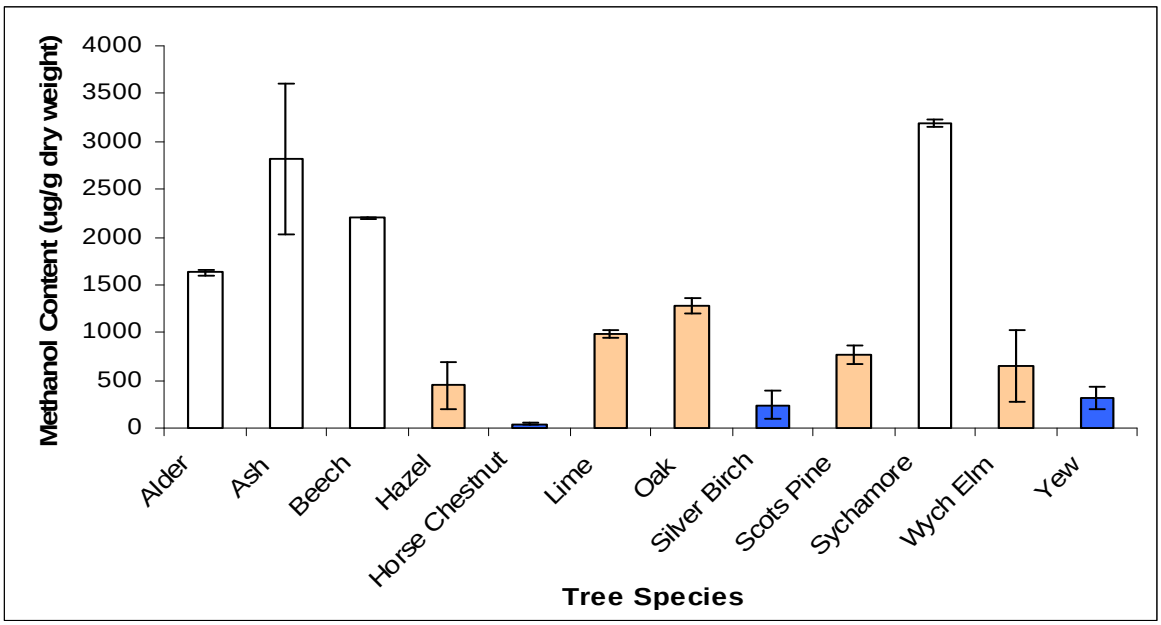


Figure 5.1 – Graphical representation of the methanol contents of the leaves of various tree species. Error bars show standard error, different colours represent significant differences at the 95% confidence interval

Figure 5.1 illustrates the considerable differences in the methyl ester content of leaves of different tree species. Comparing the data in Figure 5.1 with that in Table 2.2, it is clear that horse chestnut and silver birch leaves, both harvested from trees located within the

University campus, contained the lowest concentrations of methanol of the tree species sampled. These findings suggest that the differences observed in methanol concentrations between tree species were not due to the time lapsed between sampling and freezing the leaves, but due solely to the quantity of pectic methyl esters present in the living plant tissue. If there was some correlation between the quantity of pectic methyl esters in the leaf tissue and the length of time lapsed between harvesting and freezing the leaves, it could be argued that PME action between harvest and freezing could have had an impact on the amount of pectic methyl esters in the plant tissue.

5.3 Estimation of Methanol Emitted from Deciduous Woodland in Great Britain

5.3.1 Introduction

Results collated from the determination of potential methanol emitted from mature leaves of tree species native to the UK (Section 5.2) can be extrapolated in order to calculate an estimate for the amount of methanol potentially released into the troposphere from trees as their leaves senesce at the end of each year. In order to carry out these calculations, it is necessary to know, or at least be able to estimate the leaf biomass per hectare. Two different methods were employed for calculating the potential amount of methanol that could be introduced to the troposphere, as a result of the de-methylation of leaf pectin during the autumn and winter months in Great Britain.

The first utilised published data for the dry weights of leaf litterfall from several tree species, for which the methyl ester contents had previously been determined (see Sections 2.2.3 for methods and 5.2 for results). Data was considered to be relevant to this study if the area in which the trees were located was similar in climatic terms to Britain. Data presented in the Forestry Commission's Results National Inventory of Woodland and Trees (NIWT) for Great Britain (Anon, 2000) (Section 5.3.2), were also used in the calculations. These figures were collated and applied to the results from the determination of methanol that could potentially be released from mature leaves following abscission (Sections 2.2.3 and 5.2).

The second method utilised published data for the Leaf Area Index (LAI) of different tree species, for which the methyl ester contents had previously been determined (see Sections 2.2.3 for methods and 5.2 for results). Surface area to dry weight ratios were determined

and subsequently the approximate dry weight of leaves that would later be lost from trees of certain species during the autumn and winter months. As with the leaf litterfall data, the LAI data utilised in these calculations had to come from tree species for which leaf litterfall data and methyl ester content data were available. Results from the National Inventory of Woodland and Trees (NIWT) for Great Britain (Anon, 2000) (Section 5.3.2), were also used in these calculations. The data resulting from these calculations were applied to the results from the quantification of the pectic methyl ester content of mature leaves, as discussed in Section 5.2.

5.3.2 The National Inventory of Woodland and Trees

Between 1994 and 2000, the Forestry Commission compiled the National Inventory of Woodland and Trees (NIWT) for Great Britain (Anon, 2000). The final report, comprised of a Main Woodland Survey (MWS) covering woodlands of 2 hectares and over, and a Survey of Small Woodland and Trees (SSWT) covering small woods and groups of trees, was completed in July 2000.

The methods implemented in compiling this data varied depending on the particular country being surveyed. A digital map of England and Wales was constructed using 1:25000 scale digital photography and the particular forest types identified and recorded. In Scotland, the main survey was based on the Land Cover of Scotland (LCS) 1988 project. This land cover map was compiled using 1:25000 scale aerial photography.

5.3.2.1 Results from the National Inventory of Woodland and Trees (NIWT)

The forest types included in the survey can be categorised into eight groups. The main categories, which contribute approximately 99% of the total woodland area are coniferous, broadleaved and mixed woodland as well as felled areas and areas of open space. Coniferous and broadleaf woodland is defined as woodland containing greater than 80% by area of coniferous and broadleaved trees respectively. Mixed woodland is defined as woodland where both coniferous and broadleaved trees occupy at least 20% of the canopy each. Felled areas are considered to be woodlands that have been felled or where tree cover is less than 20% by area. Open space is classified as an area within a woodland that are not covered by trees, but is an integral part of the woodland as a whole, for example riversides.

Table 5.1 summarises the areas of different woodland types located in the different countries of Great Britain by area.

Country	Forest Type					
	Coniferous	Broadleafed	Mixed	Felled	Open Space	Total
Scotland	888317	176519	53696	23303	134130	1281472
England	280259	571051	135318	15100	71634	1096885
Wales	137474	106855	22040	8961	10902	286767
Total	1400905	868687	213982	50597	230067	2665126

Table 5.1 – Areas in hectares of different woodland types in countries of Great Britain (adapted from NIWT (Anon, 2000))

The main survey results can be summarised as follows:

- 11.6% of the total land area of Great Britain is composed of woodland of area over 0.1 hectares.
- Coniferous, broadleaf and mixed woodland constitute 49.0%, 32.1% and 7.9% of all woodland areas in Great Britain respectively.
- The main coniferous species was identified as Sitka spruce, occupying a total of 691918 hectares, or 49.2% of conifer species.
- 22.9% of all broadleaf species was identified as oak, covering 222697 hectares.

Table 5.2 lists the areas covered by woodland comprised of different principal species in Great Britain.

Species	Total Area (hectares)
Pine	407882
Sitka spruce	691918
Larch	133728
Other Conifers	154428
Mixed Conifers	17645
Total Conifers	1405604
Oak	222697
Beech	82981
Sycamore	66594
Ash	129145
Birch	159992
Elm	5150
Other Broadleaves	144644
Mixed Broadleaves	160227
Total Broadleaves	971434

Table 5.2 – A summary of woodland areas according to principal species (adapted from NIWT (Anon, 2000))

5.3.3 Available Annual Litterfall Data

The study of carbon and nutrient cycling in the environment involves measuring the major organic matter pools found in vegetation located within a particular ecosystem and determining the fluxes between the vegetation and the surrounding environment. Examples of nutrient pools include tree biomass, litter standing crop and the soil itself. Litterfall is an important pathway in the transfer of organic matter and chemical elements from vegetation to the soil surface in forest ecosystems (e.g. Ogawa, 1978) and as such, figures for annual litterfall are readily available for certain tree species growing in particular types of ecosystem. Litterfall production accounts for more than half of the above-ground net primary productivity (Olson, 1963; Clark et al, 2001), making it the single most important component in above-ground primary productivity.

Annual litterfall data is available referring to a mixed broadleaved forest in Northern Germany (Kutch et al., 2005), Kannenbruch Forest, which is unusual in that it is comprised of a mosaic of subplots dominated by particular species of tree. In this study, the 350ha deciduous forest was divided into three thirty metre square subplots dominated by oak (*Quercus robur* L.), beech (*Fagus sylvatica* L.) or alder (*Alnus glutinosa* L.) and ash (*Fraxinus excelsior* (L.), Gaertn.) trees. The characteristics for the litterfall of the three subplots were determined in terms of leaves, fruit and branches. It was concluded that oak trees contributed 382 g C m^{-2} yr^{-1}, 184 of this being contributed by leaves. The total litterfall and that contributed by leaves in the beech-dominated subplots were 254 and 130 g C m^{-2} yr^{-1} respectively. The litterfall was lowest in the alder/ash-dominated subplot where 220 and 169 g C m^{-2} yr^{-1} were observed for total litterfall and leaf fall respectively.

Rapp et al. (1999) quantified the litterfall in six sites located within deciduous and evergreen oak forests near Salamanca and Montpellier. Deciduous oak species studied were *Quercus pyrenaica* Willd. and *Quercus lanuginose* Lamk in Spain and France respectively and litterfall data was categorised into leaf litter, woody litter, fruits, flowers and residual litter. Results for leaf litterfall data are quoted for *Q. pyrenaica* and *Q. lanuginose* as between 2088 and 2829 kg ha^{-1} yr^{-1}, depending on the ecosystem in which the *Q. pyrenaica* were located, and 2466 kg ha^{-1} yr^{-1} respectively.

Foliar litterfall data for a stand of birch (*Betula pendula* Roth.) trees situated in Southern Lithuania was determined by Varnagiryte et al. (2005). On this formerly agricultural site, stands of several different tree species had been planted adjacent to each other in 1959.

Results showed that the leaf litterfall for birch in such an ecosystem was 2418 kg ha^{-1}, following the collection of abscised leaves during the autumn months of 2005.

A proportion of the available relevant litterfall data is quoted in terms of grams of carbon introduced to the soil per area per year (e.g. Kutch et al., 2005). However, there are some authors that prefer to report figures in terms of the actual dry weight of leaf material being introduced to the soil litter layer per area per year (e.g. Rapp et el., 1999, Varnagiryte et al., 2005). For the purposes of this study, data presented in the latter form are more useful. These figures can be applied directly to calculations with the intention of producing realistic data for the amount of methanol that could potentially be emitted to the troposphere through the decomposition of leaves that have been abscised. Leaf litterfall data referring to tree species closely related to those being studied in Section 2.2.3 was required for all further calculations. For this reason, it was considered prudent to regard data quoted by Kutch et al. (2005) for oak, beech and ash trees, and Varnagiryte et al. (2005) for birch trees, as being applicable to any further calculations involving litterfall data.

In order to utilise these data in calculations with the aim of estimating the amount of methanol potentially emitted to the troposphere at the end of each year, the figures quoted by Kutch et al. (2005) had to be converted to approximate values for litterfall in terms of the actual dry mass of plant material being transferred from the vegetation to the forest floor per area of forest. Love et al. (1992) determined that the percentage carbon in leaf dry matter was approximately 49%. By assuming the plant material in this study is comprised of 49% carbon, data quoted in g C m^{-2} yr^{-1} can be converted to kg biomass ha^{-1} yr^{-1}. Table 5.3 displays the results obtained by Kutch et al. (2005) and Varnagiryte et al. (2005), together with the equivalent approximate values in terms of kg biomass ha^{-1} yr^{-1} for the three tree species observed by Kutch et al. (2005).

Dominant Tree Species	Source of Data	Leaf Litterfall (gCm-2yr-1)	Leaf Litterfall (kg ha-1 yr-1)
Oak	Kutch et al., 2005	184	3755
Beech	Kutch et al., 2005	130	2653
Alder/Ash	Kutch et al., 2005	169	3449
Birch	Varnagiryte et al., 2005	N/A	2418

Table 5.3 – Leaf litterfall figures from Kutch et al. (2005) and Varnagiryte et al. (2005)

The potential amount of methanol, originating from pectic polysaccharides, released to the troposphere during the autumn and winter months in Great Britain as a result of the senescence of leaves from oak, beech, ash and birch trees was subsequently calculated (Section 5.3.4) using the leaf litterfall data above, the results of the National Inventory of Woodland and Trees (NIWT) (Section 5.3.2.1) and the results of pectic methyl ester determination of mature leaves (Section 5.2).

5.3.4 Calculations Using Leaf Litterfall Data

The approximate amount of methanol, originating from methyl ester groups on pectic polysaccharides, that could potentially be emitted from the leaves of oak, beech and ash trees located in Great Britain at the end of each year, can be calculated using the annual leaf litterfall data determined by Kutch et al. (2005) together with woodland areas detailed in NIWT (Anon, 2000) and summarised in Table 5.3. Kutch et al. (2005) quote figures for the annual leaf litterfall from stands dominated by oak, beech and alder and ash. The potential amount of methanol emitted from mature alder leaves cannot be calculated using this method as there are no figures available for the total area covered by alder-dominated woodland in Britain. However, assuming that the figure determined by Kutch et al. (2005) for the litterfall from the alder/ash stand is comparable to that of a stand dominated solely by ash trees, the potential amount of methanol emitted from leaves of oak, beech and ash trees may be calculated. The potential amount of methanol emitted from birch leaves from trees located in Britain may be calculated using figures quoted in NIWT (Anon, 2000) for the area of Great Britain occupied by birch-dominated woodland and data determined by Varnagiryte et al. (2005) for leaf litterfall.

Using the results determined for the total amount of pectic methyl ester groups present in leaves at the end of August and beginning of September, 2005 (see Section 5.2 and Figure 5.1), together with the findings presented in NIWT and the leaf litterfall data presented in Table 5.3, the potential amount of methanol that can be released to the troposphere in Britain each year from the senescence of oak, beech, ash and birch leaves can be estimated. Table 5.4 displays the results from this set of calculations.

5.3.5 Available Leaf Area Index Data

Leaf Area Index (LAI) is defined as the one-sided green leaf area per unit ground area in broadleaf canopies, or as the needle area per unit ground area in needle canopies (Chen and Black, 1992), usually quoted as $m^2\ m^{-2}$ or dimensionless. Total vegetation surface of

deciduous woodland, during periods of vegetation, is mainly composed of leaf area, but also includes, to a lesser extent twigs, branches and stems. However, vegetation cover during times of absent foliage, for example winter in temperate climates or the dry season in tropical regions, is determined by the woody parts of trees. The interaction between vegetation surface and the atmosphere, for example gas exchange or precipitation interception, is substantially determined by the vegetation surface (Monteith and Unsworth, 1990).

Various methods may be employed in the estimation of LAI. These include the use of hemispherical photography or other optical instruments, for example TRAC or LAI-2000. (Chen et al., 1997). Whole trees may also be harvested in order to determine LAI's and measurements using litter traps are also often used. Comparisons between figures for LAI of temperate forests are complicated by the fact that different authors employ different methods to measure LAI.

LAI values have been obtained using a variety of methods including hemispherical photography and leaf litterfall collection on oak/beech stands located within the Aelmoeseneie forest in Belgium, approximately 15km south-east of Gent (Mussche et al., 2001). LAI measurements were recorded throughout 1996. Maximum figures for LAI were observed in August and are quoted as being 3.47 and 5.52 $m^2 m^{-2}$ for data collected using hemispherical photography and leaf litterfall collection respectively.

Leuschner et al. (2006) studied the LAI of beech (*Fagus sylvatica* L.) stands located at a number of different woodland sites across north central Germany, via the collection of foliar biomass in a modified bucket and concluded that the average figure for LAI for the studied beech stands was 7.4 $m^2 m^{-2}$.

Tree Species	Annual Leaf Litterfall (kg ha-1 yr-1)	Methanol Content in Mature Leaves (ug g-1 leaf dry weight)	Methanol in Mature Leaves (ug ha-1 yr-1)	Area of Woodland (ha)	Potential Amount of Methanol Released from Leaves During Senescence (Gg yr-1)
Oak	3755	1282	4.82E+09	222697	0.70
Ash	3449	2822	9.73E+09	129145	0.82
Beech	2653	2201	5.84E+09	82981	0.31
Birch	2418	239	5.79E+08	159992	0.06

Table 5.4 – Results for the potential amount of methanol emitted to the troposphere from leaves of trees in Great Britain using leaf litterfall data

Tree Species	Dry Weight per Unit Area (g/cm2)	LAI (m2m-2)	Potential Weight of Leaf Litterfall (kg ha-1 yr-1)	Methanol Content in Mature Leaves (ug kg-1 leaf dry weight)	Area of Woodland (ha)	Potential Amount of Methanol Released from Leaves During Senescence (Gg yr-1)
Oak	7.45E-03	5.30	3948	1.28E+06	222697	0.73
Ash	4.54E-03	5.30	2406	2.82E+06	129145	0.57
Beech	4.86E-03	7.40	3599	2.20E+06	82981	0.43
Birch	8.26E-03	5.30	4377	2.39E+05	159992	0.11

Table 5.5 – Results for the potential amount of methanol emitted to the troposphere from leaves of trees in Great Britain using Leaf Area Index data

5.3.5.1 'Worldwide Historical Estimates of Leaf Area Index, 1932-2000

The 'Worldwide Historical Estimates of Leaf Area Index, 1932-2000' (Scurlock et al., 2001) contains approximately one thousand published estimates of LAI from about 400 field sites covering a variety of terrestrial ecosystem types located in several countries throughout the world.

Several LAI figures are included in the completed data set that refer to temperate deciduous broadleaf forest systems located within the UK. Table 5.6 displays the authors cited in the 'Worldwide Historical Estimates of Leaf Area Index, 1932-2000' (Scurlock et al., 2001), details of each study site and the estimated LAI from each particular area of study.

Author	Year of Publication	Site Location	Dominant Species	Leaf Area Index (LAI) (m2/m2)
Ovington et al.	1959	Holme Fen Nature Reserve, Lancashire	Birch	1.70 - 6.50
Bunce	1968	English Lake District	Oak, Ash, Birch	5.30
Hughes	1969	Hartlepool	N/A	3.60
Ford et al.	1970	Ham Street Woods National Nature Reserve, Kent	Sweet Chestnut	5.60, 3.60

Table 5.6 – Details of LAI values from the 'Worldwide Historical Estimates of Leaf Area Index, 1932-2000' (Scurlock et al., 2001)

Table 5.6 summarises the LAI values publicly available and cited in the 'Worldwide Historical Estimates of Leaf Area Index, 1932-2000' (Scurlock et al., 2001). The test site most closely comparable to the vegetative conditions found within the sample sites in this study is that of Bunce (1968). Four sites within the English Lake District were studied, oak being the most dominant species in all, with birch and ash also widely distributed within the areas. Considering the similarities in flora and climatic conditions between these sites and the sites located within Scotland, from which the leaves used in this study were sampled, it my be reasonable to assume that the LAI of these locations will be comparable to that concluded by Bunce.

However, as there was no data available for the LAI of beech-dominated woodland in the 'Worldwide Historical Estimates of Leaf Area Index, 1932-2000', LAI data determined by Leuschner et al. (2006) was considered suitable to utilise in any further calculations regarding the total surface area of beech leaves within beech-dominated woodland in Britain.

5.3.6 Calculations using Leaf Area Index Data

Using the LAI figures determined by Bunce (1968) and the findings from the National Inventory of Woodland and Trees, it was possible to calculate the approximate total surface area of oak, beech, ash and birch leaf present in oak-dominated woodland in Great Britain. For example, the total area of oak-dominated woodland in Great Britain is 222697 hectares. Using Bunce's figure of 5.30 $m^2 m^{-2}$ for the LAI of an oak-dominated woodland, it is reasonable to suggest that the total oak leaf surface area in the oak woodlands located within Great Britain is approximately 1180294 hectares. Similarly, using the value for beech-dominated woodland quoted in the National Inventory of Woodland and Trees, it can be estimated that the total leaf area in Great Britain of beech trees is approximately 439799 hectares. Unfortunately there are no figures available for the total area of alder trees found in Great Britain and therefore it is not possible to calculate the amount of methanol that would be released from leaves of this tree species following abscission.

A value for the total dry weight oak, beech, birch and ash leaves abscised from trees located within Great Britain was required in order to extrapolate the findings in Section 5.2 and estimate the amount of methanol released from oak and beech trees in British woodland during the autumn and winter months of each year. Five typically shaped green leaves were selected from samples of oak (*Quercus petraea*), beech (*Fagus sylvatica*), birch (*Betula pendula*) and ash (*Fraxinus excelsior*) leaves collected from various sites throughout Scotland (see Table 2.2) at the end of August and beginning of September 2005. The total surface area of the selected leaves was determined using the WinCELL program, which is primarily utilised in the calculation of cell areas. The selected leaves of each tree species were then dried and the dry weights determined. The dry weight of leaf per unit surface area of leaf could then be calculated and subsequently the approximate total amount of methanol released to the troposphere from deciduous woodland in Great Britain in one year.

Table 5.5 displays the results of the calculations for the amount of pectic methyl esters that are contained within the abscised leaves of oak, beech, ash and birch trees, which are potentially released to the troposphere in the form of methanol, following abscission during the autumn and winter months, using LAI data. Considering the potential variability in the data utilised, the two sets of calculations result in fairly close figures for the amount of methanol that may potentially be released to the troposphere from leaves during the autumn and winter months.

5.4 Leaf Cover Estimates from Mugdock Country Park

Tables 5.7 to 5.15 display the results from the estimations made for the proportions of leaves present at various stages of senescence on sample trees and on the ground in three different sites within Mugdock Country Park (Section 2.3.1), on the three different sampling dates. The difference between the two estimates is the proportion of leaf cover that is intermediate in colour.

5.4.1 On-Tree Estimates - Results

5.4.1.1 Site A Trees

Tree	% Leaf Area Present as Green	% Leaf Area Present Brown
Oak A1	92	6
Oak A2	87	8
Oak A3	95	2
Alder A1	100	0
Alder A2	90	4
Alder A3	98	1
Beech A1	40	20
Beech A2	90	1
Beech A3	91	1

Table 5.7 - Estimates of leaf cover for sample trees in Site A in October

Tree	% Leaf Area Present as Green	% Leaf Area Present Brown
Oak A1	2	30
Oak A2	15	15
Oak A3	9	10
Alder A1	15	10
Alder A2	9	10
Alder A3	25	4
Beech A1	0	100
Beech A2	0	50
Beech A3	0	48

Table 5.8 - Estimates of leaf cover for sample trees in Site A in November

Tree	% Leaf Area Present as Green	% Leaf Area Present Brown
Oak A1	0	75
Oak A2	0	79
Oak A3	0	99
Alder A1	0	0
Alder A2	0	100
Alder A3	0	0
Beech A1	0	100
Beech A2	0	100
Beech A3	0	100

Table 5.9 - Estimates of leaf cover for sample trees in Site A in December

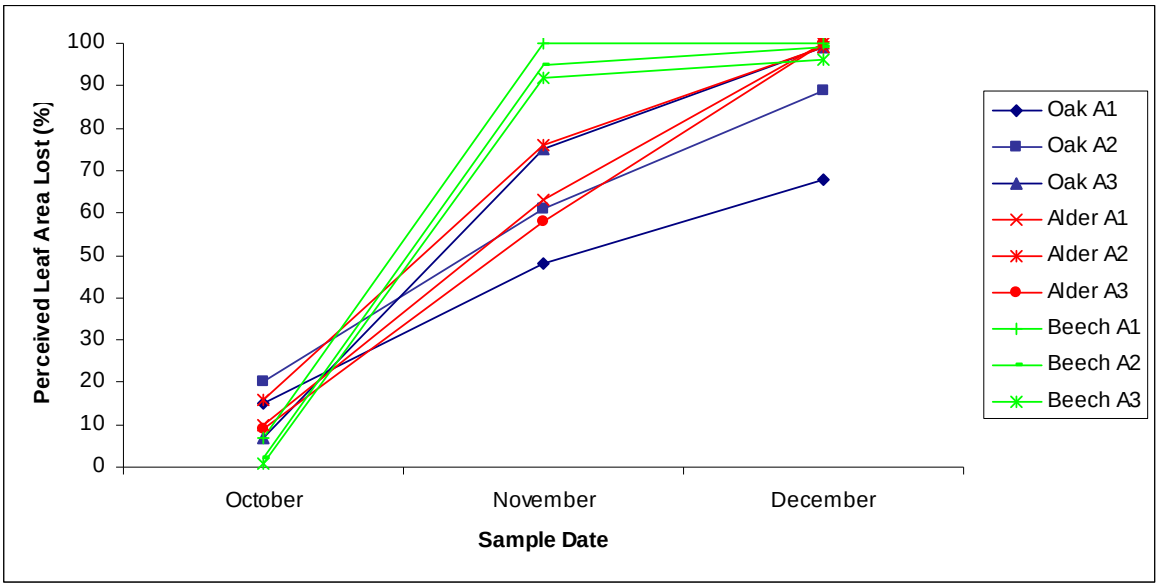


Figure 5.2 – Graphical representation of estimates for percentage leaf lost from sample trees in Site A

5.4.1.2 Site B Trees

Tree	% Leaf Area Present as Green	% Leaf Area Present as Brown
Oak B1	96	1
Oak B2	90	3
Oak B3	92	5
Alder B1	98	1
Alder B2	100	0
Alder B3	97	1
Beech B1	98	1
Beech B2	70	7
Beech B3	93	1

Table 5.10 - Estimates of leaf cover for sample trees in Site B in October

Tree	% Leaf Area Present as Green	% Leaf Area Present as Brown
Oak B1	65	5
Oak B2	4	15
Oak B3	27	12
Alder B1	7	5
Alder B2	3	18
Alder B3	2	24
Beech B1	0	50
Beech B2	0	71
Beech B3	0	65

Table 5.11 - Estimates of leaf cover for sample trees in Site B in November

Tree	% Leaf Area Present as Green	% Leaf Area Present as Brown
Oak B1	0	99
Oak B2	0	99
Oak B3	0	98
Alder B1	0	100
Alder B2	0	100
Alder B3	0	100
Beech B1	0	100
Beech B2	0	100
Beech B3	0	100

Table 5.12 - Estimates of leaf cover for sample trees in Site B in December

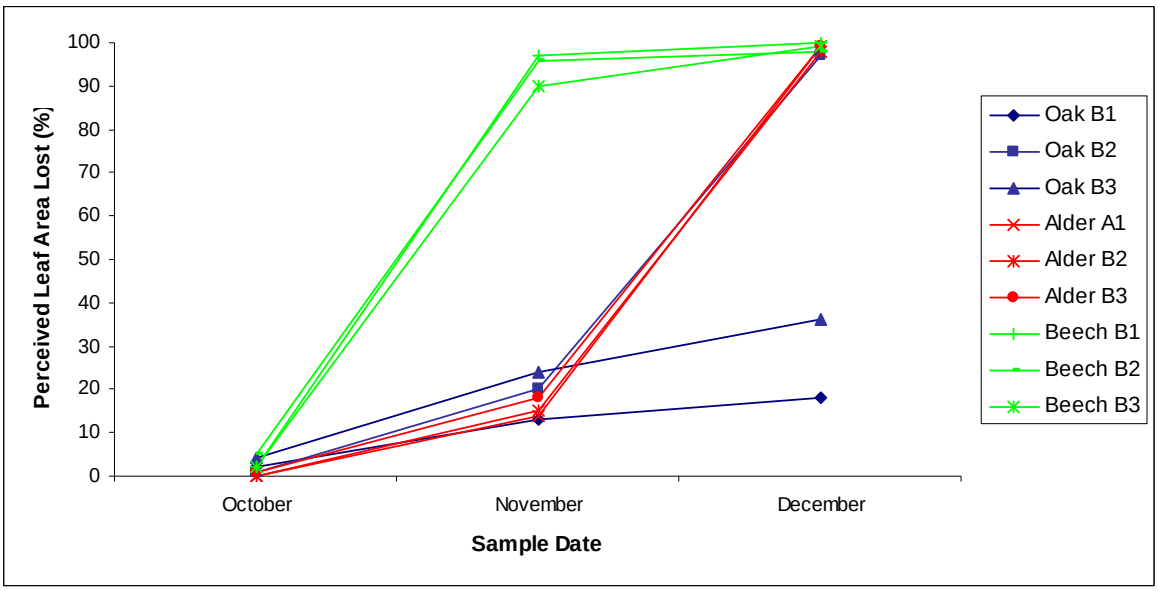


Figure 5.3 – Graphical representation of estimates for percentage leaf lost from sample trees in Site B

5.4.1.3 Site C Trees

Tree	% Leaf Area Present as Green	% Leaf Area Present as Brown
Oak C1	40	5
Oak C2	42	4
Oak C3	45	4
Alder C1	100	0
Alder C2	100	0
Alder C3	100	3
Beech C1	86	3
Beech C2	99	<1
Beech C3	98	<1

Table 5.13 - Estimates of leaf cover for sample trees in Site C in October

Tree	% Leaf Area Present as Green	% Leaf Area Present Brown
Oak C1	0	35
Oak C2	0	52
Oak C3	<1	50
Alder C1	0	0
Alder C2	0	2
Alder C3	4	5
Beech C1	0	90
Beech C2	0	95
Beech C3	0	95

Table 5.14 - Estimates of leaf cover for sample trees in Site C in November

Tree	% Leaf Area Present as Green	% Leaf Area Present as Brown
Oak C1	0	100
Oak C2	0	100
Oak C3	0	99
Alder C1	0	0
Alder C2	0	0
Alder C3	0	0
Beech C1	0	100
Beech C2	0	0
Beech C3	0	100

Table 5.15 - Estimates of leaf cover for sample trees in Site C in December

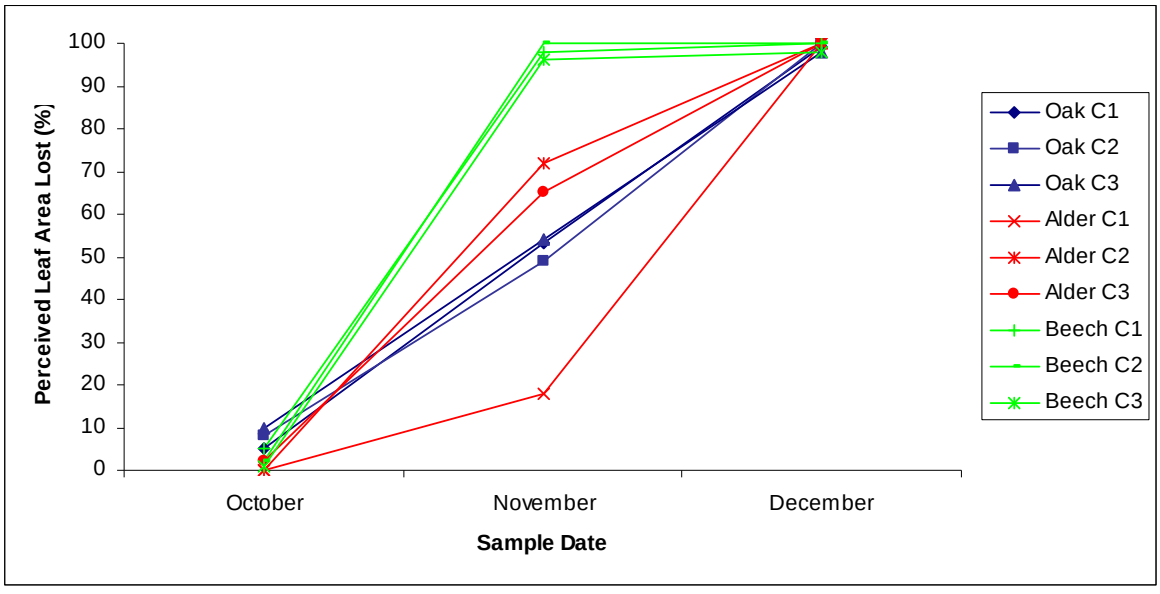


Figure 5.4 – Graphical representation of estimates for percentage leaf lost from sample trees in Site C

5.4.2 On-Tree Estimates - Discussion

It can be seen clearly from Figures 5.2 to 5.4 that the beech trees consistently lost the majority of their leaves between sampling dates in October and November, irrespective of the site in which the trees were located. Almost no leaves were abscised on any of the surveyed beech trees prior to the October sampling date and by December, surveyed beech trees had a maximum of 5% remaining leaf cover. Oak and alder trees generally appeared to exhibit similar patterns to each other in terms of the rate at which leaves were abscised, particularly between October and November sampling dates. However, this pattern also seems to be dependent, to some extent, upon the site.

Oak trees from all sites appear to have lost their leaves at fairly steady rates throughout the entire sampling period. However, the extent to which leaves remained on trees from Sites A and B at the final sampling date in December was generally variable. Oak trees in Site C had abscised almost all leaves at the final sampling date and the rate of leaf abscission was roughly constant. Sample oak trees from Site A, similar to those in Site C, shed their leaves at approximately constant rates. However, unlike those in Site C, sample oak trees in Site A abscised their leaves at different rates to each other. For example, sample oak tree A1 had a residual leaf cover of over 30% on the final sample date whereas oak tree A3 had lost almost all leaves. Sample oak trees B1 and B3 also lost their leaves at constant rates, albeit much lower rates than those oak trees in Sites A and C, with oak tree B1 having lost less than 20% of the original leaf cover by December. Sample oak tree B2 differs from all other oak trees surveyed in that the vast majority of leaves were abscised between November and December sample dates. Despite this, between October and November sample dates oak B2 lost leaves at a similar rate to both oak B1 and B3.

In terms of leaf abscission rates between October and November sample dates, the surveyed alder trees exhibited similar patterns to sample oak trees located within their respective sites. The only exception to this is sample alder tree C1, which had a significantly lower leaf abscission rate than either sample oak trees or indeed other alder trees, located within the same site. Sample alder tree C1 did however, appear to lose leaves at a similar rate to all three sample alder trees located in Site B, with between 10 and 20% of leaves having been abscised by the November sample date. Despite this, all surveyed alder trees retained less than 5% of their original leaf cover by the final sample date in December.

Alder trees from Site A underwent leaf abscission at fairly constant rates, similar to those exhibited by Site A oak trees. Alder trees located in Site B exhibited very similar abscission patterns to that of oak tree B2, with more than 80% of leaves being shed between the November and December sample dates. Alder tree C1 also conformed to this trend. However, alder trees C2 and C3 had abscised approximately 70% of the original leaf cover by the November sampling date.

The variability in leaf abscission rates in oak and alder trees from site to site could have been due to the fact that some trees were more exposed to prevailing weather conditions than others. However, the fact that beech trees exhibited such consistent trends in terms of the leaf abscission rates, irrespective of the site in which the trees were located suggests that there may have been other factors determining the rate of leaf abscission. For example, it has been previously demonstrated that drought and flooding can cause leaf senescence in plant species intolerant to extreme dry and waterlogged conditions (Kozlowski, 2002). It could be the case that, although sample trees from each of the three sites were located within the same area, there was a certain amount of variation in soil moisture contents within each site so that some trees underwent premature abscission compared to other sample trees of the same species located within the same site.

5.4.2.1 Statistical Analyses

GLM's (General Linear Models) were performed on the results obtained from the estimation of leaf cover lost from sample trees in each of the three sites over the sampling period. GLM's are statistical models that consider interactions between all combinations of variables within an experimental design. Initially a GLM was performed on a complete set of data for the percentage of leaf area lost, displayed graphically in Figures 5.2 to 5.4, in order to determine which experimental variables have an impact on the proportion of leaves abscised by sample trees throughout the sampling period. Prior to performing the statistical analyses, the raw percentage data was subjected to a transformation whereby percentage figures were converted to proper fractions. The variables in this statistical analysis were; sample date, tree species and the site from which the sample trees were located. The results confirmed that each of the three sample dates were different from each other in terms of the proportion of leaf cover lost by sample trees of each species in each of the three sample sites.

The data were subsequently separated according to the date on which the estimates were made and GLM's were performed on each of the resulting three data sets in order to

determine whether, at the 95% confidence interval, the tree species or the site in which sample trees were located had an impact on the proportion of leaf cover retained on the trees at each of the three sampling dates (see Appendix).

In October trees growing in Site A retained fewer leaves than their equivalents growing in Sites B and C. Oak trees retained a smaller proportion of their leaves than either alder or beech trees in October. On the sample date in November sample trees located in Site B retained more leaves than those located in either Site A or C. Beech trees had lost a greater proportion of leaves than oak or alder by this sample date. By the December sampling date the amount of leaves retained by trees in each of the three sites are statistically the same. However, oak trees retained a greater proportion of the original leaf cover than either alder or beech trees.

5.4.3 On-Ground Estimates - Results

5.4.3.1 Site A Quadrats

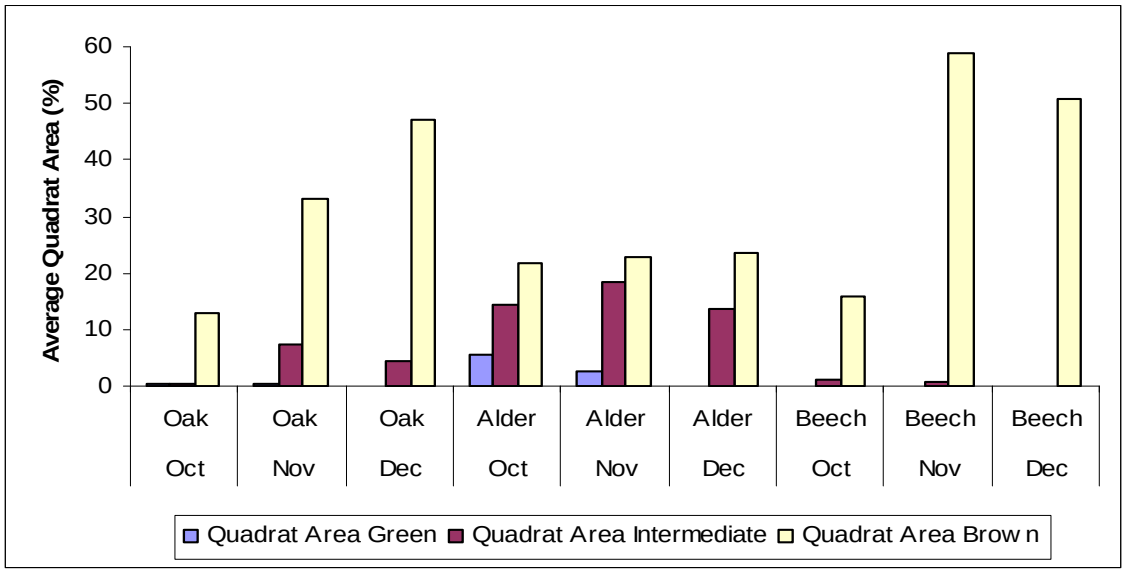


Figure 5.5 – Graphical representation of the mean quadrat areas occupied by leaves at different stages of senescence in Site A

5.4.3.2 Site B Quadrats

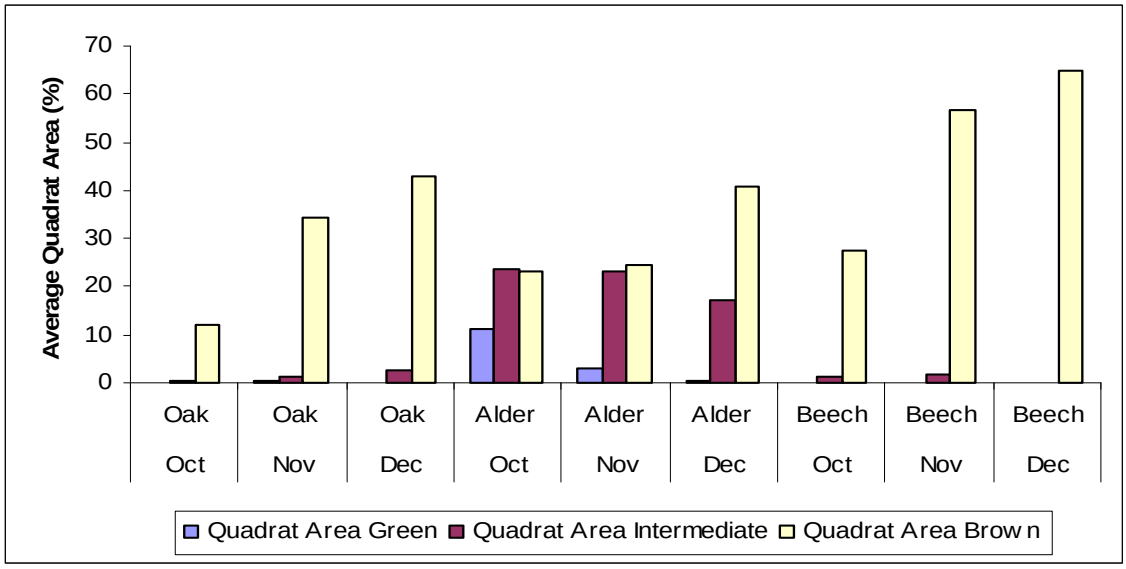


Figure 5.6 – Graphical representation of the mean quadrat areas occupied by leaves at different stages of senescence in Site B

5.4.3.3 Site C Quadrats

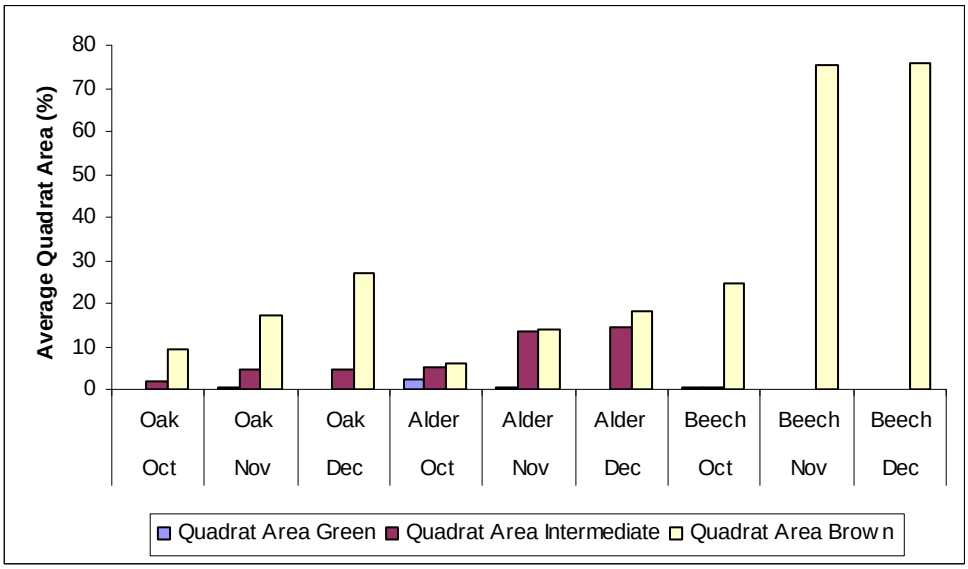


Figure 5.7 – Graphical representation of the mean quadrat areas occupied by leaves at different stages of senescence in Site C

5.4.4 On-Ground Estimates – Discussion

It can be observed from Figures 5.5 to 5.7 that, based on the colours of leaves occupying precisely, randomly positioned quadrats around the base of sample trees, oak, alder and beech trees appeared to abscise their leaves at different stages of senescence. There appear

to be consistent trends shown by each of the three sample tree species in terms of the proportions of quadrats occupied by different coloured leaves throughout the sampling period, irrespective of the Site in which sample trees are located.

Throughout the entire sampling period, quadrats associated with sample beech trees in all sample Sites contain less than 7% green and intermediate leaves. Brown beech leaves are present in much greater proportions (between 15 and 75% of quadrat areas) irrespective of the site or sampling date. Between the October and November sampling dates, the areas occupied by brown leaves increase considerably. This observation is concurrent with the observation made in Section 5.4.2, that sample beech trees lose the majority of their leaves between sample dates in October and November.

In all sample Sites, irrespective of the sampling date, quadrats associated with sample alder trees contain both green and intermediate leaves. The proportion of green leaves observed in quadrats decreases over the sampling period, which would be expected as the abscised leaves decompose and become discoloured on the ground. The proportion of quadrat areas occupied by intermediate alder leaves remains relatively constant throughout the sampling period. Brown leaves increase slightly in frequency throughout the sampling period, the quadrat area occupied being between 1 and 15% greater than that of intermediate leaves. The general trends relating to quadrat areas occupied by alder leaves at different stages of senescence are reflected by quadrats associated with sample alder trees located in all three sample Sites. However, those quadrats associated with sample alder trees located in Site C contain significantly less alder leaves at all stages of senescence than the corresponding quadrats in Sites A and B.

Quadrats associated with sample oak trees in all Sites generally contain no green leaves and a small proportion (between 0.2 and 7%) of intermediate leaves throughout the sampling period. The quadrat areas occupied by brown leaves increase steadily in all sites throughout the sampling period. This observation is in agreement with the assertion in Section 5.4.2 that oak trees abscise their leaves at a relatively steady rate throughout the sampling period. Similar to the observations regarding alder leaf colour in Site C quadrats, quadrats associated with oak trees in Site C contain a smaller proportion of brown leaves than the corresponding quadrats in Sites B and C.

An analysis of variance confirmed that alder leaves are more likely to stay green following abscission than oak, or especially beech leaves.

5.5 Determination of the Stage of Senescence at which Methanol is Lost from Leaves

The pectic methyl ester contents of leaves sampled from the three sites located within Mugdock Country Park were determined (Section 2.2.3). The methyl ester contents of both leaves sampled from the ground surrounding sample trees and those that were not yet abscised were determined. Samples were analysed in triplicate and the mean values for each calculated and analysed statistically.

5.5.1 Data Isolated According to Site

5.5.1.1 Results

Figures 5.8 to 5.10 display the results obtained following the collection of leaves from sample trees and from the ground surrounding sample trees in the three sites located within Mugdock Country Park in October, November and December of 2005 and the subsequent determination of the residual pectic methyl esters contained within these leaves. Figures 5.8 to 5.10 contain the results separated into the three individual sample sites and consequently contain data from the three sample tree species.

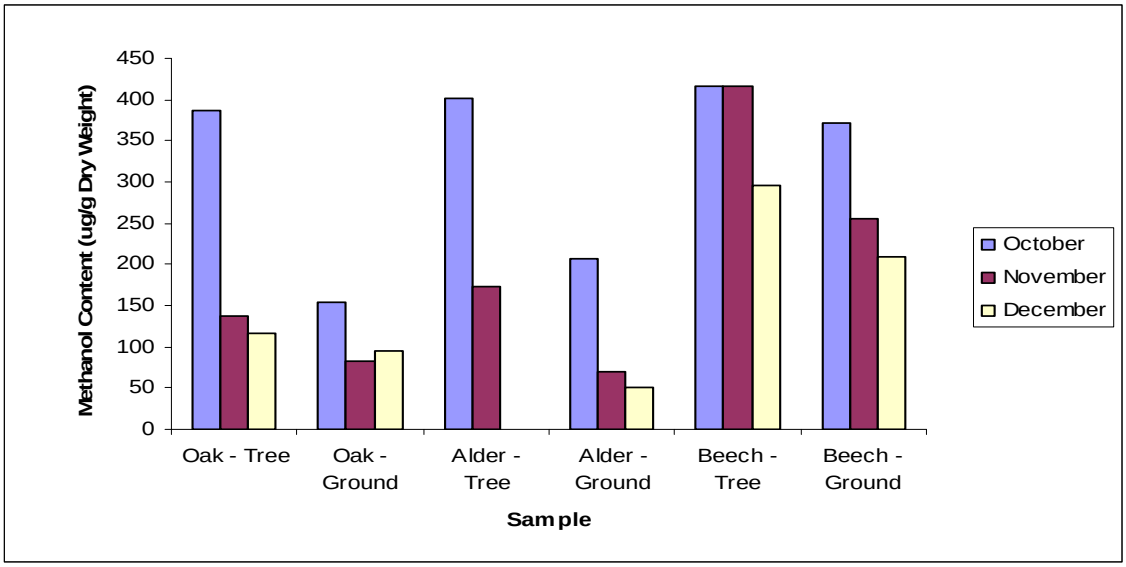


Figure 5.8 – Graphical representation of the methanol contents of leaves collected from sample trees and off the ground surrounding sample trees in Mugdock Country Park – Site A

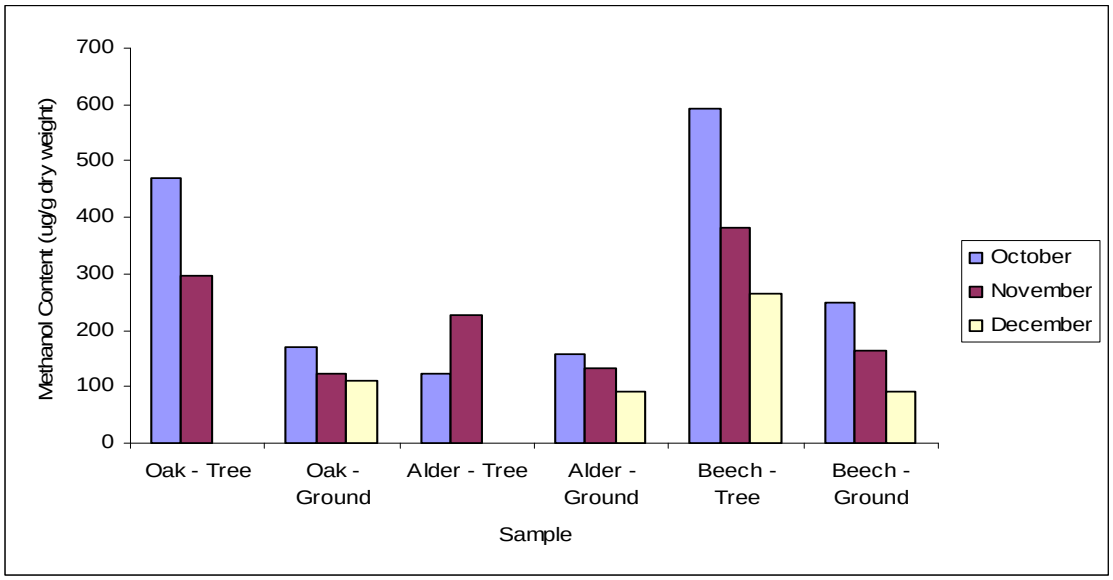


Figure 5.9 – Graphical representation of the methanol contents of leaves collected from sample trees and off the ground surrounding sample trees in Mugdock Country Park – Site B

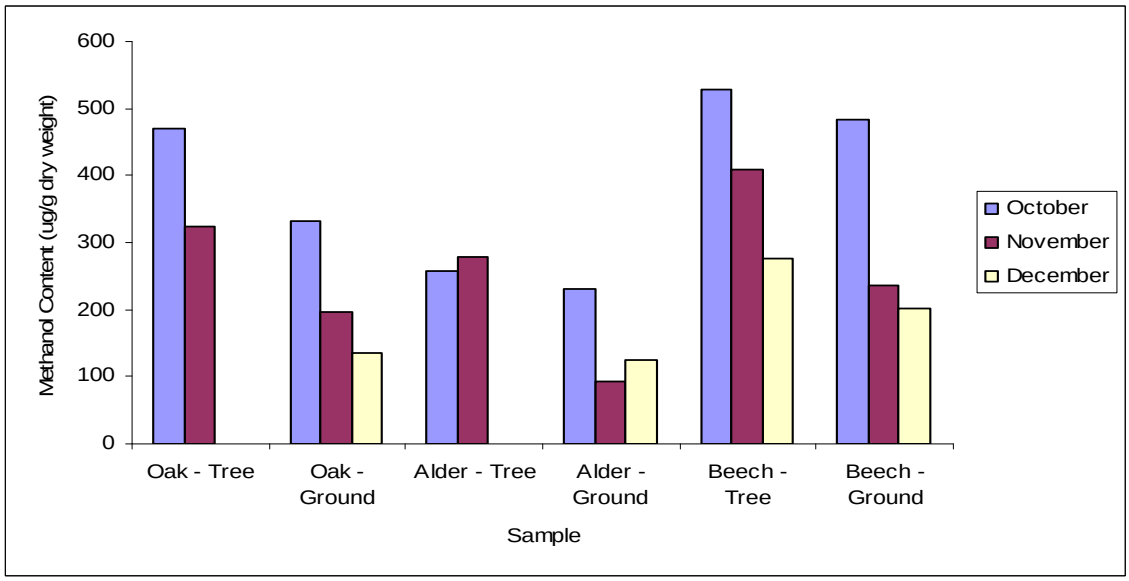


Figure 5.10 – Graphical representation of the methanol contents of leaves collected from sample trees and off the ground surrounding sample trees in Mugdock Country Park – Site C

5.5.1.2 Statistical Analyses

Due to the complex nature of the leaf-sampling strategy, a large number of variables exist within the collated data. In order to determine which variables are significant in terms of the methanol content of leaves, GLM's (General Linear Models) were carried out on data sets separated into those leaves collected from the tree and those collected from the ground. Due to the fact that, in the majority of cases, there were few, if any, leaves remaining on trees in December, any data collected as a result of on-tree sampling in December was discarded and only two sampling dates were considered. All three

sampling dates were examined in relation to leaves sampled from the ground. These GLM's were designed in order to determine, at the 95% confidence interval, which variables have a significant effect on the pectic methyl ester content of leaves, considering those sampled from the tree and those sampled from the ground individually (see Appendix).

Results obtained from GLM's carried out for pectic methyl ester content versus species, date and site for both leaves collected from the tree and those collected from the ground, show that all interactions between variables in the experiment are significant.

None of the variables within the experiment could therefore be discounted and all had to be considered as having an influence on the pectic methyl ester content of the sampled leaves.

Two further GLM's were carried out. In the first, the data was isolated in terms of tree species and in the second in terms of the site from which the leaves were sampled. These statistical analyses were designed in order to determine the extent of the influence either species of tree or the site from which the leaves were sampled, had on the amount of pectic methyl esters present in the leaves sampled at different sampling dates (see Appendix).

Results obtained from a GLM carried out for methanol content versus tree species and date of leaf collection show that leaves sampled from the ground in October contained more methanol than those collected in November and December. Leaves sampled from the ground in Sites B and C lost methanol at a relatively constant rate, whereas those corresponding leaves from Site A lost the majority of the methanol they contained between the sampling date in October and that in November.

Oak and alder leaves collected both from the tree and from the ground in Site A contained statistically similar amounts of pectic methyl esters to each other at all sampling dates. Beech leaves sampled both from the ground and from the tree in Site A, contained more methanol than the corresponding leaves sampled from either oak or alder.

All leaves sampled from the ground in Site B contained statistically similar amounts of methanol to each other, whereas those sampled from the ground in Site C contained different amounts of methanol depending on the species of tree, with beech leaves having the highest methanol content. Leaves sampled from the tree in both Sites B and C contained different amounts of pectic methyl esters, depending on the species of tree, with oak and beech both containing a greater amount of methanol than alder. Beech leaves

sampled from the tree in Sites B and C contained the highest amounts of pectic methyl esters.

5.5.1.3 Discussion

It is apparent from the data shown graphically in Figures 5.8 to 5.10, that the pectic methyl ester content of leaves collected from all tree species over the three different sites decreased over the three-month period covered by the investigation. Pectic methyl ester contents fell at various rates over this period, but all samples analysed contained a certain amount of methanol on the final sampling date. The rate of methanol loss and the residual methanol remaining after the final sampling date appear to be dependent on interactions between tree species, the particular site from which the leaves were collected and whether the leaves were collected from the ground or from the tree.

The pectic methyl ester content decreased from leaves both on the tree and on the ground throughout the autumn, presumeably releasing methanol to the atmosphere. The amounts of pectic methyl esters initially present in leaf samples collected from the trees were of a similar magnitude, irrespective of tree species and location of the tree. It therefore follows that differences in methanol contents observed in leaves collected at later dates, in November and December, were due to differences in the rate of methanol loss from the leaves. For example, beech leaves collected in December had a higher pectic methyl ester content than that of either oak or alder, irrespective of whether the leaves were collected from the ground or from the tree, suggesting that beech leaves lost the methanol they contain at the end of the summer at a much lower rate than either oak or alder leaves.

Between October and November, the pectic methyl ester contents of both oak and alder leaves appeared to decrease to approximately half their original amounts whilst the leaves remain on the tree. Both oak and alder leaves also lost methanol rapidly whilst on the ground, with approximately half of the methanol present in leaves on the ground in October being lost by December. The quantity of pectic methyl esters decreased most rapidly in Alder leaves, irrespective of the site from which the leaves were collected and consequently alder leaves contained the least methanol out of the three tree species studied by the final sample date in December.

The quantity of pectic methyl esters decreased relatively slowly in Beech leaves. This especially applies to those that were still present on the tree. Generally, over half the methanol content of leaves collected from the trees in October was still present in beech

leaves collected from the trees in December. The pectic methyl ester content in Beech leaves sampled from the ground also tended to decrease more slowly over the sampling period than either alder or oak leaves collected from the ground. An exception to this trend is shown in the results for beech leaves sampled from the ground in Site B. The total pectic methyl ester content at the beginning of the sampling period in October was significantly lower than that in leaves from both sites A and C. The rate at which the leaves lost the methanol that they contained in October was also significantly greater than the rate of methanol loss from corresponding leaves from Sites A and C. This phenomenon could be due to the fact that the beech leaves present on the ground in Site B were more exposed to prevailing weather conditions than those on the ground in Sites A and C. The beech trees in Site B have less dense foliage than those in Sites A and C and are also situated on the edge of a break between groups of trees, along which a public footpath is located. There is therefore less protection from wind and rain, thus possibly resulting in the premature loss of methanol from the beech leaves sampled from the ground in Site B.

The pectic methyl ester content in Oak leaves consistently decreased whilst they remained on the tree, with approximately half of the pectic methyl esters present in the leaves in October having been lost by November. Alder leaves sampled from the trees reflected this trend only in those leaves sampled from Site A. Methanol contents of leaves sampled from trees located in Sites B and C contained apparently higher methanol levels in November than they do in October. This could be due to the fact that, as shown in Tables 5.10 and 5.13, approximately 20% and 50% of the original leaf cover of alder trees located in Sites B and C respectively had already fallen by the November sampling date. The leaves that were subsequently available for sampling were limited to those that were within reach of the sampling team. These leaves, by November, were likely not to be a true representation of the leaves that were actually present on the alder trees located in Sites B and C but rather were likely to represent the slowest leaves to senesce on the trees. These leaves would therefore naturally contain more pectic methyl esters than, for example, a sample collected in October containing leaves at various stages of senescence.

Chapter Six -
Crop Plant Species as a Source of Atmospheric Methanol

6.1 Quantification of Methanol Emitted from Plants Using GC-FID

6.1.1 Introduction

Methanol emissions from individual leaves at different stages of development of a variety of plant species were quantified by MacDonald and Fall (1993). They concluded that methanol was emitted from all the plants species that they studied at highly variable rates. One of the most striking differences observed between the rates of methanol emission of different sample leaves was detected between young and mature leaves of the same sample species of plant. A mean rate of 13.2 ± 4.1 μg h^{-1} gdw^{-1} was observed for methanol emissions from fully expanded leaves, but the average figure for methanol emission rate from younger leaves was approximately double that rate on a dry weight basis.

Nemecek-Marshall et al. (1995) used a colorimetric enzymic method and gas chromatography (GC) to quantify methanol emissions from a variety of plant materials. Results concluded that methanol emission rate was related to the stage of leaf development, generally declining as the leaf aged following leaf expansion.

Kirstine et al. (1998) showed that VOC emissions from grass and clover varied depending, not only on climatic conditions, but also on the rate at which the plants were growing. Emission rates, of which methanol was a major constituent, contributing between 10 and 35% of the total emitted carbon, were highest during the spring and summer months when pasture growth rate is also at a maximum.

The method for the quantification of methanol emitted from growing plants developed in Chapter 3 was implemented in order to quantify the methanol released from plants commonly grown in the UK at different stages of their development. This data could then be extrapolated in order to estimate the impact on the UK troposphere of particular arable species of plant, in terms of the methanol they emit during the early stages of growth.

The data could also be compared to available data from the literature regarding the methyl ester contents of each particular plant species to determine the proportion of the methyl ester groups present in the plant tissue that are emitted as methanol as the plants develop.

6.1.2 Results

Figure 6.1 displays the total methanol emitted from two growing barley seedlings, incubated within the modified bell jar system for 24 hours, as shown in Figure 3.20 and described fully in Section 3.4.5, using Tenax-TA as the adsorbent material. Figures 6.3 and 6.5 show the results from the modified bell jar experiment quantifying the methanol emission rate from twenty growing ryegrass and oilseed rape seedlings respectively and using Tenax as the adsorbent material.

Figures 6.2, 6.4 and 6.6 display graphically the results from sets of experiments incubating barley, ryegrass and oilseed rape seedlings respectively, using Carbosieve as the adsorbent material.

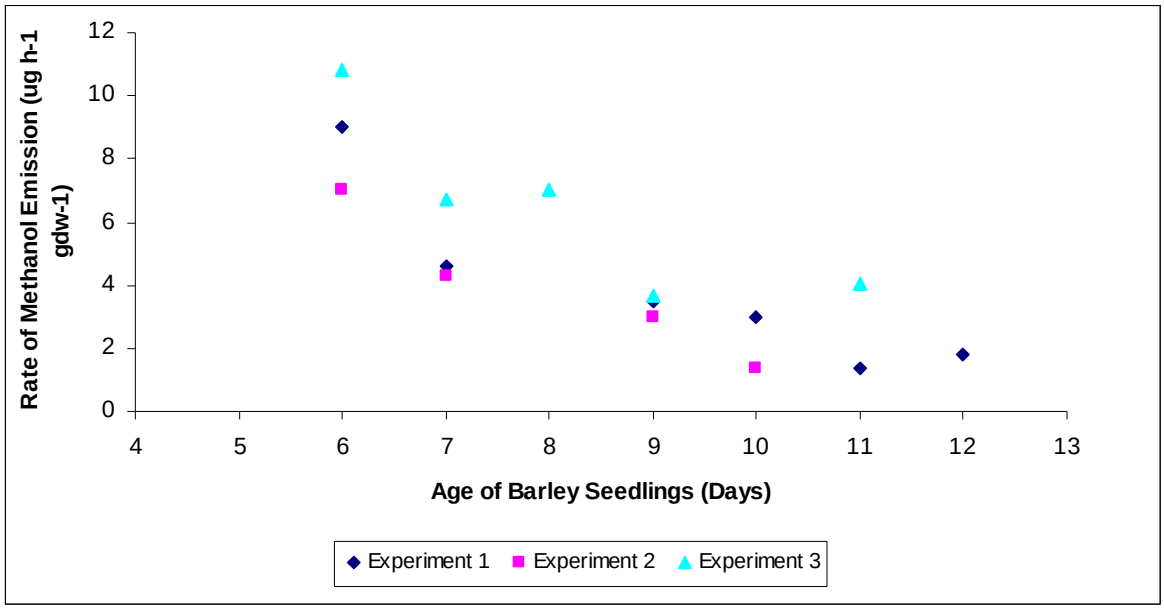


Figure 6.1 – Methanol emission rates from barley seedlings using Tenax as the adsorbent material

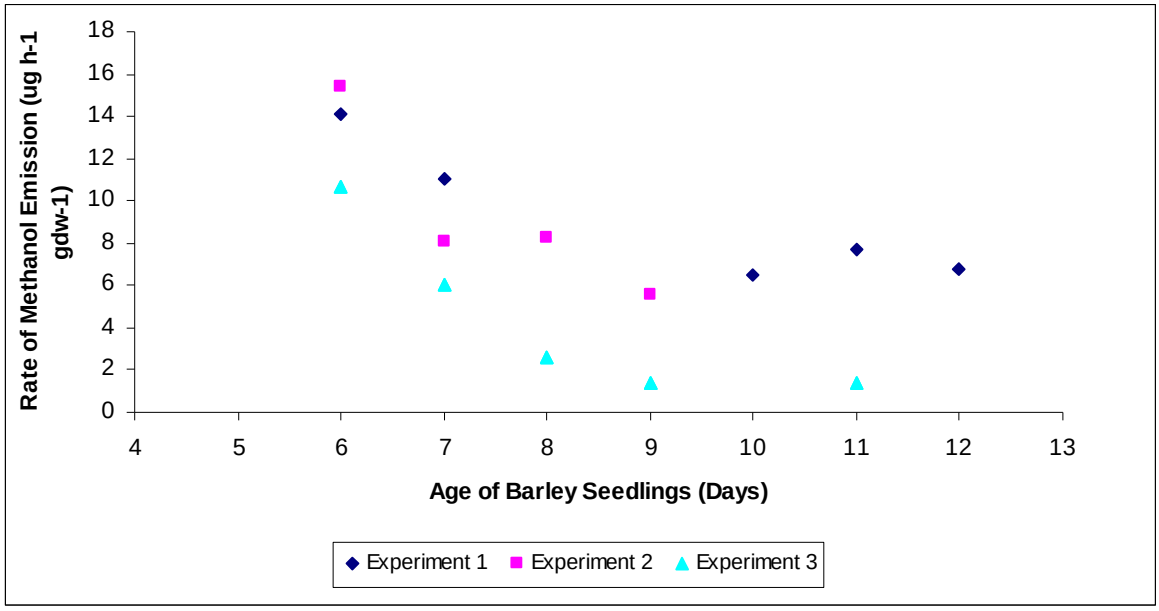


Figure 6.2 – Methanol emission rates from barley seedlings using Carbosieve as the adsorbent material

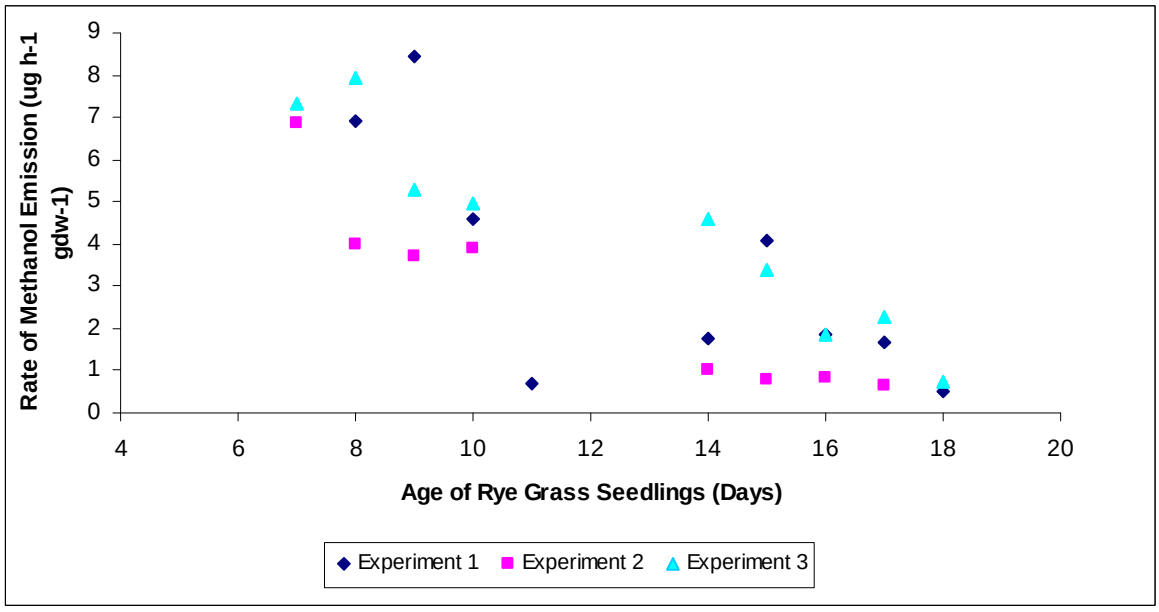


Figure 6.3 – Methanol emission rates from ryegrass seedlings using Tenax as the adsorbent material

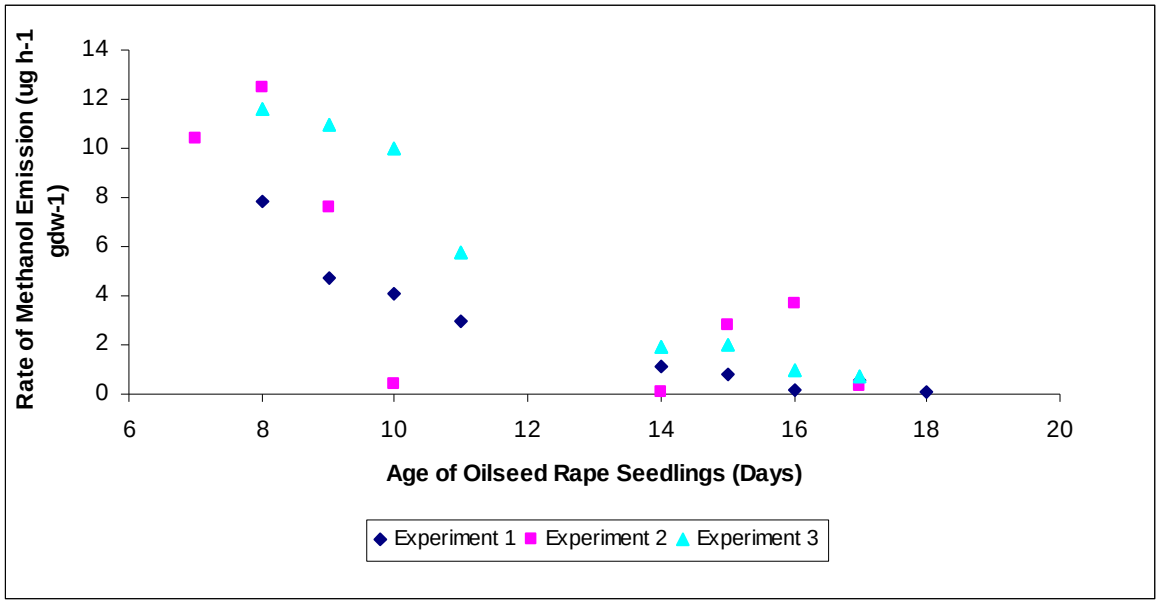


Figure 6.4 - Methanol emission rates from ryegrass seedlings using Carbosieve as the adsorbent material

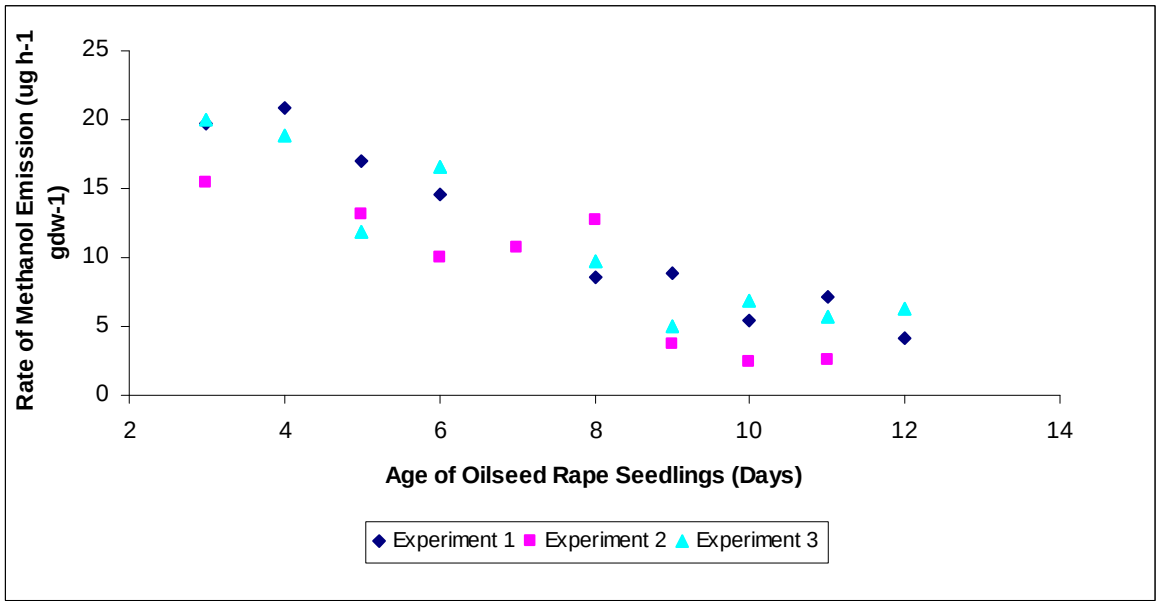


Figure 6.5 - Methanol emission rates from oilseed rape seedlings using Tenax as the adsorbent material

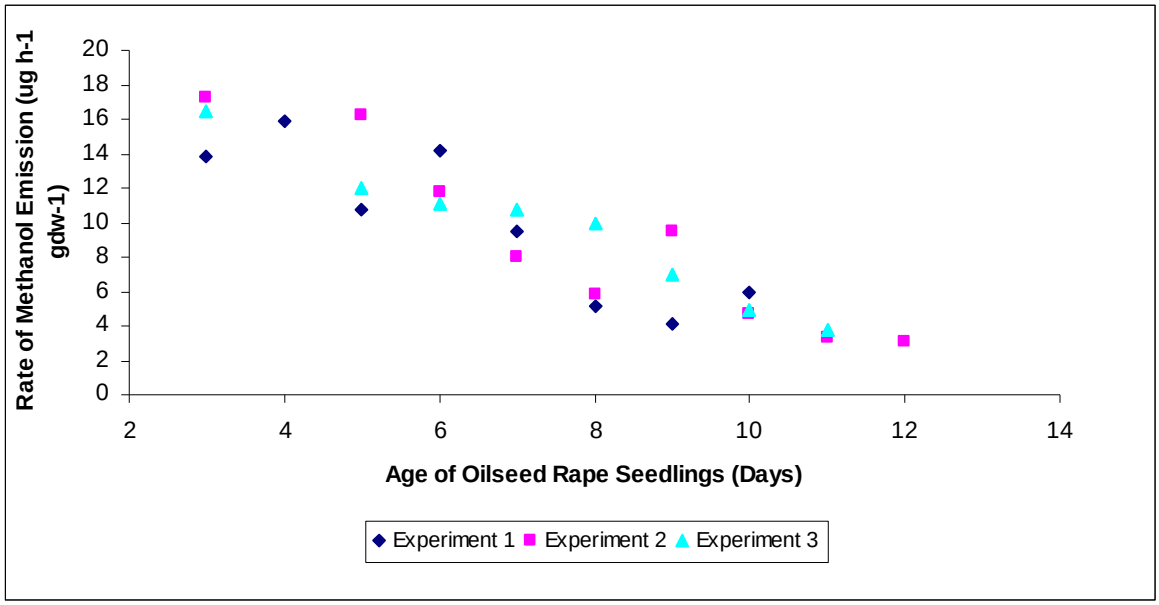


Figure 6.6 - Methanol emission rates from oilseed rape seedlings using Carbosieve as the adsorbent material

6.1.3 Discussion of Results

The results displayed graphically in Figures 6.1 to 6.6 show that the rate of methanol emission per gram of dry weight of plant material of all species studied, decreased as the age of the plants increased.

The data from each set of experiments was fitted to a quadratic function as this was considered to be the simplest function designed specifically to fit a curved linear relationship. By calculating the derivative of each of the six quadratic curves (Figures 6.1 to 6.6), the gradient of the graphs can be determined for the steepest point on the curve. The steepest point on each of the graphs equates to the earliest stage of growth at which methanol emission rate was measured for each plant species. Derivatives of quadrats with the equation $Ax^2 + Bx + C$ can be calculated by converting Ax^n to nAx^{n-1}. For example, the quadratic equation for the graph showing the results of the methanol emissions from barley seedlings using Tenax as the adsorbent material is as follows: y = 0.2227x2 - 5.0433x + 30.743. The derivative of this equation for the point at which x = 6 equates to 0.2227 x 2 x 6 – 5.0433.

Table 6.1 shows the results from calculating the derivatives for each of the six graphs displayed in Figures 6.1 to 6.6 at the steepest point in each graph.

Plant Species	Adsorbent Resin	Equation of Graph	Plant Age with Steepest Gradient (Days)	Derivative/ Gradient
Barley	Tenax	$y = 0.2227x^2 - 5.0433x + 30.743$	6	-2.3709
Barley	Carbosieve	$y = 0.6849x^2 - 13.302x + 68.255$	6	-5.0832
Rye Grass	Tenax	$y = 0.0374x^2 - 1.4663x + 15.518$	7	-0.9427
Rye Grass	Carbosieve	$y = 0.1207x^2 - 3.9802x + 33.635$	7	-2.2904
Oilseed Rape	Tenax	$y = 0.0961x^2 - 3.1905x + 28.412$	3	-2.6139
Oilseed Rape	Carbosieve	$y = 0.0254x^2 - 1.9404x + 21.998$	3	-1.788

Table 6.1 – Gradients of the steepest point on graphs shown in Figures 6.1 to 6.6

The gradients of those experiments in which Carbosieve was used as an adsorbent resin, are greater than those showing the results of experiments utilising Tenax as the adsorbent resin in the cases of barley and ryegrass. The steepest point on the graphs, corresponding to the ages of seedling at which the most rapid decrease in the rate of methanol emission occurred, varied between species. The type of adsorbent resin used in the analyses had little bearing on the point at which methanol emission was greatest.

The decrease in methanol emission rate as the plants matured was least pronounced, but the most constant, in oilseed rape seedlings. The graphs for both barley and ryegrass seedlings show that the rate of methanol emission became relatively constant as the plants matured. In the case of barley seedlings, this phenomenon occurred after about 10 days of growth and in the case of ryegrass, following approximately 15 days of growth.

Figures 6.5 and 6.6 are very similar, not only in terms of gradient, but also in terms of the quantities of methanol measured using the two different adsorbent materials as the oilseed rape seedlings matured.

6.1.3.1 Statistical Analyses

A GLM was carried out on the complete set of raw data, including methanol emission rates from all of the three plant species studied, in order to determine, at the 95% confidence interval, whether or not the adsorbent material used in the analysis caused significant differences in the measured methanol emission rates. The results of this analysis showed that there existed no significant differences between the results obtained from the modified bell jar experiments depending on the adsorbent resin used in the analyses (see Appendix).

A set of paired T-Tests was carried out on complete sets of raw data for measured methanol emission rates, examining each of the three plant species individually, to determine whether or not the results obtained from the use of the two adsorbent resins were

significantly different from one another. Results concluded that, at the 95% confidence interval, whilst the data for ryegrass did not differ significantly ($p = 0.889$), the methanol emission rates determined for barley and oilseed rape seedlings varied significantly depending on the adsorbent resin used in the analysis. However, at the 99% confidence level, differences observed between results obtained for methanol emission rates from both barley and oilseed rape did not differ significantly between adsorbent resins (p values: 0.984 and 0.952 respectively).

Despite the fact that at the 95% confidence interval adsorbent resins were resulting in statistically different methanol emission rates, based on the results of the GLM examining the complete data set, it was deemed acceptable to combine methanol emission results from each of the adsorbent resins, as this would decrease the likelihood of systematic errors being incorporated into the final set of results.

6.1.3.2 Averaged Results

Figure 6.7 displays graphically the combined results from experiments using both Tenax and Carbosieve as the adsorbent resin in the modified bell jar experiments.

Figure 6.7 illustrates that, although the methanol emission rate from oilseed rape seedlings is much greater than any measured rate of methanol emission from either of the other two species at very early stages of the plants' development, due to constraints associated with the development of the other two species of plant, there are no data to compare these emission rates to. However, the emission rates of seedlings of all species between the ages of 7 and 11 days are very similar. In effect, the rate of methanol emission from very young oilseed rape plants is a continuation of the emission graphs of both barley and ryegrass.

The rate of methanol emission from ryegrass plants appears to reach a plateau, following about 15 days of growth, at a rate of less than 2 $\mu g\ h^{-1}\ gdw^{-1}$. In the case of barley seedlings, the methanol emission rate seems to become approximately constant at between 3 and 4 $\mu g\ h^{-1}\ gdw^{-1}$ after around 10 days of growth.

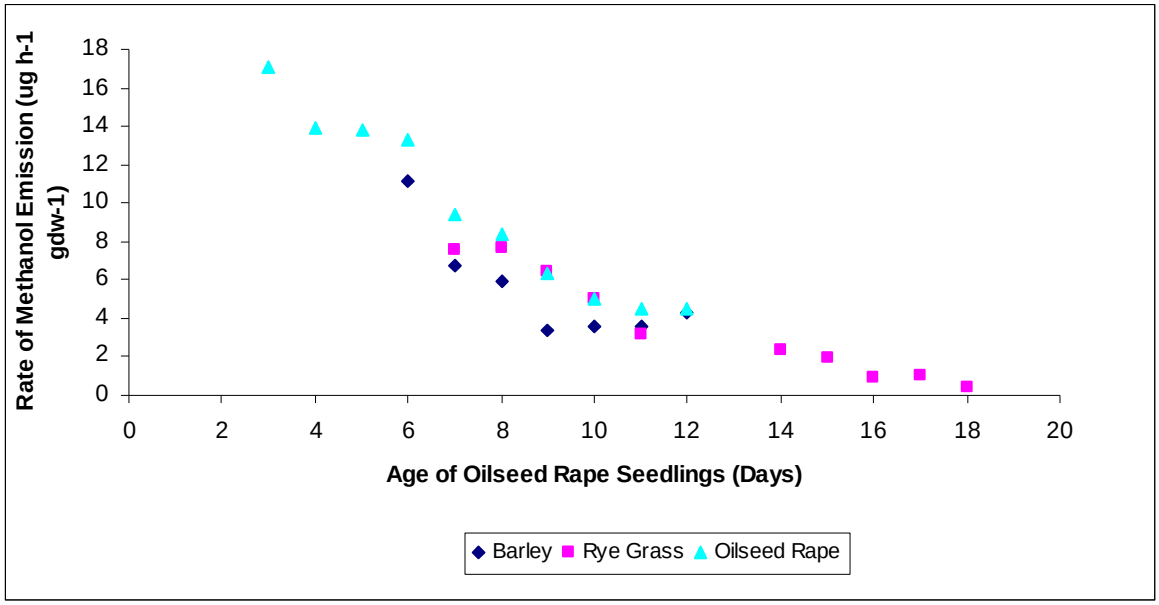


Figure 6.7 – Results of bell jar experiments averaged between experiments and adsorbent resins

With some extrapolation, it could be speculated that, similar to the other two plant species studied, the rate of methanol emission from oilseed rape seedlings becomes relatively constant once the plants have been growing for about 11 days. However, the rate at which the methanol emissions become constant is greater than either barley or ryegrass, at approximately 5 $\mu g\ h^{-1}\ gdw^{-1}$. Due to the limitations imposed by the dimensions of the modified bell jar and the size of the studied plant species, only ryegrass seedlings could be studied to an age in excess of 12 days. As a result, it is difficult to speculate how the rate of methanol emission varies between the three species after this stage.

6.1.3.3 Emission Rates Per Plant

Figures 6.8 to 6.10 display the results shown in Figures 6.1 to 6.7 in terms of methanol emission rate per plant rather than that per gram of dry plant material. The data was plotted in these terms because one of the ultimate aims of this set of experiments is to approximate the amount of methanol that is emitted to the troposphere from the growth of these three species of plant in commercial agricultural systems. Figures are readily available for the density of each of these plants in an agricultural context (Section 7.3). By calculating an approximate value for the rate of methanol emission from a growing plant and combining them with plant density figures, together with figures for the total area of Great Britain utilised in the growth of these plants, the approximate amount of methanol emitted to the troposphere as a result of the commercial production of barley, ryegrass and oilseed rape can be estimated (Section 7.3).

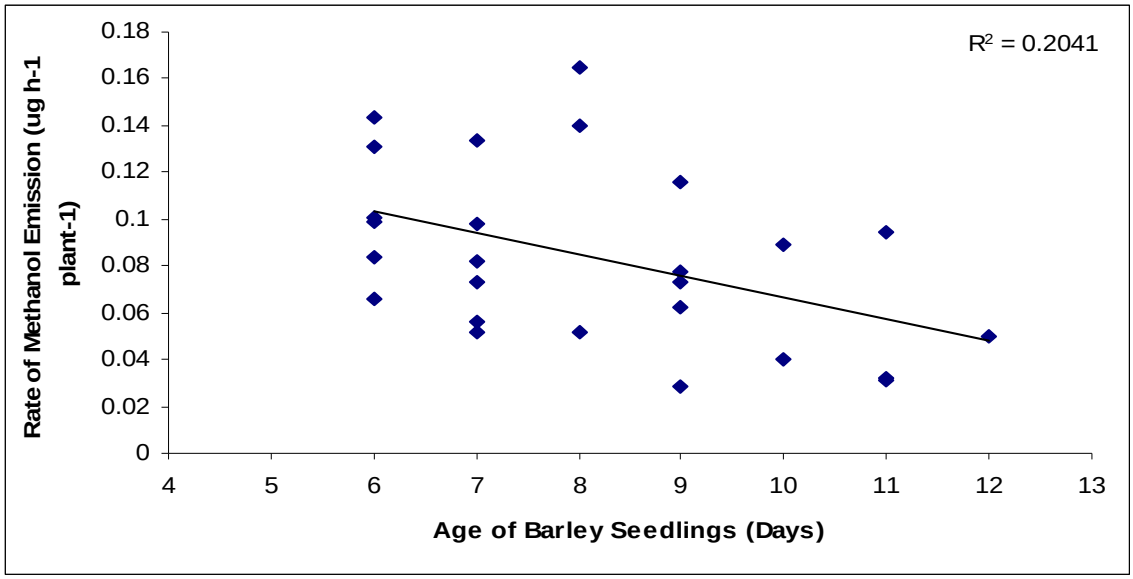


Figure 6.8 – Combined results for methanol emission rate per barley seedling from modified bell jar experiments

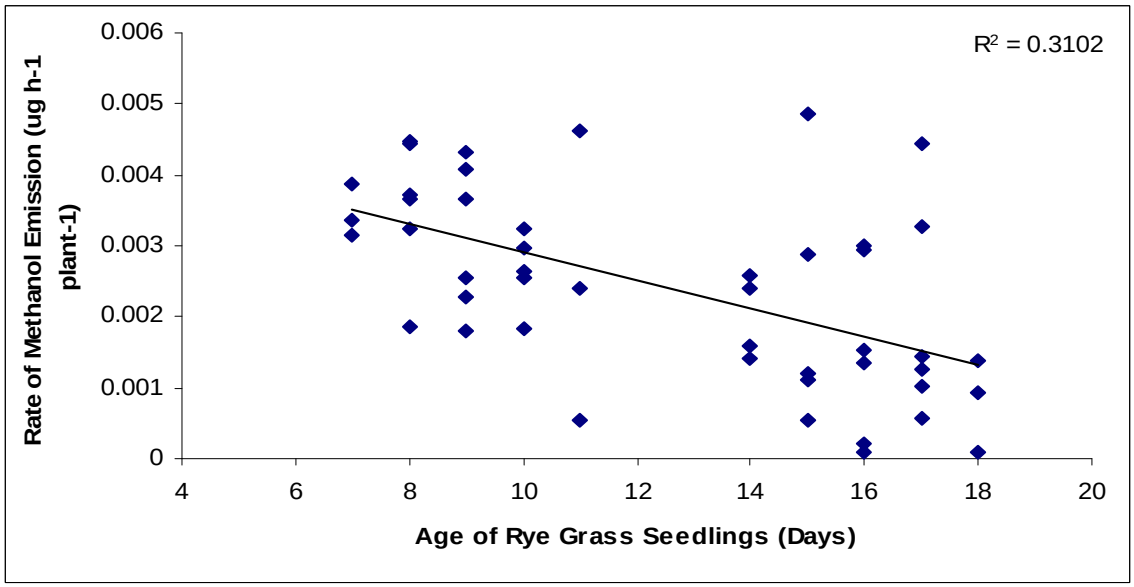


Figure 6.9 - Combined results for methanol emission rate per ryegrass seedling from modified bell jar experiments

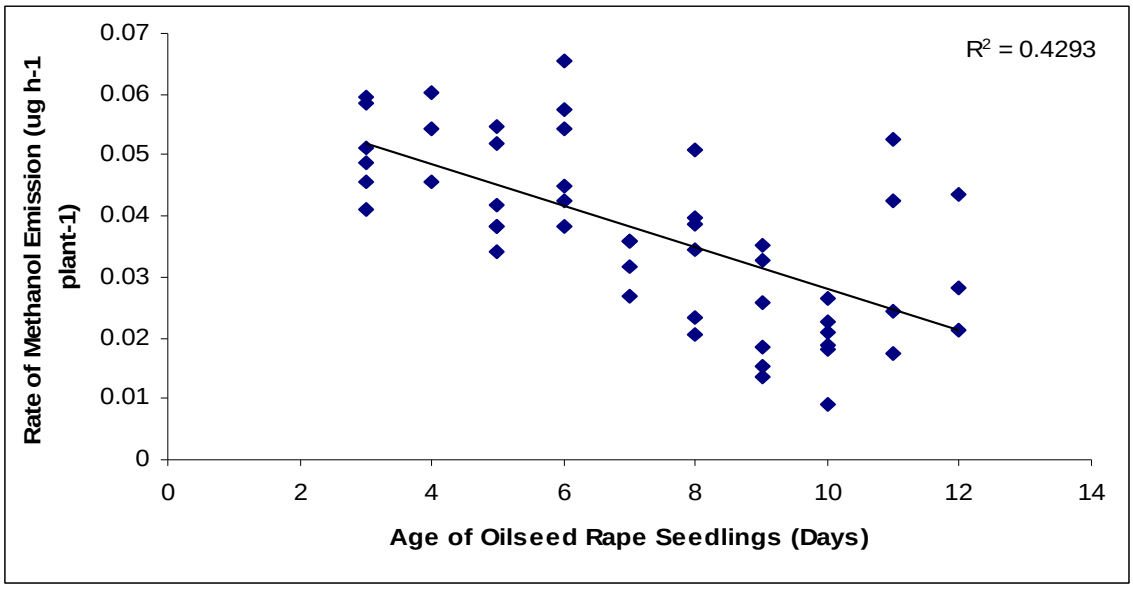


Figure 6.10 - Combined results for methanol emission rate per oilseed rape seedling from modified bell jar experiments

Figures 6.8 to 6.10 show that, when the data is plotted in terms of methanol emission rate per plant, rather than on a dry weight basis, there is a much larger degree of scatter in the data. Regression lines may be drawn on the graphs and analyses of variances were carried out to determine whether or not the association between the variables is statistically significant.

At the 95% confidence level, it was determined that there is a significant association between the age of seedlings of each species and the rate of methanol emission on a per plant basis ($p = 0.02$, $4.8*10^{-4}$, $1.32*10^{-7}$ for barley, ryegrass and oilseed rape repectively). It is therefore statistically sound to state that methanol emission rates from crop species in the early stages of development decrease as the age of seedlings increase.

In order to ultimately calculate the amount of methanol emitted to the troposphere annually as a result of the commercial production of these species of plant in the UK, a figure must be calculated for the average amount of methanol emitted per plant over a 24-hour period. This figure can then be used in calculations involving the average density of each of the three species of plant grown in commercial systems, together with figures for the total land area utilised in the production of each species (Section 7.2).

The greater degree of scattering seen in the graphs displaying the rate of methanol emission per plant (Figures 6.8 to 6.10) as opposed to emission rate per gram of dry plant material (Figures 6.1 to 6.7), suggests that, although there is a relationship between methanol emission per plant and the stage of development of the plant, there is a much closer relationship between methanol emission rate at different stages of development and the weight of the plants concerned. It can therefore be concluded that methanol emissions are greatest in young plants and that this emission rate reduces over time as the plants mature. However, the lower level of correlation between methanol emission rate per plant and the stage of development of the plant, suggests that there is some other factor controlling the emission of methanol.

The size, or surface area of the plants may have an influence on the rate of methanol emission, which would account for the increased rate in barley seedlings compared to oilseed rape or particularly ryegrass. A single ryegrass seedling will have a fraction of the surface area of that of a barley seedling of a similar age. The fact that methanol appears to be emitted from leaves via stomatal openings (Nemecek-Marshall et al., 1995) implies that, if there are fewer stomata, for example on the surface of a leaf with a relatively small surface area, the rate of methanol emission per plant will be lower that that of a larger leaf. This hypothesis does not however, account for the increased scattering displayed in Figures 6.8 to 6.10. There is evidently, an additional factor influencing the rate of methanol emissions from these immature plants, that increases the variability of emission rate when studied on a per plant basis.

Calculations utilising the finding of this set of experiments in order to estimate the methanol emissions originating from crop species produced in the UK, together with conclusions reached, can be found in Section 7.3.

6.2 Methyl Ester Contents of Barley, Ryegrass and Oilseed Rape Seedlings

6.2.1 Introduction

The methyl ester contents of barley seedlings of ages between 6 and 13 days of growth (Section 2.4) were compared to the results from the determination of methanol emitted from barley seedlings using GC-FID (Section 3.4) in order to determine how these values varied during the early stages of growth of the three plant species studied. From these

results, conclusions can be drawn on the turnover of methyl esters within the plant and the subsequent emission of methanol to the troposphere.

6.2.2 Barley Seedlings

6.2.2.1 Introduction

Approximately one third of the primary cell wall of dicots is comprised of pectic polysaccharides, whereas a much smaller fraction of the cell walls of certain monocots, such as cereal grasses, consists of these polymers. As a consequence, studies examining the pectic composition of this type of plant are far fewer than those involving dicots.

The methyl ester content of cell walls extracted from rapidly elongating maize coleoptiles was investigated in a study by Kim and Carpita (1991). It was concluded that approximately 65% of the galacturonic acid residues were esterified at the embryonic stage. This figure increased to about 80% during the rapid elongation phase and subsequently increased again to approximately 60% at the end of elongation. Despite these findings, the methyl esters associated with galactosyluronic acid residues (GalA) were found to decrease steadily over the development period, from about 50% of the total GalA to roughly 38% following 192 hours of growth (Kim and Carpita, 1991).

Gibeaut et al. (2005) studied the pectin content of barley coleoptiles and concluded that during the initial stages of cell division, pectic polysaccharides accounted for approximately 30 mol% of the total cell walls. This figure is significantly higher than the expected figure for the pectin content of typical grasses, which is considerably lower than that found in other flowering plants (Carpita and Gibeaut, 1993). The pectic polysaccharide content of the barley coleoptile cell walls decreased significantly to 10 mol% once cell elongation had ceased following 5 days of growth.

6.2.2.2 Discussion of Results

Results of methyl ester determination experiments carried out on barley seedlings 6, 9, 11 and 13 days old (Section 2.4.3) are shown in Figure 6.11.

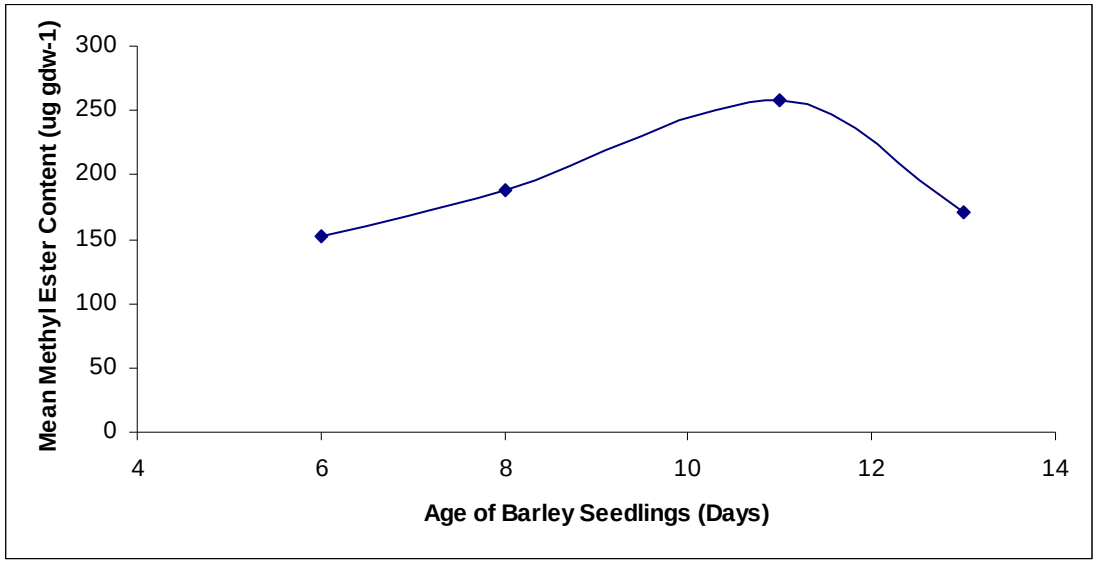


Figure 6.11 – Methyl ester contents of barley seedlings at various stages of development

The methyl ester content of barley seedlings tends to increase steadily in seedlings between 6 and 11 days old, after which time the amount of methyl esterification tends to decrease to the content found in 6 day-old seedlings as the age of seedlings increases from 11 to 13 days old. Kim and Carpita (1991) studied coleoptiles between 0 and 192 hours old. The methyl ester content of coleoptiles between the ages of 144 and 192 hours remained virtually constant, and may even have increased slightly.

From these results, it could be assumed, within the limits of this experiment, that the methyl ester content of barley plants remains relatively constant throughout the growing season. The emission of methanol from these plants does not cause the methyl ester content to diminish significantly, thus suggesting that, as methyl esters are removed from pectic polysaccharides in the cell wall, they are replaced at a similar rate.

Table 6.2 compares the mean methyl ester content of 6 day-old barley seedlings with the average total amount of methanol that is emitted from seedlings at this stage of development over a 24-hour period.

Plant Species	Age of Plant (Days)	Number of Plants in Experiment	Amount of Methanol Emitted in 24 hours (ug gdw-1)	Methyl Ester Content (ug gdw-1)
Barley	6	2	268.35	152.35

Table 6.2 – Comparison between the average amount of methanol emitted over a 24-hour period and total methyl ester content of barley seedlings

From the data in Table 6.2, it can be concluded that over a 24 hour period during the early stages of growth, the quantity of methanol emitted from barley seedlings is almost twice the total methyl ester content of the plant tissue. There is, evidently a rapid turnover of methanol within the plant tissue as it matures. As highly methylated pectic polysaccharides are laid down in elongating cell walls and the action of PME enzymes cleaves the ester bond, methanol is liberated as the cell wall stabilises and becomes cross-linked with divalent ions such as calcium. Over a seven day period, despite the fact that the quantity of methanol released is far greater than the methyl ester content of the cell walls, according to the results shown in Figure 6.11, the methyl ester content remains relatively constant. Therefore, assuming that the rate of methanol emission does not increase considerably as barley plants grow, it could be speculated that, following approximately three months of growth, the average time lapsed between sowing and the development of the granular part of the plant, at the time of harvest the quantity of methyl esters contained within the primary cell walls of the plant will be similar, on a dry weight basis, to that contained within a rapidly growing barley seedling.

It may be assumed that the methyl esters remaining in barley plant material at the stage of harvest will be released through the various processes involved in the decomposition of the barley straw. The data displayed in Table 6.2 can be utilised in calculations with the aim of estimating the total amount of methanol emitted from mature barley plants as they decompose in the form of barley straw (Section 7.2).

6.2.3 Ryegrass Seedlings

6.2.3.1 Introduction

Data detailing pectic polysaccharide composition of grasses is not readily available in the literature. The uronic acid content of the walls of mesophyll cells extracted from mature perennial ryegrass seedlings has been previously determined by Chesson et al. (1994) as being in the region of 25 mg g^{-1}. The degree of methyl esterification was found to be low, at 3.3%. Using these figures, the total amount of methanol present in the plant tissue could be estimated as being approximately 825 μg g^{-1}.

This figure is approximately five and a half times greater than the results obtained experimentally for rapidly growing barley seedlings. The considerable difference between the two sets of data is probably due to the fact that Chesson et al. (1994) used homogeneous mesophyll cell wall samples in their analyses. Mesophyll cells are known to

be atypical in terms of the fine structure of their cell walls compared to that of other cell types found in plant material. The total uronic acid and consequently the total amount of methyl esterification of a homogeneous sample consisting of only this type of cell wall is likely to be significantly different to that of a heterogeneous sample containing several different cell types. For this reason, the methyl ester content calculated for barley seedlings shown in Table 6.2 will be used as an approximate model for ryegrass seedlings of similar ages, in order to determine the approximate quantity of methanol that is present in grass harvested for the purpose of making silage or hay.

6.2.3.2 Discussion of Results

Table 6.3 displays the amount of methanol emitted over a period of 24 hours, averaged between the two adsorbent resins used in the experiments quantifying the methanol emitted from rapidly growing perennial ryegrass seedlings (Section 3.4.5). The measured average amount of methanol emitted from six day old barley seedlings in 24 hours is approximately one and a half times that from seven and a half day old ryegrass plants.

Plant Species	Age of Plant (Days)	Average Number of Plants in Experiments	Amount of Methanol Emitted in 24 hours (ug gdw-1)
Rye Grass	7.5	18	187.85

Table 6.3 – Average amount of methanol emitted over a 24-hour period from perennial ryegrass seedlings

Comparing the figures in Table 6.3 to the experimentally determined methyl ester content of rapidly growing barley seedlings (Section 6.2), it can be concluded that approximately the same quantity of methanol is emitted from rapidly growing ryegrass seedlings that is present as methyl esters in the primary cell walls at this stage of growth. Consequently, it may be concluded that, assuming that the methanol emission rate from ryegrass plants does not increase significantly at a later stage of development, the methyl ester content on a dry weight basis, of ryegrass at the stage of harvest for the purpose of silage or hay production, will be similar to that of a rapidly developing seedling.

Unlike barley, once harvested, ryegrass is used as a forage material, either in the form of silage or hay. This means that calculating the amount of residual methanol in three-month-old ryegrass plants will not be a useful contribution to determining more clearly inputs of methanol to the atmosphere. However, an approximate value for the amount of methanol

retained in grass plants at a mature stage of growth, and the subsequent release of methanol following the cutting of the grass for silage or hay production, can still be calculated. Data from the determination of the methyl ester content of barley seedlings can be utilised in these calculations, together with the assumption that the methyl ester content of mature plants will be relatively similar to that of immature seedlings on a dry weight basis.

Where applicable acreage data is available, this data may also be used to calculate the quantity methanol emitted to the troposphere per year from certain grassland systems, as a result of the maturation of the grass plants growing within it (Section 7.3).

6.2.4 Oilseed Rape Seedlings

6.2.4.1 Introduction

Broccoli (*Brassica oleracea* L var. *italica*) has been found to contain 315 g kg^{-1} uronic acid in parenchyma cell walls (Müller et al., 2003). This figure will be comparable to that of oilseed rape seedlings, as the cell walls extracted from these two species of brassica are structurally very similar to each other. Additionally, cell wall material extracted from oilseed rape seedlings contains a very small proportion of secondary xylem cell walls, which are structurally dissimilar to that of primary cell walls. Of the uronic acid residues measured, 83% were esterified hence, assuming that the majority of the carboxyl groups are esterified with methanol, the methyl ester content could be considered to be in the region of 261 mg gdw^{-1}.

The degree of esterification of galacturonic acid residues in cauliflower (*Brassica oleracea* L. var. *botrytis*) tissue has been quantified by Femenia et al. (1998). It was found that in the parenchyma tissue of the lower stem the total uronic acid content of the pectic polysaccharides was 307 mg g^{-1}, 63% of which were methyl esterified galacturonic acid residues. A total of approximately 200 mg methyl esterified galacturonic acid residues are therefore present in 1 g dried plant material.

The figures quoted by Müller et al. (2003) and Femenia et al. (1998) are both over one thousand times greater than that calculated for the methyl ester content of barley seedlings.

6.2.4.2 Discussion of Results

Table 6.4 shows the amount of methanol emitted from 3-day-old oilseed rape seedlings, on a per gram of dry weight basis, averaged between experiments and between adsorbent resins. Over a 24-hour period, rapidly developing barley seedlings emit only about 65% of the total methanol emitted from oilseed rape seedlings on a dry weight basis. Oilseed rape seedlings emit over twice as much methanol in 24 hours than do ryegrass seedlings.

Plant Species	Age of Plant (Days)	Average Number of Plants in Experiments	Amount of Methanol Emitted in 24 hours (ug gdw-1)
Oilseed Rape	3	18	410.73

Table 6.4 - Average amount of methanol emitted over a 24-hour period from perennial ryegrass seedlings

Comparing the data shown in Table 6.4 with figures for methyl ester content of Brassica species obtained from the literature (Section 6.2.4.1) of about 230 mg methyl esters per gram of dry plant material, it can be concluded that only about 0.2% of the total methyl ester content of oilseed rape seedlings is emitted to the troposphere in a 24 hour period. In three months of growth this equates to approximately 20% of the total methanol in the cell walls of oilseed rape seedlings being emitted to the troposphere. The remaining 80% will therefore presumably be retained in the primary cell walls of the plant material until the plants are harvested.

Oilseed rape crop residues, following harvest are predominantly regarded as a waste material and are left on the surface of the field in which the crops are grown. The methyl esters retained in the plant material at the stage of harvest are presumably emitted to the troposphere in the form of methanol following the cleavage of the ester bond by PME enzymes. This methanol may be quantified using a combination of data from the literature and results of the modified bell jar experiments using oilseed rape seedlings (Section 7.2).

The approximate quantity of methanol emitted to the troposphere as a result of the growth of these crops may also be calculated (Section 7.3).

6.3 Overall Results

On a dry weight basis, the methanol emission rates from each of the three plant species studied at early stages of growth are relatively similar. These differences and indeed the spread between sets of results from the same species, are much more pronounced in data plotted on a per plant basis. The degree of spread observed in Figures 6.8 to 6.10 suggests that methanol emission rate is closely related to the weight of the plant. There are large differences between plant species studied in the proportion of the methanol contained within the plants as pectic methyl esters that is emitted as methanol as the plants develop. Both monocot species emit a greater amount of methanol from the plant tissue in 24 hours than is actually present in the form of methyl esters at early stages of growth. Conversely, oilseed rape seedlings emit a tiny proportion, about 0.2%, of the methanol that is actually present in the primary cell walls in the form of pectic methyl esters at a similar stage of growth.

Table 6.5 displays the proportion of the total methyl ester content present in the immature plant tissue of barley and oilseed rape seedlings that is emitted to the troposphere over 24 hours and a full growing season of an average of three months, using the average measured maximum methanol emission rate from each species and the average methyl ester content of immature barley seedlings (Figure 6.11) in the calculations. Thus, the figures in Table 6.5 represent the maximum percentage of the total methyl ester content of each of the three crop species that is emitted to the atmosphere over a period of 24 hours.

A three-month period was considered to be applicable as the time between the first stages of growth and harvest of barley and oilseed rape plants. However, the actual time period between the emergence of the young barley seedling and harvest of the mature barley plant is likely to be greater than three months. Despite this, three months will be the approximate time taken for the vegetative part of the plant to reach maturity, prior to the development of the granular portion of the plant. Grains contain negligible amounts of pectin (Marlett, 1992) and may consequently be discounted from this series of calculations, as the granular part will contribute virtually nothing to the methyl ester content of a mature barley plant.

Plant Species	Proportion of Total Methyl Ester Content Emitted as Methanol in 24 Hours (%)
Barley	176
Rye Grass	123
Oilseed Rape	0.178

Table 6.5 – Comparison of the proportion of methyl esters emitted as methanol

The complication regarding the extrapolation of these data in terms of the proportion of the methyl esters present in ryegrass seedlings that is emitted as methanol once the crop has been harvested, lies in the fact that ryegrass is not harvested following a three month growing period. In some cases, ruminants will consume the ryegrass and the fate of the methyl esters retained in the plant tissue following consumption is the subject of much ambiguity. In the case of harvesting the grass as silage or hay, the difficulty in calculating the amount of methanol released from the ryegrass tissue as it decomposes likewise lies in the fact that the majority of the decomposition of the grass will occur in the rumen.

However, these data can be used to calculate the methanol that is present in grass plants at the stage of development at which they are harvested for use as silage or hay. This methanol will presumably be emitted to the troposphere, in one form or another, as a result of the processes that the grass will undergo, whether it be during the production of hay or silage, or decomposition following consumption by a ruminant.

The results for the quantification of methanol emitted from barley, ryegrass and oilseed rape seedlings have shown that, during the early stages of growth, dicot crop species emit only a very small proportion of the total amount of methanol present in the plant tissue in the form of methyl esters. The quantity of methanol emitted from monocot crop species over a 24-hour period totals well over the experimentally determined methyl ester content of these plants.

It may be assumed that the methyl ester content of mature monocot crop species is similar to that of rapidly growing seedlings on a dry weight basis, based on the results displayed in Figure 6.11. In the case of oilseed rape however, the quantity of methanol emitted to the troposphere, although similar in magnitude to those emissions, on a dry weight basis, from barley and ryegrass seedlings, is tiny in relation to the total methyl ester content of the primary cell walls. Extrapolating the maximum figure for methanol emission rate measured from oilseed rape seedlings, it can be estimated that at the end of a three-month growing season, approximately 80% of the methyl esters found in immature oilseed rape

remain in the plants when they are harvested. The methyl esters remaining in the plant tissue at the point at which they are harvested are presumably cleaved and emitted to the troposphere as methanol post-harvest.

6.3.1 Calculations

In the case of barley, it is possible to estimate the amount of methanol remaining in the primary cell walls of mature plants at the point of harvest. Subsequent to harvesting, these methyl ester groups are presumably cleaved from the HG backbone and released to the troposphere as methanol. Thus, a major methanol input to the troposphere may occur as a result of the use of crop residues, in this case barley straw, as a bedding material which will ultimately undergo degradation causing the release of methanol from methyl esters contained within the cell walls. An estimate as to the magnitude of this input would be valuable in approximating total inputs of methanol to the troposphere on a national and international basis.

In a similar way, extrapolations can also be made regarding the methanol input to the troposphere as a result of the decomposition of oilseed rape crop residues.

Despite the complications in calculating the amount of methanol emitted to the troposphere as a result of the harvest and subsequent possible uses of ryegrass, or indeed grass in general, the amount of methanol remaining in the grass plants at the point of harvest can be estimated.

The quantity of methanol emitted to the troposphere from the growth of barley, ryegrass and oilseed rape may be estimated using a combination of data obtained from the series of modified bell jar experiments (see Section 3.4 for methods and Section 6.1 for results) and data collated from the literature.

The calculations based on the findings of this set of experiments, together with conclusions are detailed in Section 7.3.

Chapter Seven - Projection of Methanol Emissions to a National Scale

7.1 Methanol Emissions from the Decomposition of Mature Leaves

7.1.1 Introduction

The primary purpose of carrying out methyl ester determinations on mature leaves of several species of tree growing extensively in Great Britain, was to estimate the amount of methanol that could potentially be released per year as these leaves decompose. Ultimately, this would lead to speculation as to the global impact of these emissions on the tropospheric balance of gases, bearing in mind that the estimated global tropospheric budget of methanol is 240 Tg yr^{-1}, with an estimated 23 Tg yr^{-1} originating from the decomposition of plant material (Jacob et al., 2005).

Methylotrophs are micro-organisms that can reduce one-carbon compounds as a sole source of carbon and energy. Methylotrophic bacteria are found, under some conditions, on the abaxial surface of living leaves (Section 1.4.3), as well as in soils and sediments (Anthony, 1982). Methanotrophs are a sub-group of methylotrophs that are capable of oxidising methane to methanol aerobically whereas methylotrophs can metabolise methanol either aerobically or anaerobically. Active Methylotroph populations have been detected in acidic forest soils (Radajewski et al., 2002). It is reasonable to assume, therefore that a proportion of the methanol contained within leaves that senesce on the forest floor, in the form of pectic methyl esters, will be utilised by these bacteria and therefore not be released to the troposphere. However, quantification of the processes involved in the release of methanol, resulting from the degradation of pectic methyl esters, from abscised leaves via metabolism by soil-based methylotrophs is not readily available in the literature. As a consequence, this potential sink for methanol released as a result of the decomposition of pectic polysaccharides contained within leaves at abscission will be regarded as negligible and not considered in further calculations.

7.1.2 Calculations

By utilising the LAI (leaf area index) data from the literature, an approximation of the leaf dry mass prior to abscission during the autumn and winter months was calculated. The annual leaf litterfall data was used in order to estimate the dry weight of leaves following abscission. It can be seen clearly from Tables 5.4 and 5.5 that the figures calculated for the approximate weight of leaves abscised from trees during the autumn and winter months are in close agreement with those figures obtained directly from the literature for annual leaf litterfall.

Both plant and microbial pectin methylesterase (PME) enzymes, despite remaining active under a broad range of environmental conditions, may not demethylate all methyl ester groups present on HG polysaccharides (Doong et al., 1995). The degree of demethylation depends on the source of the PME and the extent and pattern of esters on the pectic polysaccharide. Massiot et al. (1997) found that only 65% of methyl esters in apple pectin were released upon treatment with PME. This is in good agreement with earlier estimates of 60-75% for methanol release by PME (Rexová-Benková and Markovic, 1976). It therefore seems reasonable to assume that PME, whether originating from plants of from microorganisms, will remove approximately 65% of the methyl esters present in the leaves of trees during the process of senescence.

Taking this phenomenon into account, Tables 5.4 and 5.5 show that the calculated figures for the amount of methanol originating from pectic methyl esters, that could potentially be emitted to the troposphere annually as a result of the senescence of oak, ash, beech and birch leaves in Great British woodlands, using the two methods described in Sections 5.3.4 and 5.3.6 are very similar. The calculated total amount of methanol emitted to the troposphere as a result of the decomposition of oak, ash, beech and birch leaves is 1.89 and 1.84 Gg yr^{-1} using leaf litterfall data and LAI data respectively. The calculated figures for the potential amount of methanol released from the senescence of oak leaves using leaf litterfall and LAI data are particularly close (0.70 and 0.73 Gg yr^{-1} respectively).

Assuming that the majority of pectic methyl esters in the leaves at the time of abscission are released, oak and ash trees are responsible for the largest contribution of tropospheric methanol in Great Britain. Birch leaves, despite the fact that the land area covered by birch-dominated woodland is second only to oak, may contribute the least amount of methanol.

It has been speculated that the global contribution to tropospheric methanol from the decomposition of plant material is 23 Tg yr^{-1} (Jacob et al., 2005). According to the results from the determination of methanol content of mature leaves, recorded in Section 5.2 and utilised in subsequent calculations, the average amount of methanol emitted to the troposphere in Great Britain as a result of the decomposition of abscised leaves from the four most widespread deciduous tree species, is between approximately 0.06 and 0.8 Gg yr^{-1} for each species (Tables 5.4 and 5.5). The total amount of methanol emitted from these trees at the end of every year is approximately 1.9 Gg yr^{-1}. The total area of Great Britain occupied by woodland dominated by these species is roughly 595 thousand hectares (Anon, 2000). These data can be compared to the estimated global figures for methanol being introduced to the troposphere as a result of plant decomposition, and the total forested area on Earth, which are quoted as being in the region of 23 Tg yr^{-1} methanol (Jacob et al., 2005) and approximately 39.52 x 10^8 hectares (Anon, 2005) respectively.

In this case, it could be estimated that the average amount of methanol being emitted to the troposphere as a result of plant decomposition globally is 5.82 kg methanol per hectare of forested area.

According to the results displayed in Tables 5.4 and 5.5, the annual amount of methanol emitted to the troposphere as a result of the decomposition of leaves from oak, ash, beech, and birch trees in Great Britain is approximately 3.19 kg methanol per hectare of forest dominated by these four species of tree.

7.1.3 Discussion

This figure is in close agreement with the global estimates quoted by Jacob et al. (2005) considering the variables involved in the calculations. There is no doubt that, between different forest zones on the planet, there will be some variation between certain data used in calculations detailed in Section 5.3 and the actual values. This will most certainly be the case for the LAI and litterfall data, although not necessarily as pronounced for the pectic methyl ester contents of leaves. Despite the fact that there exists an immense variation in the species of forest vegetation thriving across different regions of the globe, the average global figures for the methanol emissions from the decomposition of plant matter appears to be in close accordance with the figures measured in this study for the methanol content of mature leaves of the four most widespread native broadleafed tree species in Great Britain.

7.2 Estimated Methanol Emissions from the Decomposition of Crop Residues

7.2.1 Introduction

Methyl ester determinations on barley seedlings during early stages of growth (Section 2.4) were carried out in order to determine obvious trends in the methyl ester content of pectic polysaccharides in barley seedling primary cell wall material as the plants matured. Using these data, together with data from the literature for methyl ester contents of Brassica species, it is possible to estimate the amount of methanol that will be present in both barley and oilseed rape plants at the end of a growing season, immediately prior to harvest. The amount of methanol present in the residual plant material following harvest could subsequently be estimated, hence the quantity of methanol that may potentially be released to the troposphere as a result of the senesce of these crop residues.

The methyl ester content of barley seedlings will give a good estimation of the amount of methanol present in the primary cell walls of grass seedlings. However, unlike barley, grass, when harvested does not produce an above-ground residue which subsequently decomposes, releasing the methanol contained within it to the troposphere. Therein lies the complication in estimating the amount of methanol emitted to the atmosphere as a consequence of the harvest and subsequent decomposition of grass. Despite this, certain assumptions can be made and calculations undertaken in order to obtain a rough estimation for the amount of methanol that will be present in grass when it is harvested for use as silage or hay. The methanol contained within the plant material, whether it is used as a livestock forage in the form of silage or hay, will presumably be emitted to the troposphere in one form or another, whether as a result of the mechanical cutting of the grass, the fermentation process in silage production, the drying process involved in hay production, or the degradation of the plant material within the rumen of livestock animals. General conclusions may be drawn from these calculations as regards the implications that these emissions have on the global troposphere.

7.2.1.1 Elevated Methanol Emissions Following Harvest

Significant increases in the rate of methanol emissions have been observed in a number of different plant species after the plants have been cut (Section 1.4.5.2.4). The observed increase in methanol emission following the harvest of these plant species will obviously

reduce the total quantity of methyl ester groups present in the plant material following harvest. However, because the exact magnitude of these emissions is a source of debate, it is difficult to speculate exactly what proportion of the methanol present in for example, mature grass plants prior to harvest remains there during the silage-making process.

However, based on the results of the experiments described in Section 1.4.5.2.4, the majority of the methanol present in monocot plants, for example barley and ryegrass, at the point of harvest remains in the plant material and is subsequently emitted to the troposphere, whether through decomposition in the case of barley straw, through the degradation of cell wall material during the silage-making process or the enzymic degradation of cell walls in the rumen in the case of pasture.

In spite of the fact that dicots appear to emit methanol at a vastly elevated rate following abscission, the assumption remains that the methanol present in the plant tissue at the time of harvest is released to the troposphere, either as a direct result of wounding, or as a result of the prolonged activity of PME enzymes during the period following harvest. Estimates for the overall methanol release from oilseed rape to the troposphere at the time of harvest, together with the period following harvest can therefore be calculated.

7.2.2 Barley

7.2.2.1 Introduction

The results of the modified bell jar experiments measuring methanol emission rates from growing barley seedlings on a dry weight basis (Section 6.1.2), together with the results from the quantification of methyl esters in young barley seedlings (Section 6.2.1) can be used to estimate the amount of methanol retained in mature barley plants at the time of harvest. As the vast majority of barley straw, like oilseed rape straw, is not used as a forage material due to its low digestibility and relatively high absorbency, making it more suitable as a bedding material, estimates can subsequently be calculated for the amount of methanol emitted to the troposphere following the harvest of barley in Scotland as a result of both the harvest process itself and the decomposition of the resulting crop residue.

As plant primary cell walls mature, cell expansion ceases and, in more mature cells of certain plants, including monocots such as barley, the secondary cell wall is laid down next to the plasma membrane of the primary cell wall (Carpita and Gibeaut, 1993). The secondary cell wall is composed of cellulose, comprising approximately 40-50% of the

total dry weight, lignin, hemicelluloses and extractives (Core et al., 1979). Secondary walls are comprised of three layers that can be distinguished from each other by the differences in the orientation of their respective cellulose microfibrils. The point at which the synthesis of the secondary cell wall commences can be identified by the discontinuation of the deposition of pectic polysaccharides in the cell wall and the deposition of cellulose, hemicellulose and lignin on the inner surface of the primary cell wall.

Thus, as barley plants mature, the proportion of the total cell wall content comprised of secondary cell wall material will increase. One consequence of this process continuously taking place as the plants mature, is that the total methanol content per gram of plant material decreases due to the fact that secondary cell walls contain a much reduced proportion of pectic polysaccharides as compared to primary cell walls (Carpita and McCann, 2000). This phenomenon must be taken into account when carrying out calculations with the aim of estimating the amount of methanol contained within mature barley plants immediately prior to harvest.

7.2.2.2 Calculations

During the growth of plants, it is reasonable to assume that the total quantity of cell walls increases. The amount of primary cell wall material will also increase but this will constitute a smaller proportion of the total amount of cell wall material in the plant. As the cell walls mature and pectic methyl esters are removed as a result of PME activity, the relative amount of methyl ester groups contained within the pectic fraction of these cell walls will decrease. The result of the combination of these processes, as suggested by the findings in Section 6.2.2.2, is that the methyl ester content of plants, at least during the early stages of growth, does not diminish significantly with increasing age of the plant. Assuming that the total methanol content in a mature cell wall is similar to that in a rapidly growing seedling the approximate proportion of primary cell wall material in a mature plant may be calculated using forage crop nutritional data.

The forage component of commercially grown crops is the structural portion of the plant that may be fed to animals, particularly ruminants which, due to the presence of rumen bacteria, possess the ability to digest this plant matter and render it useful primarily as energy and nitrogen. Forage fibre components are cellulose, hemicellulose, lignin, pectin, cutin and silica. Cellulose and hemicellulose are digestible, while lignin, cutin and silica are not. Pectin is highly digestible but, due to its chemical characteristics and analytical

methods most commonly used in quantifying cell wall components in forage crops, data regarding pectin content is not widely available.

Several methods of analyses, for example near infrared reflectance spectroscopy (NIRS) or chemical based analyses, may be carried out on various parts of a forage plant species in order to determine the digestibility and therefore the nutritional value of the plant. The principal fractions determined by such analyses include neutral detergent fibre (NDF), acid detergent fibre (ADF) and crude fibre (CF).

The NDF fraction is an estimate of the total amount of cell wall, both digestible and non-digestible, and consequently gives a good indication of the intake potential of a forage. The ADF portion of a forage primarily represents the amount of cellulose and lignin, the non-digestible fraction, contained within the cell walls. ADF figures are used to determine the digestibility and therefore the nutritional value of a forage. The CF is approximately equivalent to the ADF fraction, but the analysis procedure for CF determination underestimates the cell wall content in the forage as it solubilises a significant portion of the total lignin, cellulose and hemicellulose. Therefore, data for the NDF and ADF fractions will be utilized in these calculations.

By subtracting figures for the mean ADF fraction in a typical barley straw from a typical value for NDF, the approximate primary cell wall content of barley straw may be determined. Assuming the amount of methyl esters present in the primary cell walls of mature barley is similar to that of seedlings, the approximate potential amount of methanol released to the troposphere as a result of the decomposition of barley straw may be calculated.

Values were obtained from Colorado State University for typical ADF and NDF contents of barley straw. Figures were quoted as being 57 and 82% for ADF and NDF respectively, in terms of total dry matter (Stanton and LeValley, 2006). Alfredo DiCostanzo (1993) of the Department of Animal Science at the University of Minnesota quotes the ADF and NDF of barley straw as being 49 and 80% dry weight respectively, in good agreement with the figures determined by Stanton and LeValley (1996).

Hence, mean figures for the NDF and ADF fractions of barley straw could be considered to be approximately 81 and 53% respectively in terms of dry weight. The primary cell wall content of barley straw will therefore approximate 28% of the total dry matter. The results discussed in Section 6.2.1.2 show that the methyl ester content of rapidly developing

barley seedlings is approximately 152 μg gdw^{-1}. Assuming that the methyl ester content of a mature barley plant immediately prior to harvest is similar to this on a dry weight basis, the total amount of methyl esterified GA residues at the end of a growing season may be approximately 152 μg gdw^{-1}. However, the amount of primary cell wall will be a significantly lower proportion of the total biomass of mature barley plants compared to rapidly growing seedlings. In fact, according to the NDF and ADF values, only 28% of the biomass of mature barley plants will be comprised of primary cell wall material. So in mature barley plants there will be only about 34 μg methyl esters per gram of mature barley plant material on a dry weight basis. This is equivalent to about 5.7 μg methanol per g dry plant material. Therefore, in 1 g dry barley straw, there will be around 0.28 g primary cell wall material and approximately 5.7 μg methanol.

As discussed in Section 7.1.2, it has been found that PME releases only about 65% of the methyl esters in pectin (Rexová-Benková and Markovic, 1976; Massiot et al., 1997). It therefore seems reasonable to assume that PME, whether originating from plants or from microorganisms, will remove approximately 65% of the methyl esters present in barley straw at the stage of harvest. Hence, it can be estimated that 3.7 mg methanol will be emitted from 1kg barley straw following harvest.

Data on the amount of straw produced from the harvesting of crops in Scotland is not readily available. However, the approximate amount of straw can be estimated by multiplying available data for the total land area utilised in the production of the major straw-producing crops in Scotland (those applicable to this study being winter and spring barley and oilseed rape) by the reported average straw yield. Table 7.1 displays figures obtained from the Scottish Excecutive's 'Potential of Biomass Feedstocks for Energy Generation in Scotland' (Anon, 2006a).

Crop	Planted area (ha)	Straw yield (oven dried tonnes ha^{-1})	Total Straw production (oven dried tonnes)
Winter Barley	259,670	5.6	1,454,152
Spring Barley	56,790	4.1	232,839
Oilseed rape	39,400	2.5	98,500

Table 7.1 – Estimated straw production in Scotland in 2004

According to the data in Table 7.1, the total amount of barley straw produced in Scotland in 2004 was 1,686,991 tonnes dry weight. If the concentration of methanol, in the form of

pectic methyl esters, in mature barley plants is 5.7 mg kg^{-1}, but only 3.7 mg kg^{-1} of this is emitted as a result of the action of PME enzymes, it can be extrapolated that the amount of methanol emitted to the troposphere as a result of the decomposition of barley straw produced in Scotland will be in the region of 6240 kg yr^{-1}.

7.2.2.3 Discussion

Comparing this figure to that quoted by Jacob et al. (2005) for the amount of methanol introduced to the atmosphere as a result of plant decay of 23 Tg yr^{-1}, it is immediately apparent that the figure calculated above for the potential amount of methanol emitted to the troposphere as a result of the decomposition of barley straw in Scotland is a relatively small proportion of this. The calculated amount of methanol that could potentially be emitted to the troposphere as a result of the decomposition of barley straw produced in Scotland constitutes approximately $2.7x10^{-5}$% of the total amount of methanol that is emitted to the troposphere as a result of plant decomposition globally.

In 2006 a combined total of 881,000 hectares of spring and winter barley was grown in the UK (Anon, 2007a). According to the yield data in Table 7.1, this constitutes approximately 4.3 million tonnes of straw. From this, about 15,800 kg or 0.016 Gg methanol may be emitted.

Using figures obtained from the United States Department of Agriculture, figures for the contribution of methanol from the degradation of barley straw produced in Canada and the USA can also be estimated. Areas of 405,000 and 4,981,500 hectares of barley were produced in the USA and Canada in 2006 and 2002 respectively. Using the data calculated in Section 6.2.2.2, it can be estimated that approximately 97,000 kg, 0.097 Gg, methanol will be released to the troposphere annually as a result of the decomposition of barley straw produced in the US and Canada per year. This figure constitutes approximately 0.0004% of the estimate for methanol introduced to the troposphere as a result of plant decomposition quoted by Jacob et al. (2005).

Global barley harvest was estimated at 57,000,000 hectares in 2005 (Anon, 2000-2006). Using the average straw yield from spring and winter barley grown in Scotland as a rough estimate for global barley straw yield, it can be speculated that about 276 Tg barley straw would be produced as a by-product of this process. If each kg barley straw emits 3.7 mg methanol, the total amount of methanol released to the global troposphere as a result of the

degradation of barley straw is about 1 Gg yr^{-1}, approximately 0.0044% of the estimated total quoted by Jacob et al. (2005).

7.2.3 Ryegrass

7.2.3.1 Introduction

Ryegrass is grown extensively across the country (Section 3.4.1.2) and globally. Ryegrass rough pasture, by its very nature, is not harvested, but remains as a growing plant throughout the year. Estimates on the quantity of methanol emitted as a result of this growing process are calculated and discussed in Section 7.3.3.

Ryegrass is also grown extensively for use as silage or hay, owing to the fact that ryegrass has a particularly high sugar content, which is beneficial in the anaerobic fermentation stage of the silage production process. Silage production obviously involves the initial mowing of grass. This process, in wounding the plant material, has been found to enhance the emission of typical biogenic VOC's, including methanol (de Gouw, 1999) (Section 7.2.1.1). Immediately following the cutting of grass, it was found that there was an initial burst of VOC emission, dominated by methanol and (*Z*)-3-hexenal. Silage production also requires the grass to be wilted following cutting, which will in itself cause an elevation in the rate of methanol release. In spite of this, the assumption that the methanol present in the grass material at the time of cutting is emitted to the troposphere, whether in a large pulse immediately following harvet, as a more prolonged period of methanol release during drying, or as the result of decomposition in the rumen of livestock, still stands.

The findings in Section 6.2.2.2, that the methyl ester content of barley seedlings, at least during the early stages of growth, does not diminish significantly with increasing age of the plant, suggests that methanol is released to the troposphere at such a rate that, as highly esterified cell walls are laid down in rapidly growing regions of the plant and mature cell walls constantly lose methyl esters as a result of the action of PME, the quantity of methyl ester groups contained in the plant on a dry weight basis remains relatively constant. The majority of the methyl esters present at early stages of growth, based on the findings for barley seedlings and the methanol emission rates from ryegrass seedlings, therefore remain within the cell walls of plants until they are harvested.

From results calculated in Section 6.2.2, it can be speculated that three-month-old ryegrass plants contain approximately the same amount of methylated GA residues on a dry weight

basis as rapidly growing plants. Therefore, on a dry weight basis, the quantity of methanol contained in ryegrass seedlings is similar to that of plants when they are cut, be it for the production of hay or silage. The fate of pectic methyl esters in the rumen of livestock is the source of much speculation and is outwith the scope of this study. Consequently, grass grown as permanent pasture will not be taken into consideration and calculations and estimates in this section will be made only for the amount of methanol emitted to the troposphere as a result of the harvest of grass for the production of hay or silage.

On the assumption that the methyl ester content of graminaceous plants remains relatively constant, on a dry weight basis, throughout the growth of the plant as new cell walls are continuously laid down in the growing region of the stem and methanol is emitted as a result of the action of PME enzymes, calculations based on these data can be undertaken in order to determine the amount of methanol emitted to the troposphere as a result of the production of silage and hay from grass in the UK.

7.2.3.2 Calculations

According to the results from the quantification of methyl esters in rapidly growing barley seedlings, together with the findings for the rate of methanol emission from ryegrass seedlings, it could be assumed that the overall methyl ester content of ryegrass plants remains relatively constant as they mature. It could therefore be stated that, at the point of ryegrass harvest for the purpose of either hay or straw production, the methyl ester content of the plant material could be approximately 152 $\mu g\ gdw^{-1}$.

The Ministry of Agriculture, Food and Rural Affairs (Anon, 1999) have conducted experiments to determine the forage quality of annual ryegrass grown at three different sites across Ontario, USA. The ryegrass was cut and ADF and NDF values calculated. Average figures for ADF and NDF of first cut annual ryegrass over the three sites are quoted as 34.4 and 53.6% respectively. Using the same principles as were used in the calculations involving barley (Section 7.2.2.2), it could therefore be approximated that the total amount of primary cell wall material in ryegrass after about 60 days of growth is 19.2% on a dry weight basis.

The total methyl ester content of mature ryegrass could be estimated at 152 $\mu g\ gdw^{-1}$. At this stage of growth the primary cell wall content will be of a much smaller proportion of the total call wall material than that in a rapidly growing seedling. To take this into account in the calculations, the difference between the ADF and NDF values can be used

as an approximation of primary cell wall content. Therefore, in mature ryegrass plants, there will be around 29 μg methyl esters per gram of plant material on a dry weight basis. This equates to approximately 4.91 μg methanol per g dry plant material. Therefore, in 1 g dry ryegrass at the point of harvest, there will be around 0.19 g primary cell wall material and approximately 4.91 μg methanol contained within the plant material, the majority of which will presumably be emitted to the atmosphere in one form or another, be it as a result of the hay or silage-making process, or as a consequence of being consumed by ruminants.

Accurate values for UK land area used in the production of silage and hay are not readily available. The total area used for the production of grass in the UK is quoted by UK Agriculture as being around 6,904,000 hectares (Anon, 1999-2007), excluding rough grazing. Of this, it is estimated that about two-thirds, 4,602,667 hectares, is used in the production of silage or hay in the spring of each year, the vast majority being harvested as silage. Figures for grass yield are likewise elusive. O'Kiely (2000) found that average silage yields from a perennial ryegrass sward and from a productive old pasture were 10.8 and 10.5 tonnes ha^{-1} respectively on a dry weight basis. The Irish Agriculture and Food Development Authority (O'Loughlin, 2001) has published similar data regarding silage yield. Under good management, a first and second cut silage yield of 6 to 7 and between 4.5 and 5.0 t ha^{-1} respectively, on a dry weight basis, was attainable.

It could therefore be reasonable to state that an approximation for the yield of grass from a perennial ryegrass sward, grown with the purpose of harvesting for silage, would be in the region of 11 t ha^{-1} on a dry weight basis. This equates to UK production of silage of approximately 51 Tg yr^{-1}. It follows that, assuming the methyl ester content of ryegrass is similar to that of barley plants, about 0.25 Gg methanol is contained within the cell walls of the grass cut every year for the purpose of producing silage in the UK.

7.2.3.3 Discussion

The amount of methanol contained in the tissue of ryegrass cut for the purpose of producing silage in the UK equates to approximately sixteen times the calculated quantity of methanol emitted as a result of the decomposition of barley straw. However, due to the complex nature of the processes that the plant material may undergo following harvest, it is likely that a proportion of this methanol will not be emitted to the atmosphere, or it will perhaps be emitted in a different form. For these reasons, comparing this figure to that

quoted by Jacob et al. (2005) for the total quantity of methanol released to the global troposphere as a result of the decomposition of plant material will serve little purpose.

The quantity of methanol contained in grass at the stage of growth at which it is harvested for silage is perhaps surprisingly large. However, it is worth noting that the area used for the growth of grass for the sole purpose of silage production in the UK is five times greater than that used for the growth of spring and winter barley combined. It is also the case that grass DM yield from a silage harvest alone is over double that of barley straw from a barley crop.

7.2.4 Oilseed Rape

7.2.4.1 Introduction

The results of the modified bell jar experiments measuring methanol emission rates from rapidly growing oilseed rape seedlings on a dry weight basis (Section 6.1.2) can be used, together with the average results of the quantification of methyl esters in broccoli stems and cauliflower tissue (Section 6.2.3) to estimate the amount of methanol retained in oilseed rape plants at the stage at which they are harvested. Using these data, estimates can subsequently be calculated for the amount of methanol emitted to the troposphere as a result of the decomposition of oilseed rape straw following the harvest in Scotland.

Assuming that the methyl ester content of broccoli and cauliflower tissues are similar to that of a mature oilseed rape plant, the amount of methanol retained in oilseed rape plants following harvest can be estimated. By subtracting the potential amount of methanol released from oilseed rape plants over a growing season of approximately three months, calculated from the results of the modified bell jar experiment (for methods see Section 3.4, for results see Chapter 6), from the data obtained from the literature regarding the methyl ester content of broccoli and cauliflower, an approximate value for the total amount of methanol present in oilseed rape plants when they are harvested can be calculated. Oilseed rape straw residues are typically thought to be of little nutritional value as a feedstuff for ruminants in an unmodified form. For this reason oilseed rape residues are generally left on the surface of the field and ploughed back into the soil following harvest. It can be assumed therefore, that the vast majority of the methanol present in the plant at the harvest stage is released to the atmosphere as the plant material decomposes.

7.2.4.2 Calculations

As with barley in Section 7.2.1.2, the approximate proportion of primary cell wall material in a mature oilseed rape plant may be calculated using forage crop nutritional data, specifically figures for ADF and NDF. By subtracting figures for the mean ADF fraction in an oilseed rape plant from a typical value for NDF, the approximate primary cell wall content of oilseed rape plants may be determined. The approximate potential amount of methanol released to the troposphere as a result of the decomposition of oilseed rape crop residues following harvest may be calculated using approximate values for the primary cell wall content of oilseed rape straw together with data from the literature for the quantification of methyl esters in Brassica species, assuming the methyl ester contents of these Brassicca species is similar to that of oilseed rape.

McCluskey et al. (1984) quantified the total cell wall yields and indigestible cell wall content of twelve cultivars of kale, including several species of winter kale types with thin stems, considered to be anatomically comparable to oilseed rape plants. Average values were in the region of 48 and 22 for values for total cell wall yield and indigestible cell wall material respectively on a % dry weight basis. Taking the former value as an approximation of the NDF value of these types of kale and the latter as that of the ADF value, it could be concluded from this study that the primary cell wall content of these plants is in the region of 26%.

The composition and rumen degradability of straw from three oilseed rape varieties were compared by Alexander et al. (1987). Between the three varieties, the average NDF and ADF values were found to be about 75% and 60% dry matter respectively. This would suggest a primary cell wall component of approximately 15%.

Trinsoutrot et al. (2001) investigated the biochemical composition of individual components of oilseed rape crop residues. Techniques proposed by Van Soest (1963) were implemented in order to quantify soluble, cellulose, hemicellulose and ‘lignin-like compound’ fractions in each of the studied residue materials. The mean value for total cell wall material, generally speaking constituting the NDF fraction, of oilseed rape stems was determined to be approximately 61.3% of the total dry matter. Around 43.6% of the total cell wall material was found to consist of cellulose and lignin. This figure gives a good approximation for the ADF value. According to this study, the approximate primary cell wall content of oilseed rape stems is therefore approximately 18% of the total dry matter.

The mean methyl ester content of Brassica species can be considered to be approximately 230 mg methyl esters per gram of dry plant material based on figures obtained from the literature (Section 6.2.3.1). Taking into account the findings that approximately 20% of the total methyl ester content in rapidly developing oilseed rape plants would be lost to the atmosphere as methanol following a growing season of approximately three months of growth, the total amount of methyl esterified GA residues at the end of a growing season could be approximated at 184 mg gdw^{-1}. The primary cell wall content of mature oilseed rape plants prior to harvest will constitute a smaller proportion of the total biomass than that of a rapidly growing seedling. In fact, according to the literature, the primary cell wall content may be approximately 17% on a dry weight basis. In 1g dry oilseed rape straw, there will be around 0.17g of primary cell wall material and approximately 31 mg of methyl esterified GA residues. This equates to around 5.2 mg methanol in 1g straw from the harvest of oilseed rape. Therefore, in 1 g dry oilseed rape straw, there will be around 0.17 g primary cell wall material and approximately 5.2 mg methanol.

As discussed in Section 7.2.1.2, it has been found that, on average, PME enzymes will liberate only about 65% of the total methyl esters as methanol into the atmosphere as plant material decomposes (Doong et al., 1995; Massiot et al., 1997; Rexová-Benková and Markovic, 1976). Therefore, it can be reasonably estimated that the amount of methanol emitted from 1kg straw produced from the harvest of oilseed rape will be 3.4g.

From the data shown in Table 7.1, it can be seen that oilseed rape yields a considerable straw volume. The quantification of this portion of the crop is complicated however, by the fact that it disintegrates readily during harvest, so that the collectable straw yield is probably lower than the actual amount of straw produced (Garrad-Hassan 2001). More recent figures for the area of land used in the production of oilseed rape in Scotland are available from the Scottish Executive (Anon, 2006b). These figures estimate this area to be in the region of 34,000 hectares. This area, together with the yield value quoted in Table 7.1 (Anon, 2006a) will be used in subsequent calculations.

Assuming the concentration of methanol in mature oilseed rape seedlings is in the region of 5.2 g kg^{-1}, 3.4 g kg^{-1} of which is emitted to the atmosphere as the straw decomposes, it can be extrapolated that 0.29 Gg methanol will be emitted to the troposphere as a result of the decomposition of oilseed rape residues produced in Scotland per year.

7.2.4.3 Discussion

The amount of methanol that is potentially released to the troposphere as a result of the decomposition of oilseed rape straw residues produced in Scotland constitutes approximately 0.0013% of the total amount of methanol emitted to the troposphere as a result of plant decomposition as proposed by Jacob et al. (2005).

DEFRA recently published data for the area, yield and production of oilseed rape in the UK (Anon, 2007a) and concluded that 575,000 hectares were used for oilseed rape production in the UK. Assuming 3.4 g methanol is emitted from every kg of oilseed rape straw produced, the total amount of methanol emitted to the troposphere as a result of the decomposition of oilseed rape straw produced in the UK will be in the region of 4.9 Gg yr^{-1}.

In 1997, the estimated worldwide production of oilseed rape was approximately 22 million hectares and by 2003 this area had increased to around 23 million hectares (Anon, 2004). Taking the figure for oilseed rape straw yield for rape grown in Scotland as being an approximate global average, it can be stated that the amount of straw produced globally as a result of oilseed rape harvests is around 57.5 Tg. 3.4 g methanol may be released as a result of the decomposition of 1 kg oilseed rape straw; therefore 57.5 Tg oilseed rape straw may be responsible for the introduction of 0.195 Tg methanol to the global methanol budget annually. This figure constitutes approximately 0.85% of the total proposed by Jacob et al. (2005) for the tropospheric methanol contribution made by the decomposition of plant material.

7.2.5 Overall Discussion and Conclusions

The total contribution to the global atmospheric methanol budget by the decomposition of forage crop residues of the species studied here is substantial. However, despite the extensive nature of global barley production, the decomposition of barley straw may only contribute about 0.0044% of the total amount of methanol released from the decomposition of plant material as proposed by Jacob et al. (2005). The decomposition of oilseed rape straw may be responsible for around 0.85% of the total methanol in the troposphere as a result of plant matter decomposition per year. This quantity is huge in relation to the equivalent amount contributed by barley straw, despite the area used in the global production of barley being about two and a half times that of oilseed rape.

Both oilseed rape and barley straw may be utilised in several different ways following crop harvest, depending upon the agricultural system in which it is produced. In both of these cases, a small proportion of the residual straw produced from the harvest process may be utilised as a forage material. The fate of the methyl esters contained within the forage material once consumed by ruminants is unknown, however it is possible that a smaller proportion of the methyl esters present in the plant material is released as methanol following digestion in the rumen than that released as a result of decomposition. Or perhaps the methanol is emitted from ruminants in the form of a different compound, for example methane.

The calculated global figures for methanol release from the decomposition of residues from the harvest of barley and oilseed rape are estimates based on both results of analyses carried out by the author and data obtained from the literature. Assumptions have been made that these data are reliable and therefore calculated figures can be regarded as rough estimates only. Based on these assumptions, it can be stated that annually, about 196 Gg methanol is emitted to the troposphere from the decomposition of barley and oilseed rape straw produced throughout the world, with 195 Gg of this being contributed by oilseed rape straw. This quantity constitutes approximately 0.85% of the estimated methanol emitted globally as a result of the decomposition of plant material proposed by Jacob et al. (2005).

This study highlights the fact that the quantity of methanol present in both the graminaceous and non-graminaceous crop species studied at harvest time is comparable to that during early stages of development. This suggests that, as methanol is released from the plant material as a result of the action of PME enzymes on cell wall polysaccharides, the elevated methyl ester content within new cell wall material continuously being produced as the plants grow, results in the overall methyl ester content remaining relatively constant.

Due to the complex nature of the fate of cell walls and the methyl esters contained within them following mastication and digestion by ruminants, the quantity of methanol released from grass after it has been harvested cannot be estimated with any degree of accuracy. However, on the assumption that the methyl ester content of mature grass plants is comparable, on a dry weight basis, to that of seedlings, the amount of methanol potentially stored in the cell walls of grass harvested as silage or hay annually in the UK has been estimated. About 0.25 Gg methanol is present in the grass harvested as silage in the UK

annually. The fate of this methanol and the proportion of it subsequently emitted to the troposphere is difficult to speculate.

7.3 Estimation of Methanol Emissions from Growing Crop Species in the UK

7.3.1 Introduction

Methanol emissions from seedlings of three commonly grown crop species have been quantified in a series of experiments using the modified bell jar (see Section 3.4 for methods, Chapter 6 for results). These quantities may be extrapolated to estimate the amount of methanol emitted to the troposphere as a result of the growth these crops in the UK every year.

In the cases of barley and oilseed rape, these calculations can then be used to estimate the amount of methanol emitted to the troposphere from the growth of these crops annually on a global scale, using data determined from the modified bell jar experiments together with figures obtained from the literature for areas of land used in the production of the particular crop species. Extrapolating the quantity of methanol emitted from these crop species during the growth of the plants on a global scale is difficult without the inclusion of a large degree of uncertainty. This is particularly the case when dealing with ryegrass. Ryegrass, a C3 plant, is grown extensively throughout the UK and Europe. However, in warmer climates, grass species that use C4 carbon fixation are grown more effectively. For this reason, the amount of methanol emitted by all grass species grown globally cannot be estimated.

7.3.2 Barley

7.3.2.1 Introduction

According to the data in Table 7.1, 316,460 hectares of spring and winter barley combined were grown in Scotland in 2004 (Anon, 2006a). In 2006 a combined total of 881,000 hectares of spring and winter barley was grown in the UK (Anon, 2007a). The ideal population of winter barley in the spring is quoted by the Agriculture and Rural Development Department of the Northern Ireland Government as being between 250 and 300 plants per m^2 (Anon, 2006c), this being equivalent to approximately 2.75 million

plants per hectare. Figures of between 300 and 325 plants per square metre are quoted by the Scottish Farmer as being the optimum plant population of spring barley (Anon, 2007b). Mike Peel, North Dakota State University extension agronomist, quotes the recommended plant population for barley as being about 1.25 million plants per acre. This is equivalent to approximately 3.1 million plants per hectare.

The maximum and minimum methanol emission rates from barley plants were measured using GC-FID when the plants were six and twelve days old respectively (Section 6.1.3.3). It was decided that the figures calculated for the rate of methanol emission per plant rather than on a dry weight basis would be more useful in calculations with the aim of determining the amount of methanol emitted from barley crops as they grow in the UK every year.

7.3.2.2 Calculations

The average maximum emission rate of methanol from 6-day-old barley seedlings on a per plant basis is approximately 0.1 μg methanol hr^{-1} $plant^{-1}$ (Figure 6.8). Figures from the literature quote the optimum plant density for barley plants at about 2.9 million barley plants per hectare. At this plant density, 1 hectare of barley seedlings will emit 290 mg methanol hr^{-1}, with approximately 91.8 kg methanol being emitted from barley plants growing in Scotland per hour. It is worth noting that this figure applies only to seedlings that were sown 6 days previously and also that this emission rate steadily decreases to approximately half of that of 6-day-old seedlings during the subsequent 6 days of growth.

The methanol emission rate from plants of an age greater than 12 days is not known. It can therefore only be assumed that, for the majority of the three months or so that barley plants are growing before the grain part of the plant develops, the average methanol emission rate is in the region of 0.05 μg hr^{-1} $plant^{-1}$. This being the case, over 3 months, the barley growing in Scotland will have emitted a total of approximately 0.11 Gg methanol. From the entire population of growing barley plants in the UK, throughout the growth of the leafy portion of the plant, it can be considered reasonable to suggest from the results of these experiments that about 0.31 Gg methanol is emitted from these barley plants every year.

7.3.2.3 Discussion

In 2006, the USA produced 405,000 hectares of barley, whilst in 2002, 4,981,500 hectares of barley were produced in Canada. This equates to the emission of about 1.87 Gg methanol emitted to the troposphere per year from the growth of barley.

As mentioned in Section 7.2.2.3, the global barley harvest was estimated at 57,000,000 hectares in 2005 (Anon, 2000-2006). If the average rate of methanol emission from a growing barley crop is approximately 145 mg hr^{-1} ha^{-1}, over three months of growth, the global barley crop will emit in the region of 19.8 Gg methanol to the troposphere.

The predicted global tropospheric methanol contribution made by the growth of plants is thought to be in the region of 128 Tg yr^{-1} (Jacob, 2005). Only a tiny proportion of this, approximately 0.016%, is due to the growth of barley.

7.3.3 Ryegrass

7.3.3.1 Introduction

Ryegrass accounts for approximately 70% of forage grass seed sales in the UK. Although perennial ryegrass is not the only variety of grass grown in the UK, it may be reasonable to assume that grasses of different varieties and species, considering the similarities in general physiology, emit similar quantities of volatiles from their leaves as they grow.

The same cannot be said, however, for grass species grown globally. C3 plant species, of which ryegrass is one, constitue over 95% of the plant life on the planet. However, 1% of all known plant species, equating to about 5% of the earth's plant biomass, use C4 carbon fixation methods. C4 plants exist and thrive in regions where there are high air temperatures, a tendency for drought conditions and a limited supply of carbon or nitrogen. Their mechanism for photosynethsis efficiently delivers CO_2 to the first enzyme in the Calvin cycle, reducing the requirement for photorespiration, which effectively wastes energy. Plant species of commercial significance that use C4 carbon fixation include sugarcane, maize, sorghum, finger millet, amaranth and switchgrass. The leaf anatomy of C4 plants is unique and differs greatly from that of C3 plants. Two layers of cells, the bundle sheath layer and the mesophyll layer, surround the vascular bundles within the leaf. It has been found that the digestibility of C4 plants is characteristically low (Wilson, 1983), suggesting that the proportion of total cell wall biomass comprised of primary cell walls

may be lower than in C3 plants of a similar stage of growth (Carpita, 1996) . If there is a lower proportion of primary cell walls in a C4 plant species, it is reasonable to assume that the pectin, and consequently the pectic methyl ester content, of the cell walls is lower than an equivalent plant of a C3 species. This may have a great influence on the amount of methanol that is emitted from these plants as they grow. As a result, it is considered prudent not to include in the calculations an extrapolation to estimate the amount of methanol emitted by grass species on a global scale.

The germination rate of perennial ryegrass seeds and therefore the density of grass plants in a crop, perhaps unsurprisingly, varies considerably depending on, among other variables, regional climate, soil fertility and fertiliser application rate. Figures for the germination rate and therefore the density of ryegrass plants in either a hay or silage, or a pasture system are not readily available, which discounts the use of data for methanol emission rate per ryegrass plant from subsequent calculations. Instead, data for the average yield of dry weight from each of these systems can be utilised, together with methanol emission rates from ryegrass seedlings determined from the modified bell jar experiments (see Section 3.4 for methods, Section 6.1.2 for results).

O'Kiely (2000) concluded that the average silage yield from a perennial ryegrass sward and that from a productive old pasture were 10.8 and 10.5 tonnes ha^{-1} respectively on a dry weight basis. Using these figures, together with results from the modified bell jar experiments involving ryegrass seedlings, it will be possible to calculate the approximate amount of methanol emitted to the UK troposphere from these two grassland systems over the time period during which they are growing.

7.3.3.2 Calculations

Calculations with the aim of quantifying methanol emissions from growing ryegrass plants will not involve an easily defined time period for the growing season, after which the plants will be harvested. However, Defra have developed a model called the Poaching Risk Assessment Model (PRAM 2.0), designed for the quantification of the potential for an autumn extension of the standard summer grazing season (Anthony and Lyons). The growing season for grass is defined as the period during which soil temperatures at a depth of 30 cm are greater than the 6 ^{o}C. The growing season calculated using the model, averaged over five sites located in England and Wales, was concluded to be approximately 185 days. Thus, the time period during which methanol will be emitted at a similar rate to

that observed in the experiments described in Section 3.4 could be considered to be in the region of 185 days.

Accurate land area values for UK silage and hay production are difficult to come by in the literature. The total area used for the production of grass in the UK, excluding rough grazing, is quoted by UK Agriculture as being around 6,904,000 hectares (Anon, 1999-2007). Of this, it is estimated that about two-thirds, 4,602,667 hectares, is used in the production of silage or hay in the spring of each year, the vast majority being harvested as silage.

Figures for grass yield are likewise elusive, but O'Kiely (2000) found that average silage yields from a perennial ryegrass sward and that from a productive old pasture were 10.8 and 10.5 tonnes ha^{-1} respectively on a dry weight basis. The Irish Agriculture and Food Development Authority (O'Loughlin, 2001) has published similar data regarding silage yield. Under good management, a first and second cut silage yield of 6 to 7 and between 4.5 and 5.0 t ha^{-1} respectively, on a dry weight basis, was attainable. Using these figures, an approximate yield of grass from a perennial ryegrass sward, grown with the purpose of harvesting for silage, would be in the region of 11 t ha^{-1}. The grass yield from a mature pasture may be considered to be around 10.5 t ha^{-1}.

According to the data above, the total weight of grass produced as silage in the UK per year is approximately 51 Tg. If the remainder of the total area of the UK used in the production of grass can be considered mature pasture, then the approximate amount of this type of grass grown in this country will be in the region of 24 Tg. Thus, the cumulative weight of grass grown for silage production and that grown for mature pasture may be roughly speaking 75 Tg dry weight.

In order to make calculated values for methanol emissions from all three crop species studied here comparable, the average minimum, rather than average, measured emission rate from ryegrass plants will be used in subsequent calculations. The methanol emission rate from plants of a greater age than this has not been determined. It can therefore only be assumed that the steady methanol emission rate from mature ryegrass plants will be roughly 0.001 μg hr^{-1} $plant^{-1}$ (Section 6.1.3.3) and that on a dry weight basis, the methanol emission rate will be in the region of 0.5 μg hr^{-1} gdw^{-1} (Section 6.1.3.2).

Using the figure above for the dry weight of grass produced in the UK annually in both pastoral and silage-producing systems, the total mount of methanol emitted to the

troposphere annually as a result of the growth of grass could be as much as 167 Gg. However, this will be a vast over-estimation as these calculations were based on the methanol emission rate on a dry weight basis, rather than that on a per plant basis. This consequently assumes that the amount of methanol emitted will increase as the dry weight of the ryegrass plants increase. This is almost certainly not the case. In fact, the results of the modified bell jar experiments (Section 6.1) suggest that, as the size of the plant increases with increasing age, the methanol emission rate per gram dry weight decreases significantly.

The dry weight of the average 18-day-old ryegrass plant is about 1.9mg. However, the age of the grass plants in this case will be closer to 60 days old (O'Kiely, 2000). Assuming that the dry weight of ryegrass plants increases at a similar rate to that of plants between the ages of 6 and 18 days, at 60 days, the dry weight of the average plant may be approximately 21.83 mg. If the combined total weight of grass grown for the purpose of silage production, together with that growing as mature pasture, is about 75 Tg, it could be calculated that there will be in the region of 3.43×10^{15} grass plants growing in these systems. Based on this assumption, the results of the modified bell jar experiments calculated on a per plant basis can be used to determine the approximate quantity of methanol emitted from areas of grass that are grown in the UK for silage production and that classed as mature pasture. Assuming grass plants are effectively growing for 185 days of the year, it can be estimated that methanol introduced from grass grown for silage and that in mature pasture systems will emit approximately 15.1 Gg.

7.3.3.3 Discussion

The figure calculated here for the quantity of methanol emitted to the troposphere annually from the growth of ryegrass, both in the form of mature pasture and for the production of silage, is approximately 15.1 Gg. The quantity of methanol emitted from the growth of barley in the UK is only about 2% of this value. One may have predicted this to be the case, given the vast area of the UK utilised in the growth of grass and the relative densities in which these two plant species are grown.

It is worth noting that this set of calculations considers only grass grown for the purpose of silage production and that growing in the form of mature pasture. In the UK, the total amount of grass growing will be greater than this and consequently the amount of methanol emitted as a result of grass growth will be greater than the figure quoted above. However, despite the fact that land area utilised for rough and hill grazings will be several

times larger than the area used for silage production and mature pasture, the total grass biomass produced in the former grazing systems will be comparatively small due to the relatively low biomass production rate. The weight of grass grown globally cannot be estimated due to the vast variety of growing conditions, types of grass and the constant flux in land areas concerned.

7.3.4 Oilseed Rape

7.3.4.1 Introduction

Figures dating from the 2006 harvest quote the total acreage of oilseed rape grown in Scotland as 34,000 hectares and in the UK as 575,000 hectares (Anon, 2007a). VOC's emitted from rape plants as they grow will therefore be likely to contribute significantly to the overall composition of the UK stratosphere. Dr Jo Bowman, a plant breeder at Nickersons Seeds Ltd., Lincolnshire, has conducted trials using a range of different oilseed rape varieties. It was concluded that 60 to 80 plants per square metre gave the highest economic return (Nickerson (UK) Ltd.).

The minimum measured methanol emission rate from growing oilseed rape seedlings will be used in these calculations as an approximate average rate of emission from growing plants, from emergence to harvest.

7.3.4.2 Calculations

According to the data displayed graphically in Figure 6.10 (Section 6.1.3.3), the minimum measured rate of methanol emission from growing oilseed rape plants in the modified bell jar experiments occurs when the plants are 12 days old. This emission rate is approximately 0.02 $\mu g\ hr^{-1}\ plant^{-1}$ and, due to the lack of data for emission rates from plants of a greater age, will be used in subsequent calculations.

The optimum density of oilseed rape plants is around 700,000 plants per hectare. According to figures from the literature mentioned above, 34,000 hectares of oilseed rape was grown in Scotland in 2006. Over the growing season of three months, these plants will have produced a total of approximately 1142 kg methanol. In the 575,000 hectares of land used for the growth of oilseed rape in the UK, there will therefore be in the region of $4.0x10^{11}$ oilseed rape plants being grown annually in the UK. During an entire growing

season of approximately 3 months, the UK's oilseed rape crop will have produced about 0.02 Gg methanol.

7.3.4.3 Discussion

In 2003 it was estimated that globally, approximately 23 million hectares of oilseed rape were grown (Anon, 2004). If the average rate of methanol emission from a growing oilseed rape crop is approximately 14 mg hr^{-1} ha^{-1}, over three months of growth, the global oilseed rape crop will emit in the region of 0.77 Gg methanol to the troposphere.

It is thought that the input of methanol to the global troposphere originating from growing plants is approximately 128 Tg yr^{-1} (Jacob, 2005). An almost negligible proportion of this is contributed by the growth of oilseed rape crops ($6x10^{-4}$%).

7.3.5 Overall Discussion and Conclusions

A very small proportion of the total amount of methanol emitted to the troposphere as a result of the growth of plants is contributed to by the growth of the crop species studied here. From the calculations above, it could be estimated that about 20.6 Gg methanol is emitted to the global troposphere as a result of the worldwide growth of barley and oilseed rape crops. This constitutes a mere 0.016% of the value estimated by Jacob et al. (2005) for the total quantity of methanol emitted to the troposphere from growing plants.

The input made by the growth of grass is vast in comparison to the other two crop species studied here. For example, the calculated quantity of methanol emitted to the troposphere from grass grown for silage production and grass growing as mature pasture in the UK alone is estimated as being approximately 15.1 Gg. It can only be speculated as to the magnitude of the total amount of methanol that is emitted from the lowland pastures, silage and hay crops, rough upland pastures, golf courses and parklands across the globe and the impact that this has on the global balance of volatile species in the troposphere.

7.4 Comparison Between Methanol Emissions from the Decomposition and Growth of Crop Species

Tables 7.2 and 7.3 display the data calculated in Sections 7.2 and 7.3 respectively for the quantities of methanol emitted to the troposphere from the decomposition of crop residues and those emitted from growing crop plants. Figures were calculated using results from

experiments quantifying the methyl ester content of barley seedlings and those quantifying the methanol emission rates from barley, oilseed rape and ryegrass, combined with data from the literature.

Plant Species	Quantity of Methanol Emitted Through Decomposition of Residues (Gg yr-1)			
	Scotland	UK	US and Canada	Global
Barley	0.00624	0.016	0.097	1
Rye Grass	*	0.25 (a)	*	*
Oilseed Rape	0.29	4.9	*	195

*** Figure not calculated**
(a) Methanol contained in plant material at point of harvest for silage

Table 7.2 – Calculated quantities of methanol emitted from residue decomposition

Plant Species	Quantity of Methanol Emitted from Growing Plants (Gg yr-1)			
	Scotland	UK	US and Canada	Global
Barley	0.11	0.31	1.87	195
Rye Grass	*	15.12 (a)	*	*
Oilseed Rape	0.00114	0.0193	*	0.77

*** Figure not calculated**
(a) Grass growing as mature pasture and for the production of silage

Table 7.3 – Calculated quantities of methanol from crop plant growth

It can be concluded from the data in Tables 7.2 and 7.3 that the quantity of methanol emitted to the troposphere from the growth of monocot crop species, both nationally and globally, is generally on a much greater scale than the amount of methanol available for release during the decomposition of the same plants. These finding are concurrent with the conclusion that the methanol released from monocot plants over a 24-hour period as young, rapidly growing plants develop constitutes a greater quantity than is actually present as total methyl esters in the primary cell walls.

However, the converse is true of oilseed rape, the only non-graminaceous crop plant studied. These plants contain a much greater quantity of methyl esterified GA residues than the graminaceous plant species studied here. The result of this, as shown in Table 7.2, is that, at the time at which the plants are harvested, the graminaceous species contain a very small amount of pectic methyl esters compared to the oilseed rape plants.

The methanol released from the growth of the oilseed rape seedlings on a per plant basis is comparable to that of barley seedlings but, due to the fact that barley is grown over a much larger area globally and barley plant densities are greater than that of rape, as Table 7.3 shows, the methanol emitted from these plants as they grow totals over 250 times that of

methanol emitted from growing oilseed rape plants. It is worth noting that these experiments were carried out on rapidly growing, immature seedlings. The trend in methanol emission rates and the variation in methyl ester content of these plants as they reach maturity can only be speculated.

The fact that the figures for methanol emissions from ryegrass during the growth of the plants and the amount of methanol available for release during decomposition are so much greater than those calculated for the other graminaceous crop species, is likely to be indicative of the vast quantities of grass produced in the UK. The area utilised in the growth of grass is about five times that used for both spring and winter barley crops combined. The biomass produced by these grassland systems is also very large in comparison to that produced from a barley crop. It is also worth noting that the growing season of grass was taken as being almost twice that of both barley and oilseed rape in these calculations, 185 and 100 days respectively, which will also contribute to the higher values seen in the case of methanol released from growing ryegrass plants.

7.5 Comparison Between Methanol Emissions from Decomposing Mature Leaves and Crop Residues

According to the figures calculated from LAI data, leaf litterfall data and the methyl ester content of mature leaves of native tree species, about 2.9 Gg methanol may potentially be released as a result of the decomposition of mature leaves of oak, beech and ash trees during the autumn and winter months in Great Britain. Table 7.4 shows the areas occupied by respective plant species studied here, the approximate biomass that this area of production represents and the calculated mean quantity of methanol that this weight of biomass may contain in the form of pectic methyl esters. This methanol, or perhaps the majority of it, may be emitted to the troposphere following the harvest or following the abscission of leaves in the case of tree species. Figures quoted in the cases of methanol emitted from leaves following abscission are averaged between calculated values from leaf litterfall (Section 6.2.4) and LAI data (Section 6.2.6).

Plant Material	Area of Plant Grown ('000 Hectares)	Biomass Produced (Tg yr-1)	Methyl Ester Content of Plant Material (mg gdw-1)	Mean Quantity of Methanol Emitted (Gg yr-1)
Barley Straw	881.0	4.273	0.12	0.016
Rye Grass*	4602.7	51.00	0.12(a)	0.25(b)
Oilseed Rape Straw	575.0	1.438	184.0	4.90
Mature Oak Leaves	222.7	0.858	1282.4	0.71
Mature Ash Leaves	129.1	0.378	2822.2	0.69
Mature Beech Leaves	83.0	0.125	2201.2	0.37
Mature Birch Leaves	160.0	0.544	239.4	0.08

*** Grass grown for the purpose of silage production**
(a) Assuming a similar methyl ester content to barley seedlings
(b) Methanol contained in grass harvested as silage as methyl esters

Table 7.4 – Methanol emitted from the decomposition of different plant materials grown in the UK

It can be seen from Table 7.4 that the relatively low pectic methyl ester content of barley and ryegrass plants results in a smaller contribution of methanol inputs made to the troposphere by these monocots as they decompose, despite the vast areas over which they are grown. Ryegrass plants make by far the largest contribution of biomass but, due to the low methyl ester content of these plants, this does not translate in the figure for the amount of methanol contained in grass plants when harvested for use as silage.

Oilseed rape crop residues potentially contribute the greatest amount of methanol to the troposphere as they decompose and pectic methyl ester groups are liberated. Compared to the mature leaves, the methyl ester content of these non-graminaceous plants is low, but because oilseed rape is planted over a much greater area than any of the tree species studied, the biomass of rape straw is larger and the amount of methanol available for release from the decomposition of the plant material greater.

Three different situations are examined here. Firstly, it has been shown that annual grasses, such as rye grass, barley, and potentially all graminaceous species, produce methanol as they grow. It has also been shown that non-graminaceous crop species emit a quantity of methanol as they grow, but that a larger amount of methanol is emitted to the troposphere as they decompose. This has also been shown to be the case for deciduous tree species.

Chapter Eight - Conclusions and Perspective

Pectic non-methyl esters were detected in the cell walls of potato tubers and pea epicotyls. There was no obvious correlation between duration of storage following harvest and the amount of pectic non-methyl esterification, suggesting that the occurrence of methyl and non-methyl esters does not account for the deterioration in the texture of potato tubers over the course of the storage season. There were however, significant varietal differences in overall pectin composition between the two cultivars studied.

The growth of crop plant species in the UK is a major contibuter to the country's output of methanol in the troposphere and, assuming that pectic methyl esters remaining in crop plants at the time of harvest release methanol into the surrounding environment, it can be concluded that the decomposition of crop plant residues can also be considered a significant source of tropospheric methanol. Measured methanol emission rates from barley and ryegrass seedlings, together with the pectic methyl ester contents of primary cell wall material from these plants, suggested that the quantity of methanol emitted to the troposphere from the growth of graminaceous crop species, is generally on a much greater scale than the amount of methanol available for release during the decomposition of the same plants. The converse is true of non-graminaceous plants. Oilseed rape seedlings contained a much greater quantity of methyl esterified GA residues than either barley or ryegrass plants. The consequence of this is that, at the time at which the plants are harvested, the graminaceous species contain a very small amount of pectic methyl esters compared to oilseed rape plants.

Pectic methyl esters were found in the primary cell walls of the leaves of tree species that grow extensively in Great Britain. These esters, if released to the troposphere during the decomposition of abscised leaves, would constitute a major source of biogenic methanol production in Great Britain. The annual amount of methanol emitted to the troposphere as a result of the decomposition of leaves from oak, ash, beech, and birch trees in Great Britain is approximately 5 kg methanol per hectare of broadleafed woodland.

A variety of further studies could be carried out using the modified bell jar capture and purge technique developed here to quantify methanol emissions from plant material of different origins, for example decaying plant tissue. Methanol emitted from the decomposition of plant material such as residues from harvested crop species, leaves abscised from native trees species or needles shed from evergreen tree species grown

extensively in Great Britain, particularly Sitka spruce, could be quantified and the resulting data scaled up to a national level. The conditions within the jar could also be varied, for example so as to determine the effect of prevailing environmental conditions, for example moisture level, pH and temperature, on the amount and rate of methanol release from plant tissue of different types.

Stable isotopic measurements could be utilised in work of this nature to both trace the fate of methanol and source the location of methanol emitted from plants in the troposphere, and also to determine the origin of different components of the volatile emissions from plants. For example, thermal desorption-gas chromatography-isotope ratio mass spectrometry (TD-GC-IR-MS) method was developed by Turner et al. (2006) to determine stable carbon isotopic compositions of low molecular-weight volatile organic compounds(VOCs) in airborne samples as a means of determining their respective sources in the environment. In this case, only anthropogenic sources of these volatiles were investigated. Schmidt et al. (2004) reviewed the techniques currently utilising gas chromatography-isotope ratio-mass spectrometry (GC-IR-MS) for carbon stable isotope analysis. This technique has successfully been used in the following areas: the allocation of contaminant sources on a local, regional and global scale, the identification and quantification of biotransformation reactions, and the characterisation of elementary reaction mechanisms that govern product formation. However, the investigated compounds are comprised mainly of monoaromatics such as benzene and toluene, polycyclic aromatic hydrocarbons (PAHs), and chlorinated hydrocarbons such as tetrachloromethane, trichloroethylene, and polychlorinated biphenyls (PCBs) (Schmidt et al., 2004). By using compound-specific isotope analysis (GC-MS-IR-MS), Keppler et al. (2004) found that, by measuring the carbon isotopic ratios in VOC's, the methoxyl groups of pectin and lignin represented the predominant source of biogenic C-1 compounds such as methanol, chloromethane and bromomethane in the troposphere (Keppler et al., 2004). A more incisive approach here could also include labelling pectic polysaccharides within a plant with ^{13}C and analysing VOC's subsequently emitted from the plant, the ^{13}C tracer would allow the source of particular volatile emissions could be determined. Further work of this nature would improve our knowledge and understanding of the origins of biogenic methanol in the troposphere and allow us to present a more accurate global methanol budget.

In order to identify the nature of volatile compounds other than methanol being emitted from sample plant material, GC could be coupled with mass spectrometry. This analytical process could improve our understanding of the impact of environmental conditions on the

rates of biogenic VOC emissions from growing or decomposing plant material. By incorporating a motor-driven sampling and injection system into the modified bell jar headspace sampling method, as described in Section 3.2.11.2, air samples could be taken from the bell jar and immediately injected onto a GC column automatically, increasing sample through-put and possibly improving reproducibility as the need for an adsorbent resin would be negated.

The scaling-up of figures determined in small-scale experiments inevitably incurs errors, especially when dealing with parameters in nature, where the extent of variability is largely unknown. However, scaling-up is an important process in drawing meaningful conclusions from small-scale studies. In this case for example, scaling-up allows the estimation of the amount of methanol that could potentially be emitted to the troposphere as a result of the growth of a number of crop species and the senescence of leaves from certain species of tree, on a global scale. It has been shown here that the seasonal pattern of methanol emission from plants is extremely complex and likely to be due, not only to the prevailing environmental conditions, but also due to the proportion of growing plants in a region that are grasses and cereals.

Natural variability in the quantity of methanol emitted from both growing and decaying plant material will no doubt occur, depending on the plant species and the environs in which the plants are growing or senescing. Extensive further investigation, such as described above, must be carried out in order to quantify these variations and obtain a more accurate model for global inputs of methanol to the troposphere contributed by plants. However, within these constraints, this thesis has contributed to the current understanding of methanol emissions from plants and speculated on potential global inputs to the troposphere from the growth and senescence of certain plant species and thus goes some way to furthering insite to the global methanol cycle.

Current global methanol budgets (e.g. Singh et al., 2000; Galbally and Kirstine, 2002; Heikes et al., 2002; Horowitz et al., 2003; Lathière et al., 2006) quote estimated biogenic methanol emissions at anywhere between 37 (Galbally and Kirstine, 2002) and 280 Tg yr^{-1} (Heikes et al., 2002). With such a vast number of uncertainties being incorporated into the calculations, it is perhaps unsurprising that such an enormous difference exists between estimates. However, by considering the processes that ultimately control the rate of methanol release from vegetation to the troposphere, it may be possible to predict more accurately the global inputs of tropospheric methanol contributed to by plants and perhaps how projected changes in global climate may affect these inputs.

Appendix – Statistical Data

General Linear Model: October - Leaf Cover Vs Tree Species, Site

```
Factor     Type Levels Values
Site      fixed      3 1 2 3
Tree Spe  fixed      3 1 2 3

Analysis of Variance for Leaf Cover, using Adjusted SS for Tests

Source          DF     Seq SS     Adj SS     Adj MS       F      P
Site             2    0.63870    0.63870    0.31935   17.00  0.000
Tree Spe         2    0.31969    0.31969    0.15984    8.51  0.003
Site*Tree Spe    4    0.42684    0.42684    0.10671    5.68  0.004
Error           18    0.33807    0.33807    0.01878
Total           26    1.72330

Least Squares Means for Leaf Cover

Site               Mean   SE Mean
1                -0.976   0.04568
2                -1.337   0.04568
3                -1.250   0.04568
Tree Spe
1                -1.036   0.04568
2                -1.286   0.04568
3                -1.240   0.04568
Site*Tree Spe
1    1           -0.818   0.07912
1    2           -0.879   0.07912
1    3           -1.231   0.07912
2    1           -1.275   0.07912
2    2           -1.504   0.07912
2    3           -1.231   0.07912
3    1           -1.015   0.07912
3    2           -1.476   0.07912
3    3           -1.259   0.07912

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Leaf Cover
All Pairwise Comparisons among Levels of Site

Site = 1 subtracted from:

Site     Lower    Center     Upper  --+---------+---------+---------+----
2      -0.5258   -0.3609   -0.1960   (------*-----)
3      -0.4390   -0.2741   -0.1092      (------*------)
                                   --+---------+---------+---------+----
                                   -0.50     -0.25      0.00      0.25


Site = 2 subtracted from:

Site     Lower    Center     Upper  --+---------+---------+---------+----
3     -0.07811   0.08680    0.2517                    (-----*------)
                                   --+---------+---------+---------+----
                                   -0.50     -0.25      0.00      0.25


Tukey Simultaneous Tests
Response Variable Leaf Cover
All Pairwise Comparisons among Levels of Site

Site = 1 subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
2           -0.3609     0.06460    -5.586     0.0001
3           -0.2741     0.06460    -4.243     0.0014

Site = 2 subtracted from:
```

```
Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
3           0.08680     0.06460     1.344     0.3904

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Leaf Cover
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Tree Spe     Lower    Center     Upper  -+---------+---------+---------+-----
2          -0.4153   -0.2503  -0.08543  (-------*--------)
3          -0.3693   -0.2044  -0.03950     (-------*-------)
                                         -+---------+---------+---------+-----
                                       -0.40     -0.20     -0.00      0.20


Tree Spe = 2 subtracted from:

Tree Spe     Lower    Center     Upper  -+---------+---------+---------+-----
3          -0.1190   0.04593    0.2108                (-------*--------)
                                         -+---------+---------+---------+-----
                                       -0.40     -0.20     -0.00      0.20


Tukey Simultaneous Tests
Response Variable Leaf Cover
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
2               -0.2503     0.06460    -3.875     0.0030
3               -0.2044     0.06460    -3.164     0.0141

Tree Spe = 2 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
3               0.04593     0.06460    0.7110     0.7602
```

General Linear Model: November - Leaf Cover Vs Tree Species, Site

```
Factor     Type Levels Values
Site      fixed      3 1 2 3
Tree Spe  fixed      3 1 2 3

Analysis of Variance for Leaf Cover, using Adjusted SS for Tests

Source          DF     Seq SS     Adj SS     Adj MS       F      P
Site             2     2.5372     2.5372     1.2686   16.40  0.000
Tree Spe         2    11.1147    11.1147     5.5573   71.84  0.000
Site*Tree Spe    4     0.7909     0.7909     0.1977    2.56  0.074
Error           18     1.3923     1.3923     0.0774
Total           26    15.8351

Unusual Observations for Leaf Cover

Obs  Leaf Cover       Fit      SE Fit  Residual   St Resid
 22  -0.69450   0.02193     0.16057  -0.71643     -3.15R

R denotes an observation with a large standardized residual.

Least Squares Means for Leaf Cover

Site          Mean   SE Mean
1           0.5960   0.09271
```

```
2          -0.1088   0.09271
3           0.4680   0.09271
Tree Spe
1          -0.1331   0.09271
2          -0.1375   0.09271
3           1.2257   0.09271

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Leaf Cover
All Pairwise Comparisons among Levels of Site

Site = 1 subtracted from:

Site     Lower    Center     Upper  --------+---------+---------+--------
2       -1.039   -0.7048   -0.3701   (----*-----)
3       -0.463   -0.1280    0.2067             (-----*----)
                                    --------+---------+---------+--------
                                          -0.60      0.00      0.60


Site = 2 subtracted from:

Site     Lower    Center     Upper  --------+---------+---------+--------
3       0.2421    0.5768    0.9114                       (-----*----)
                                    --------+---------+---------+--------
                                          -0.60      0.00      0.60


Tukey Simultaneous Tests
Response Variable Leaf Cover
All Pairwise Comparisons among Levels of Site

Site = 1 subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
2           -0.7048      0.1311    -5.375     0.0001
3           -0.1280      0.1311    -0.976     0.6007

Site = 2 subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
3            0.5768      0.1311     4.399     0.0010

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Leaf Cover
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Tree Spe     Lower    Center     Upper  ------+---------+---------+---------+
2          -0.3391  -0.00438    0.3303  (-----*-----)
3           1.0242   1.35885    1.6935                       (-----*----)
                                    ------+---------+---------+---------+
                                         0.00      0.60      1.20      1.80


Tree Spe = 2 subtracted from:

Tree Spe     Lower    Center     Upper  ------+---------+---------+---------+
3            1.029     1.363     1.698                       (-----*----)
                                    ------+---------+---------+---------+
                                         0.00      0.60      1.20      1.80


Tukey Simultaneous Tests
Response Variable Leaf Cover
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
```

```
2              -0.00438      0.1311   -0.0334     0.9994
3               1.35885      0.1311   10.3643     0.0000

Tree Spe = 2 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
3                 1.363      0.1311     10.40     0.0000
```

General Linear Model: December - Leaf Cover Vs Tree Species, Site

```
Factor     Type Levels Values
Site      fixed      3 1 2 3
Tree Spe  fixed      3 1 2 3

Analysis of Variance for Leaf Cover, using Adjusted SS for Tests

Source          DF     Seq SS     Adj SS     Adj MS       F      P
Site             2     1.3245     1.3245     0.6622    4.27  0.030
Tree Spe         2     2.5945     2.5945     1.2973    8.36  0.003
Site*Tree Spe    4     1.4557     1.4557     0.3639    2.35  0.094
Error           18     2.7917     2.7917     0.1551
Total           26     8.1664

Unusual Observations for Leaf Cover

Obs  Leaf Cover       Fit      SE Fit  Residual   St Resid
 10  -0.69450   0.08145     0.22737  -0.77594     -2.41R
 11   1.22263   0.08145     0.22737   1.14118      3.55R

R denotes an observation with a large standardized residual.

Least Squares Means for Leaf Cover

Site          Mean   SE Mean
1           1.2505    0.1313
2           0.9445    0.1313
3           1.4855    0.1313
Tree Spe
1           0.7896    0.1313
2           1.4725    0.1313
3           1.4185    0.1313

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Leaf Cover
All Pairwise Comparisons among Levels of Site

Site = 1 subtracted from:

Site     Lower    Center     Upper  ------+---------+---------+---------+
2      -0.7799   -0.3060    0.1679  (---------*--------)
3      -0.2390    0.2349    0.7088            (---------*--------)
                                   ------+---------+---------+---------+
                                       -0.50      0.00      0.50      1.00


Site = 2 subtracted from:

Site     Lower    Center     Upper  ------+---------+---------+---------+
3      0.06707    0.5410     1.015                  (---------*--------)
                                   ------+---------+---------+---------+
                                       -0.50      0.00      0.50      1.00


Tukey Simultaneous Tests
Response Variable Leaf Cover
All Pairwise Comparisons among Levels of Site

Site = 1 subtracted from:
```

```
Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
2           -0.3060      0.1856    -1.648     0.2518
3            0.2349      0.1856     1.266     0.4319

Site = 2 subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
3            0.5410      0.1856     2.914     0.0239

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Leaf Cover
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Tree Spe     Lower    Center     Upper  -+---------+---------+---------+-----
2           0.2090    0.6829     1.157                (---------*--------)
3           0.1550    0.6289     1.103              (---------*--------)
                                      -+---------+---------+---------+-----
                                    -0.50      0.00      0.50      1.00


Tree Spe = 2 subtracted from:

Tree Spe     Lower    Center     Upper  -+---------+---------+---------+-----
3          -0.5279  -0.05402    0.4199  (---------*--------)
                                      -+---------+---------+---------+-----
                                    -0.50      0.00      0.50      1.00


Tukey Simultaneous Tests
Response Variable Leaf Cover
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
2                0.6829      0.1856     3.679     0.0047
3                0.6289      0.1856     3.388     0.0088

Tree Spe = 2 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
3              -0.05402      0.1856   -0.2910     0.9545
```

General Linear Model: Methyl Ester Content Vs Tree Species, Date of Collection, Tree/Ground Sample, Site

```
Factor     Type Levels Values
Tree Spe  fixed      3 1 2 3
Date of   fixed      2 1 2
Tree/Gro  fixed      2 1 2
Site      fixed      3 1 2 3

Analysis of Variance for Methyl Ester, using Adjusted SS for Tests

Source                    DF     Seq SS     Adj SS     Adj MS       F      P
Tree Spe                   2     593836     593836     296918  117.28  0.000
Date of                    1     337921     337921     337921  133.47  0.000
Tree/Gro                   1     556774     556774     556774  219.91  0.000
Site                       2      96099      96099      48049   18.98  0.000
Tree Spe*Date of           2      26627      26627      13314    5.26  0.007
Tree Spe*Tree/Gro          2      32073      32073      16037    6.33  0.003
Tree Spe*Site              4      73845      73845      18461    7.29  0.000
Date of*Tree/Gro           1          1          1          1    0.00  0.983
```

Date of*Site	2	20366	20366	10183	4.02	0.022
Tree/Gro*Site	2	21298	21298	10649	4.21	0.019
Tree Spe*Date of*Tree/Gro	2	37547	37547	18773	7.42	0.001
Tree Spe*Date of*Site	4	82330	82330	20582	8.13	0.000
Tree Spe*Tree/Gro*Site	4	80337	80337	20084	7.93	0.000
Date of*Tree/Gro*Site	2	28576	28576	14288	5.64	0.005
Tree Spe*Date of*Tree/Gro *Site	4	41547	41547	10387	4.10	0.005
Error	72	182290	182290	2532		
Total	107	2211468				

Unusual Observations for Methyl Ester

Obs	Methyl Ester	Fit	SE Fit	Residual	St Resid
1	282.669	387.134	29.051	-104.465	-2.54R
2	473.163	387.134	29.051	86.030	2.09R
9	493.982	400.592	29.051	93.390	2.27R
32	698.304	593.052	29.051	105.252	2.56R
35	157.338	248.593	29.051	-91.256	-2.22R
40	239.191	332.720	29.051	-93.530	-2.28R
42	492.181	332.720	29.051	159.460	3.88R
72	340.022	254.649	29.051	85.374	2.08R

R denotes an observation with a large standardized residual.

Least Squares Means for Methyl Ester

	Mean	SE Mean
Tree Spe		
1	262.1	8.386
2	196.1	8.386
3	375.6	8.386
Date of		
1	333.9	6.847
2	222.0	6.847
Tree/Gro		
1	349.7	6.847
2	206.1	6.847
Site		
1	256.0	8.386
2	257.7	8.386
3	320.1	8.386

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Tree Spe	Lower	Center	Upper	----------+---------+---------+-----
2	-94.38	-66.04	-37.69	(-*--)
3	85.17	113.52	141.86	(-*--)
				----------+---------+---------+-----
				0 100 200

Tree Spe = 2 subtracted from:

Tree Spe	Lower	Center	Upper	----------+---------+---------+-----
3	151.2	179.6	207.9	(--*--)
				----------+---------+---------+-----
				0 100 200

Tukey Simultaneous Tests
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Level Tree Spe	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
2	-66.04	11.86	-5.568	0.0000
3	113.52	11.86	9.572	0.0000

```
Tree Spe = 2 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
3                 179.6       11.86     15.14     0.0000

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Date of

Date of = 1 subtracted from:

Date of     Lower    Center     Upper  ---+---------+---------+---------+---
2          -131.2    -111.9    -92.57  (----*----)
                                       ---+---------+---------+---------+---
                                        -120       -80       -40         0


Tukey Simultaneous Tests
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Date of

Date of = 1 subtracted from:

Level       Difference       SE of             Adjusted
Date of       of Means  Difference   T-Value    P-Value
2               -111.9       9.684    -11.55     0.0000

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Tree/Gro

Tree/Gro = 1 subtracted from:

Tree/Gro     Lower    Center     Upper  ---+---------+---------+---------+--
2           -162.9    -143.6    -124.3  (---*---)
                                       ---+---------+---------+---------+--
                                        -150      -100       -50         0


Tukey Simultaneous Tests
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Tree/Gro

Tree/Gro = 1 subtracted from:

Level        Difference       SE of             Adjusted
Tree/Gro       of Means  Difference   T-Value    P-Value
2               -143.6       9.684    -14.83     0.0000

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Site

Site = 1 subtracted from:

Site     Lower    Center     Upper  --------+---------+---------+--------
2       -26.62     1.726     30.07  (-------*--------)
3        35.78    64.123     92.47                    (-------*-------)
                                    --------+---------+---------+--------
                                            0        35        70


Site = 2 subtracted from:

Site     Lower    Center     Upper  --------+---------+---------+--------
3        34.05     62.40     90.74                    (-------*-------)
                                    --------+---------+---------+--------
                                            0        35        70


Tukey Simultaneous Tests
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Site
```

```
Site = 1 subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
2             1.726       11.86    0.1455     0.9884
3            64.123       11.86    5.4068     0.0000

Site = 2 subtracted from:

Level    Difference       SE of             Adjusted
Site       of Means  Difference   T-Value    P-Value
3             62.40       11.86     5.261     0.0000
```

General Linear Model: Site A - Methyl Ester Content Vs Tree Species, Date of Collection – On Ground Samples

```
Factor     Type Levels Values
Tree Spe  fixed      3 1 2 3
Date of   fixed      3 1 2 3

Analysis of Variance for Methyl Ester, using Adjusted SS for Tests

Source             DF     Seq SS     Adj SS     Adj MS       F      P
Tree Spe            2     170855     170855      85428   39.23  0.000
Date of             2      84330      84330      42165   19.36  0.000
Tree Spe*Date of    4      10641      10641       2660    1.22  0.336
Error              18      39193      39193       2177
Total              26     305020

Unusual Observations for Methyl Ester

Obs  Methyl Ester       Fit      SE Fit  Residual   St Resid
 18   340.022   254.649      26.941    85.374      2.24R
 26   112.150   209.346      26.941   -97.196     -2.55R

R denotes an observation with a large standardized residual.

Least Squares Means for Methyl Ester

Tree Spe      Mean   SE Mean
1            110.4     15.55
2            109.4     15.55
3            278.6     15.55
Date of
1            244.6     15.55
2            135.5     15.55
3            118.4     15.55

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Tree Spe     Lower    Center     Upper  --------+---------+---------+-------
2           -57.22    -1.070     55.08   (------*------)
3           112.06   168.210    224.36                    (------*------
                                        --------+---------+---------+-------
                                                0        80       160



Tree Spe = 2 subtracted from:

Tree Spe     Lower    Center     Upper  --------+---------+---------+-------
3           113.1     169.3     225.4                     (------*------
```

```
                                          --------+---------+---------+-------
                                                  0        80       160


Tukey Simultaneous Tests
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
2               -1.070       22.00  -0.04866     0.9987
3              168.210       22.00   7.64694     0.0000

Tree Spe = 2 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
3                169.3       22.00     7.696     0.0000

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Date of

Date of = 1 subtracted from:

Date of     Lower    Center     Upper  -+---------+---------+---------+-----
2          -165.2    -109.1    -52.92     (---------*--------)
3          -182.3    -126.2    -70.03   (--------*--------)
                                       -+---------+---------+---------+-----
                                      -180      -120       -60         0


Date of = 2 subtracted from:

Date of     Lower    Center     Upper  -+---------+---------+---------+-----
3          -73.25    -17.10     39.05                    (--------*--------
                                       -+---------+---------+---------+-----
                                      -180      -120       -60         0


Tukey Simultaneous Tests
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Date of

Date of = 1 subtracted from:

Level       Difference       SE of             Adjusted
Date of       of Means  Difference   T-Value    P-Value
2               -109.1       22.00    -4.959     0.0003
3               -126.2       22.00    -5.736     0.0001

Date of = 2 subtracted from:

Level       Difference       SE of             Adjusted
Date of       of Means  Difference   T-Value    P-Value
3               -17.10       22.00   -0.7775     0.7212
```

General Linear Model: Site A - Methyl Ester Content Vs Tree Species, Date of Collection – On Tree Samples

```
Factor     Type Levels Values
Tree Spe  fixed      3 1 2 3
Date of   fixed      2 1 2

Analysis of Variance for Methyl Ester, using Adjusted SS for Tests
```

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Tree Spe	2	82738	82738	41369	10.79	0.002
Date of	1	114217	114217	114217	29.78	0.000
Tree Spe*Date of	2	56986	56986	28493	7.43	0.008
Error	12	46018	46018	3835		
Total	17	299958				

Unusual Observations for Methyl Ester

Obs	Methyl Ester	Fit	SE Fit	Residual	St Resid
1	282.669	387.134	35.753	-104.465	-2.07R

R denotes an observation with a large standardized residual.

Least Squares Means for Methyl Ester

Tree Spe	Mean	SE Mean
1	262.5	25.28
2	286.5	25.28
3	416.8	25.28
Date of		
1	401.6	20.64
2	242.3	20.64

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Tree Spe	Lower	Center	Upper	--------+---------+---------+-------
2	-71.33	23.98	119.3	(--------*---------)
3	59.00	154.31	249.6	(--------*---------)
				--------+---------+---------+--------
				0 100 200

Tree Spe = 2 subtracted from:

Tree Spe	Lower	Center	Upper	--------+---------+---------+-------
3	35.01	130.3	225.6	(--------*---------)
				--------+---------+---------+-------
				0 100 200

Tukey Simultaneous Tests
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Level Tree Spe	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
2	23.98	35.75	0.6708	0.7844
3	154.31	35.75	4.3159	0.0027

Tree Spe = 2 subtracted from:

Level Tree Spe	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
3	130.3	35.75	3.645	0.0087

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Date of

Date of = 1 subtracted from:

Date of	Lower	Center	Upper	--+---------+---------+---------+----
2	-222.9	-159.3	-95.71	(--------*--------)
				--+---------+---------+---------+----

```
                                        -210      -140       -70         0
```

```
Tukey Simultaneous Tests
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Date of

Date of = 1 subtracted from:

Level       Difference       SE of             Adjusted
Date of       of Means  Difference   T-Value    P-Value
2               -159.3       29.19    -5.457     0.0002
```

General Linear Model: Site B - Methyl Ester Content Vs Tree Species, Date of Collection – On Ground Samples

```
Factor     Type Levels Values
Tree Spe  fixed      3 1 2 3
Date of   fixed      3 1 2 3

Analysis of Variance for Methyl Ester, using Adjusted SS for Tests

Source             DF     Seq SS     Adj SS     Adj MS       F      P
Tree Spe            2       8521       8521       4260    3.39  0.056
Date of             2      40580      40580      20290   16.14  0.000
Tree Spe*Date of    4       9310       9310       2327    1.85  0.163
Error              18      22631      22631       1257
Total              26      81042

Unusual Observations for Methyl Ester

Obs  Methyl Ester       Fit      SE Fit  Residual   St Resid
  8   157.338   248.593      20.472   -91.256     -3.15R
  9   317.822   248.593      20.472    69.229      2.39R

R denotes an observation with a large standardized residual.

Least Squares Means for Methyl Ester

Tree Spe      Mean   SE Mean
1           135.00     11.82
2           127.78     11.82
3           168.55     11.82
Date of
1           192.96     11.82
2           140.17     11.82
3            98.21     11.82

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Tree Spe     Lower    Center     Upper  ---+---------+---------+---------+---
2           -49.88    -7.215     35.45   (---------*----------)
3            -9.11    33.555     76.22             (---------*----------)
                                       ---+---------+---------+---------+---
                                         -40         0        40        80


Tree Spe = 2 subtracted from:

Tree Spe     Lower    Center     Upper  ---+---------+---------+---------+---
3           -1.898     40.77     83.44              (---------*----------)
                                       ---+---------+---------+---------+---
                                         -40         0        40        80
```

```
Tukey Simultaneous Tests
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
2               -7.215       16.72   -0.4316     0.9029
3               33.555       16.72    2.0075     0.1391

Tree Spe = 2 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
3                 40.77       16.72     2.439     0.0624

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Date of

Date of = 1 subtracted from:

Date of     Lower    Center     Upper  -----+---------+---------+---------+-
2           -95.5    -52.80    -10.13            (----------*---------)
3          -137.4    -94.76    -52.09   (---------*----------)
                                       -----+---------+---------+---------+-
                                          -120       -80       -40         0


Date of = 2 subtracted from:

Date of     Lower    Center     Upper  -----+---------+---------+---------+-
3          -84.63    -41.96    0.7087               (----------*---------)
                                       -----+---------+---------+---------+-
                                          -120       -80       -40         0


Tukey Simultaneous Tests
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Date of

Date of = 1 subtracted from:

Level       Difference       SE of             Adjusted
Date of       of Means  Difference   T-Value    P-Value
2              -52.80       16.72    -3.159     0.0143
3              -94.76       16.72    -5.669     0.0001

Date of = 2 subtracted from:

Level       Difference       SE of             Adjusted
Date of       of Means  Difference   T-Value    P-Value
3              -41.96       16.72    -2.510     0.0543
```

General Linear Model: Site B - Methyl Ester Content Vs Tree Species, Date of Collection – On Tree Samples

```
Factor     Type Levels Values
Tree Spe  fixed      3 1 2 3
Date of   fixed      2 1 2

Analysis of Variance for Methyl Ester, using Adjusted SS for Tests

Source             DF     Seq SS     Adj SS     Adj MS       F      P
Tree Spe            2     303403     303403     151702   72.22  0.000
Date of             1      39401      39401      39401   18.76  0.001
Tree Spe*Date of    2      88320      88320      44160   21.02  0.000
```

```
Error              12      25207      25207       2101
Total              17     456332

Unusual Observations for Methyl Ester

Obs  Methyl Ester       Fit      SE Fit  Residual   St Resid
  8   698.304   593.052      26.461   105.252      2.81R

R denotes an observation with a large standardized residual.

Least Squares Means for Methyl Ester

Tree Spe      Mean   SE Mean
1            384.1     18.71
2            175.2     18.71
3            487.3     18.71
Date of
1            395.7     15.28
2            302.1     15.28

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Tree Spe     Lower    Center     Upper  ----+---------+---------+---------+--
2          -279.4    -208.9    -138.3  (---*--)
3            32.7     103.3     173.8                (--*---)
                                       ----+---------+---------+---------+--
                                         -200         0       200       400


Tree Spe = 2 subtracted from:

Tree Spe     Lower    Center     Upper  ----+---------+---------+---------+--
3            241.6     312.1     382.7                          (---*--)
                                       ----+---------+---------+---------+--
                                         -200         0       200       400


Tukey Simultaneous Tests
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
2               -208.9       26.46    -7.893     0.0000
3                103.3       26.46     3.902     0.0055

Tree Spe = 2 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
3                312.1       26.46     11.80     0.0000

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Date of

Date of = 1 subtracted from:

Date of     Lower    Center     Upper  ------+---------+---------+---------+
2          -140.6    -93.57    -46.50   (-----------*----------)
                                       ------+---------+---------+---------+
                                           -120       -80       -40         0


Tukey Simultaneous Tests
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Date of
```

```
Date of = 1 subtracted from:

Level       Difference       SE of             Adjusted
Date of       of Means  Difference   T-Value    P-Value
2               -93.57       21.61    -4.331     0.0010
```

General Linear Model: Site C - Methyl Ester Content Vs Tree Species, Date of Collection – On Ground Samples

```
Factor     Type Levels Values
Tree Spe  fixed      3 1 2 3
Date of   fixed      3 1 2 3

Analysis of Variance for Methyl Ester, using Adjusted SS for Tests

Source             DF     Seq SS     Adj SS     Adj MS       F      P
Tree Spe            2     112015     112015      56007   14.07  0.000
Date of             2     206847     206847     103424   25.99  0.000
Tree Spe*Date of    4      28310      28310       7077    1.78  0.177
Error              18      71632      71632       3980
Total              26     418804

Unusual Observations for Methyl Ester

Obs  Methyl Ester       Fit      SE Fit  Residual   St Resid
  3   492.181   332.720      36.421   159.460      3.10R

R denotes an observation with a large standardized residual.

Least Squares Means for Methyl Ester

Tree Spe      Mean   SE Mean
1            221.0     21.03
2            149.5     21.03
3            307.0     21.03
Date of
1            349.0     21.03
2            174.7     21.03
3            153.8     21.03

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Tree Spe     Lower    Center     Upper  ---+---------+---------+---------+---
2           -147.5    -71.54     4.368   (-----*-----)
3             10.1     86.01   161.919               (-----*-----)
                                       ---+---------+---------+---------+---
                                        -120         0       120       240


Tree Spe = 2 subtracted from:

Tree Spe     Lower    Center     Upper  ---+---------+---------+---------+---
3            81.64     157.6     233.5                     (-----*-----)
                                       ---+---------+---------+---------+---
                                        -120         0       120       240


Tukey Simultaneous Tests
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
2               -71.54       29.74    -2.406     0.0666
```

```
3                86.01       29.74     2.892     0.0251

Tree Spe = 2 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
3                157.6       29.74     5.298     0.0001

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Date of

Date of = 1 subtracted from:

Date of     Lower    Center     Upper  --------+---------+---------+--------
2          -250.3    -174.4     -98.5     (-------*------)
3          -271.1    -195.2    -119.3   (------*-------)
                                       --------+---------+---------+--------
                                             -200      -100         0


Date of = 2 subtracted from:

Date of     Lower    Center     Upper  --------+---------+---------+--------
3          -96.75    -20.84     55.07                   (-------*-------)
                                       --------+---------+---------+--------
                                             -200      -100         0


Tukey Simultaneous Tests
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Date of

Date of = 1 subtracted from:

Level       Difference       SE of             Adjusted
Date of       of Means  Difference   T-Value    P-Value
2              -174.4       29.74    -5.864     0.0001
3              -195.2       29.74    -6.564     0.0000

Date of = 2 subtracted from:

Level       Difference       SE of             Adjusted
Date of       of Means  Difference   T-Value    P-Value
3              -20.84       29.74   -0.7007     0.7661
```

General Linear Model: Site C- Methyl Ester Content Vs Tree Species, Date of Collection – On Tree Samples

```
Factor     Type Levels Values
Tree Spe  fixed      3 1 2 3
Date of   fixed      2 1 2



Analysis of Variance for Methyl Ester, using Adjusted SS for Tests

Source             DF     Seq SS     Adj SS     Adj MS       F      P
Tree Spe            2     123800     123800      61900   45.56  0.000
Date of             1      30336      30336      30336   22.33  0.000
Tree Spe*Date of    2      24489      24489      12244    9.01  0.004
Error              12      16304      16304       1359
Total              17     194929

Least Squares Means for Methyl Ester

Tree Spe      Mean   SE Mean
1            396.4     15.05
2            269.0     15.05
```

```
3            469.7     15.05
Date of
1            419.4     12.29
2            337.3     12.29

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Tree Spe     Lower    Center     Upper  ------+---------+---------+---------+
2          -184.2    -127.4    -70.69   (---*----)
3            16.6      73.3    130.03                (----*----)
                                       ------+---------+---------+---------+
                                           -120         0       120       240


Tree Spe = 2 subtracted from:

Tree Spe     Lower    Center     Upper  ------+---------+---------+---------+
3            144.0     200.7     257.5                           (----*---)
                                       ------+---------+---------+---------+
                                           -120         0       120       240


Tukey Simultaneous Tests
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Tree Spe

Tree Spe = 1 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
2               -127.4       21.28    -5.988     0.0002
3                 73.3       21.28     3.444     0.0125

Tree Spe = 2 subtracted from:

Level        Difference       SE of             Adjusted
Tree Spe       of Means  Difference   T-Value    P-Value
3                200.7       21.28     9.432     0.0000

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Date of



Date of = 1 subtracted from:

Date of     Lower    Center     Upper  -----+---------+---------+---------+-
2          -120.0    -82.11    -44.25   (----------*---------)
                                       -----+---------+---------+---------+-
                                          -105       -70       -35         0


Tukey Simultaneous Tests
Response Variable Methyl Ester
All Pairwise Comparisons among Levels of Date of

Date of = 1 subtracted from:

Level       Difference       SE of             Adjusted
Date of       of Means  Difference   T-Value    P-Value
2              -82.11       17.38    -4.725     0.0005
```

General Linear Model: Mean Methanol Emission Vs Crop Species, Adsorbent

```
Factor     Type Levels Values
Species   fixed      3 1 2 3
Adsorben  fixed      2 1 2

Analysis of Variance for Mean Met, using Adjusted SS for Tests

Source             DF     Seq SS     Adj SS     Adj MS       F      P
Species             2     542.59     542.59     271.30   20.19  0.000
Adsorben            1       2.48       0.92       0.92    0.07  0.794
Species*Adsorben    2      50.76      50.76      25.38    1.89  0.162
Error              48     644.85     644.85      13.43
Total              53    1240.67

Unusual Observations for Mean Met

Obs  Mean Met       Fit      SE Fit  Residual   St Resid
 35   18.3725   10.8097      1.1591    7.5628      2.17R
 36   19.8485   10.8097      1.1591    9.0388      2.60R
 45   15.8550    7.8565      1.1591    7.9985      2.30R
 46   15.8542    7.8565      1.1591    7.9977      2.30R

R denotes an observation with a large standardized residual.

Least Squares Means for Mean Met

Species       Mean   SE Mean
1            1.979    0.9796
2            3.527    0.8196
3            9.333    0.8196
Adsorben
1            5.079    0.7154
2            4.814    0.7154

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Mean Met
All Pairwise Comparisons among Levels of Species

Species = 1 subtracted from:

Species     Lower    Center     Upper  -----+---------+---------+---------+-
2          -1.540     1.548     4.637   (-------*--------)
3           4.266     7.354    10.443                  (--------*--------)
                                       -----+---------+---------+---------+-
                                           0.0       3.5       7.0      10.5


Species = 2 subtracted from:

Species     Lower    Center     Upper  -----+---------+---------+---------+-
3           3.003     5.806     8.609               (-------*-------)
                                       -----+---------+---------+---------+-
                                           0.0       3.5       7.0      10.5


Tukey Simultaneous Tests
Response Variable Mean Met
All Pairwise Comparisons among Levels of Species

Species = 1 subtracted from:

Level       Difference       SE of             Adjusted
Species       of Means  Difference   T-Value    P-Value
2                1.548       1.277     1.212     0.4518
3                7.354       1.277     5.758     0.0000

Species = 2 subtracted from:

Level       Difference       SE of             Adjusted
Species       of Means  Difference   T-Value    P-Value
3                5.806       1.159     5.009     0.0000
```

```
Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Mean Met
All Pairwise Comparisons among Levels of Adsorben

Adsorben = 1 subtracted from:

Adsorben     Lower    Center     Upper  ----------+---------+---------+------
2           -2.299   -0.2650     1.769   (-----------------*----------------)
                                        ----------+---------+---------+------
                                               -1.2       0.0       1.2


Tukey Simultaneous Tests
Response Variable Mean Met
All Pairwise Comparisons among Levels of Adsorben

Adsorben = 1 subtracted from:

Level        Difference       SE of             Adjusted
Adsorben       of Means  Difference   T-Value    P-Value
2               -0.2650       1.012   -0.2619     0.7945
```

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