Ph.D. THESIS

A STUDY OF PHENOLIC-CARBOHYDRATE LINKAGES IN THE GRAMINEAE.

BY

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DECLARATION.

I hereby declare that this thesis has been composed by myself and has not been presented or accepted in any previous application for a degree and is a record of work carried out by myself unless stated otherwise. All quotations have been distinguished by quotation marks and all sources of information acknowledged.

G.Wallace.

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ABSTRACT.

The complex phenolic polymer lignin is generally accepted as having a strong influence over the biodegradability of plant cell walls. The precise mechanisms of this phenomenon, however, are not well understood and a number of factors may be of particular importance *i.e* the size and structure of the lignin molecules, their distribution within the cell wall and their interactions with other cell wall components as lignin-carbohydrate complexes. To date, relatively little is known about these factors. The purpose of this work was to study the interactions between phenolics and carbohydrate in graminaceous cell walls.

Barley straw (*Hordeum vulgare* c.v Golden Promise) and a 'non-mesophyll' preparation of perennial ryegrass (*Lolium perenne* c.v. Perma) cell walls were subjected to the fractionation scheme demonstrated in Figure 1. The carbohydrate and phenolic components of the resulting fractions were analysed by a number of 'classical' wet chemical techniques and several physical methods (Pyrolysis-MS (and GC-MS); NMR and Infrared spectroscopy).

It was found that oxalic acid hydrolysis solubilised much of the arabinose and ferulic acid content of both. A high proportion of the phenolic content (phenolic acids and lignin) was solubilised from ryegrass, whereas much less was solubilised from barley straw. Fragments, consisting of ferulic acid ester linked through the *O*-5 of arabinose and *p*-coumaric acid ester linked to arabinose were isolated and characterised.

Extraction of the hydrolysed materials with dimethyl sulphoxide (DMSO) was found to solubilise a high molecular weight lignin-

carbohydrate complex, the carbohydrate component consisting, predominantly, of a ß 1-4 xylan with small amounts of mixed linked ß D-glucan. The LCC's differed considerably in their response to alkali hydrolysis, suggesting differences in content of ester and ether linked phenolics.

Enzymic hydrolysis of the residues with a commercial cellulase preparation, 'Driselase' was then found to solubilise much of the remaining sugar to leave a lignin 'core'. Solid state NMR also suggested differences in ester/ether linked phenolics between barley and ryegrass.

Analytical pyrolysis techniques were then used for further structural analysis. Time resolved platinum filament pyrolysis-mass spectrometry (electron impact) suggested differences in the structure of the lignin moieties in the DMSO soluble and residue fractions. These differences were characterised by thioacidolysis analysis, which showed that the DMSO soluble lignin (type 1 lignin) consists of either small and/or highly branched molecules with a high condensed lignin content (90%), and is associated with a pentosan fraction whereas the residual lignin (type 2 lignin), in ryegrass at least, is either large and/or straight chained lignin, have a much lower condensed lignin content (59%) and is associated with a pentosan/hexosan fraction. It is also suggested that two different xylan components are present in the starting materials.

It was suggested that the two different lignin moieties may have originated from different areas within the cell wall, type 1 lignin originating from the secondary cell wall and type 2 lignin from primary layers of secondary thickened cell walls.

The distribution of the major phenolic and carbohydrate components of the cell walls through the fractionation scheme is simplified in table 1.

TABLE 1.

FRACTION	BARLEY STRAW	RYEGRASS
UNTREATED	2% Ara, 20% Xyl 0.29% *p*-CA, 0.22% FA 9.5% lignin	4.7% Ara, 22% Xyl 0.4% *p*-CA, 0.68% FA 4.7% lignin
OXALATE HYDROLYSED	Most of original Xyl, FA, *p*-CA and lignin. Little Ara	Most Xyl, some Ara little FA & *p*-CA
OXALATE MeOH	Most of original Ara and solubilised phenolics	Similar to Barley straw but higher phenolic acid content (esp FA 2.6%)
OXALATE H_2O	Essentially the same as MeOH but more Glu, less xylose and phenolics. Xyl mostly oligomeric.	Very similar to barley straw but a slightly higher Ara content.
DMSO SOLUBLE	Mostly β 1-4 xylan little phenolic acid (mostly *p*-CA) high lignin (28.6%). Lignin highly condensed, poorly branched (or high M_{w-t}) S/G 0.79.	Mostly β 1-4 xylan higher phenolic acid (1% FA, 0.6% *p*-CA) low lignin. Lignin highly condensed and highly branched (or low M_{w-t}) S/G 0.71
DMSO RESIDUE	Very similar to oxalate hydrolysed.	Very similar to oxalate hydrolysed.
DRISELASE SOLUBLE	36% of DMSO residue some Xyl and Glu little phenolics.	76% of DMSO residue some Xyl and Glu little phenolics.

DRISELASE RESIDUE	40% of original. Mostly Xyl and Glu most of original phenolics	12.4% of original Mostly Xyl and Glu very little of original phenolics. Lignin poorly branched (or high M_{w+}) poorly condensed. S/G 0.5.

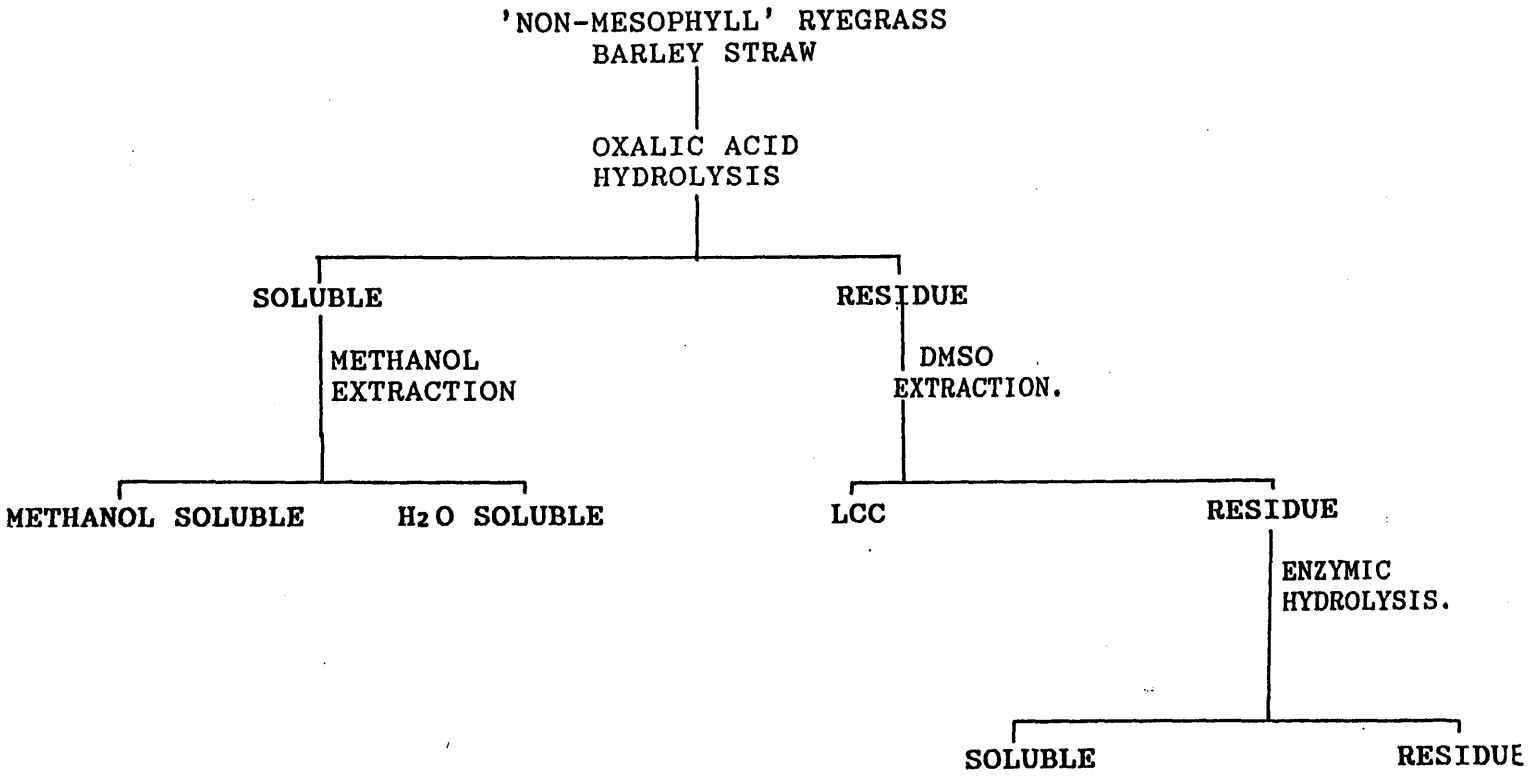

Figure 1 Fractionation scheme.

CONTENTS.

ABBREVIATIONS.

Ar - Aryl

Ara - Arabinose.

BS - Barley straw.

BSTFA - bis-(trimethylsilyl)trifluoroacetamide.

p-CA - *p*-Coumaric acid

CI - Chemical ionisation.

COSY - Comparative spectroscopy.

CP/MAS - Cross polarization and magic angle spinning.

d - Daltons.

2,4-D - 2,4-Dichlorophenoxyacetic acid.

DFA - Diferulic acid.

DMSO - Dimethyl sulphoxide.

DP - Degree of polymerisation.

EI - Electron impact ionisation.

ESR - Electron spin resonance.

FA - Ferulic acid.

f - Furanose.

Gal - Galactose.

GalCOOH - Galacturonic acid.

Glu - Glucose.

GluCOOH - Glucuronic acid.

G - Guaiacyl.

G.C - Gas chromatography.

H - *p*-Hydroxyphenyl.

HPLC - High performance liquid chromatography.

i.d - Internal diameter.

I.R - Infrared.

L.C - Liquid chromatography.

LCC - Lignin-carbohydrate complex.

Man - Mannose.

Me - Methyl.

MeOH - Methanol.

MIR - Mid infrared.

M.S - Mass spectrometry.

MWL - Milled wood lignin.

NIR - Near infrared.

NMR - Nuclear magnetic resonance.

OD - Optical density

Pa - Pascals.

PC - Paper chromatography.

p - Pyranose.

Py - Pyrolysis.

Rha - Rhamnose.

rf - Response factor.

RG - Ryegrass.

RG I & II - Rhamnogalacturonan I & II.

RRT - Relative retention time

RT - Retention time.

S - Syringyl.

SIM - selected ion monitoring.

T - Tesla.

TA - Truxillic acid.

TEM - Transmission electron microscopy.

TMS - Trimethyl silyl.

TLC - Thin layer chromatography.

TMAH - Tetra methyl ammonium hydroxide.

UNME - Unmethylated.

U.V - Ultra violet.

V_o - Void volume.

V_I - Inclusion volume.

Xyl - Xylose.

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1.0 INTRODUCTION.

1.1 GENERAL INTRODUCTION.

The plant cell wall is a complex amorphous matrix of neutral and acidic polysaccharides surrounding a "skeleton" of cellulose microfibrils (Fry, 1986). It acts as a barrier to infection (Bell, 1981) and degradation and also helps the cell, and plant, maintain its shape. It controls the rate of growth of the plant (McNeil *et al.*, 1984) and is a basic source of energy and fibre in the food chain (Evans *et al.*, 1984). For these reasons, a thorough knowledge of the structure and chemistry of the plant cell wall is essential (Darvill *et al.*, 1980).

At present, our knowledge of the chemistry of cell walls is restricted to the structure and physical properties of individual components e.g the hemicelluloses, lignin, cellulose and pectins and relatively little is known about how these components are interconnected within the cell wall (Fry, 1986). Many of the problems associated with answering such questions, however, may now be overcome with the advent of improvements in analytical methodology and the application of high powered analytical and chromatographic techniques (Himmelsbach, 1989; Boon, 1989; Chesson & Murison, 1989).

Alkali has been used to increase the digestibility of plant feed stuffs since the beginning of this century (Kellner & Kohler, 1900, for original reference see Tenrud, 1987). The precise

mechanisms of this phenomenon have been a controversial subject for many years and have only, relatively, recently come to light.

Alkaline treatment has been shown to cause a wide range of effects on the cell wall e.g solubilisation of silica, hemicellulose and phenolics and decreasing the crystallinity of cellulose (Jackson, 1977; Evans, 1979). Only the solubilisation of phenolics has been shown to have a high correlation with increasing cell wall digestibility (Chesson, 1981).

The importance of phenolics (phenolic acids and lignin) in limiting cell wall digestibility is now generally accepted as beyond doubt (Brice & Morrison, 1982; Chesson, 1988). The most important questions to be answered now, are those pertaining to the precise nature, and extent of these linkages within cell walls.

The purpose of this research was to study the nature, degree and distribution of phenolic-carbohydrate linkages in Graminaceous cell walls.

The following sections will briefly describe many of the techniques used in cell wall studies, and our present knowledge of cell wall structure.

Botanical taxonomy divides the angiosperms (flowering plants) into two subclasses, the monocotyledons (monocots) and dicotyledons (dicots). As this project was concerned with the Gramineae (grasses and cereals), a subgroup of the monocots, the following description of the structure of cell walls will concentrate primarily on those of the monocots. However, as much of our present knowledge and

understanding of cell walls has stemmed from research on dicots, the structure and functions, where known, of the cell wall components of both will be presented.

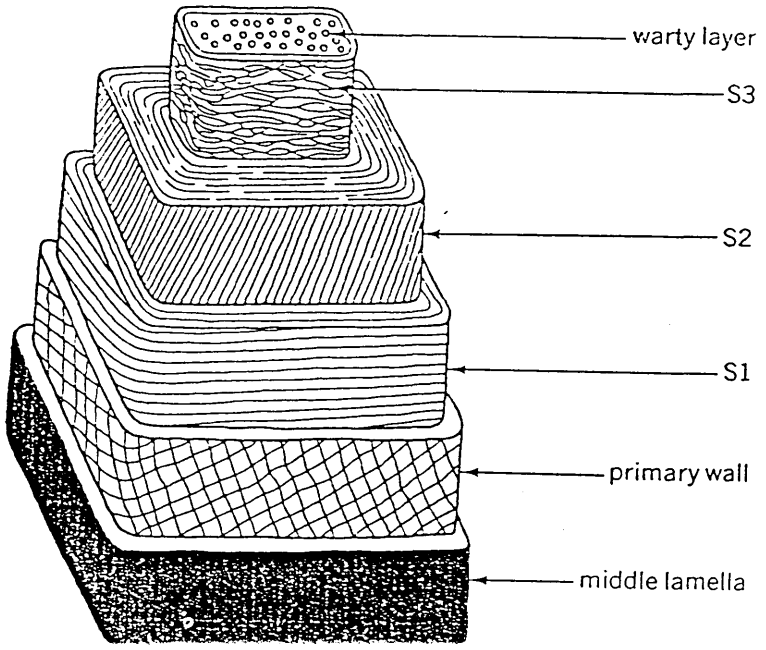
As this project was primarily concerned with phenolic-carbohydrate linkages and their possible role in the limitation of cell wall digestibility, these subjects will receive special consideration.

1.2 CELL WALL ULTRASTRUCTURE AND DEVELOPMENT.

Histological examination of plant material clearly reveals a variation in cell wall types. This variation can be simply classified into primary and secondary cell walls.

As the name implies, primary walls are those formed first and are found on actively growing undifferentiated cells. The primary wall consists of hemicellulose and randomly arranged cellulose fibrils which are connected to each other, in dicots, via an intercellular, pectin rich region called the middle lamella (Nordkvist, 1987). As the cell differentiates the cell wall undergoes secondary thickening to produce a secondary cell wall and it is at this point that lignification begins (Selvendran, 1983). The newly formed wall material is deposited on the inside of the primary wall and has a more complex structure (Esau, 1977). (Figure 1.2.1).

Figure 1.2.1 General scheme of the structure of the plant cell wall.



As can be seen from figure 1.2.1, the secondary wall is a multilayered structure with each layer having a different orientation of its cellulose fibrils (Higuchi, 1981). The S3 layer is often considered distinct enough from the S1 and S2 to be called the tertiary layer (Esau, 1977). On the inside of the S3 layer there is another much thinner layer which can often be easily overlooked. This is the so-called 'warty layer' and is thought to be composed of remnants of the protoplast (Engels & Brice, 1985). This has been found to be particularly resistant to celluloytic attack and could be of considerable importance in cell wall degradation.

1.3 CELL WALL METHODOLOGY.

In order to understand the techniques used and difficulties encountered in cell wall research it was felt necessary to discuss some of the old techniques and some of the newer methods now available for cell wall studies.

1.3.1 Cell Wall Preparation.

The growing plant is a complex mixture of different cell types and consequently cell wall types. The different cell wall types each contain their own relative concentrations and, possibly, types of individual components. In order to fully understand the significance of these components in relation to the plant itself and degradation in particular, we must have means whereby we can specify the cell wall types that a component has been isolated from. Care must also be taken in knowing the cultivar, the stage of growth, the state of the plant, the growing conditions and the history between harvesting and analysis (Wilkie, 1979). Cell culture techniques have been widely used for a number of species and have yielded valuable results (Bauer *et al.*, 1973: Fry, 1982, 1983, 1987: McNeil *et al.*, 1980: Kieliszewski & Lamport, 1987). One significant problem in cell culture techniques however is the difficulty in obtaining a pure cell line with identical secondary cell walls, therefore this method has only been of real value for primary cell wall structure. Another, more widely used approach, is to actually isolate the cell walls of

interest from whole plant material. A number of approaches can be taken in this respect e.g microdissection of plant material (Hinton, 1947). This technique is severely restricted by the quantities of material needed for an extensive study. A mixture of sieving and differential centrifugation is a more common approach and has been widely used to good effect (Gordon & Bacon, 1981: Gordon *et al.*, 1985: Lomax *et al.*, 1983: Chesson *et al.*, 1985: Wilson *et al.*, 1988: Clements, 1979: Stevens, 1973: Mares & Stone, 1973: Bacic & Stone, 1981).

1.3.2 Cell Wall Fractionation.

Much of our classification of cell wall components comes from their fractionation procedures i.e different components extracted by the same technique tend to be classified together. A number of different solvents have been used for cell wall fractionations the most common being water, alkali and chelating agents such as EDTA. More recently however, as more questions on how the cell wall polymers are put together are being asked, a wide variety of extraction solvents are now being used. This strategy involves using specific reagents to extract material bound by specific bond types e.g specific enzymes, 8M urea or guanidine hydrochloride to break hydrogen bonds or hydrophobic interactions and mild acid treatment to cleave weak glycosidic linkages. This whole subject has been extensively discussed by Fry, (1986) who emphasises the questions a researcher must ask himself prior to fractionation.

Once isolated, the cell wall components can be separated and purified, in preparation for structural analysis, by a wide variety of chromatographic techniques. Gel filtration is one of the most common methods applied here and can rapidly yield relatively large amounts of 'purified' material. Adsorption of some cell wall components onto the gel, especially those containing phenolics (Sarkanen *et al.*, 1981), is a common experience and care should be taken before applying the sample. Other methods commonly employed are selective precipitation (Redgewell & Selvendran, 1986: Yamaoka & Chiba, 1983: Meier, 1965: Gaillard, 1961), ion exchange chromatography (Darvill *et al.*, 1980) and membrane ultra filtration (Conchie *et al.*, 1988).

1.3.3 Chemical Structural Analysis.

1.3.3.1 Polysaccharides.

The monomer composition of an isolated and 'purified' polysaccharide can simply be determined by GC, usually as their alditol acetates (Blakeney *et al.*, 1983). For GC analysis uronic acids must be reduced prior to GC analysis (York *et al.*, 1985). However due to the notorious difficulties involved with this, it is often much simpler to determine total uronides colorimetrically (Blumenkrantz & Asboe-Hansen, 1973).

The next step in polysaccharide analysis is to determine the linkages present by methylation analysis, usually by the Hakomori technique (Hakomori, 1964). The free hydroxyl groups on the

polysaccharide are ionised then methylated by methyl iodide, the polysaccharide is then hydrolysed into the monomers, acetylated and analysed by GC. The positions of the methyl groups on the monomers tell us the linkage positions in the polysaccharide. For more detailed analysis of the arrangement of the monomers in the polysaccharide a mixture of techniques e.g mild acid hydrolysis, enzymic hydrolysis and periodate oxidation, with characterisation of the products are needed (Fincher & Stone, 1986).

Specific substituents can be analysed by a number of techniques e.g acetyl groups (Bethge & Lindstrom, 1973), phenolic acids (Hartley & Buchan, 1979: Salmonsson *et al.*, 1978) and methyl esters (Wood & Siddiqui, 1971). The positions of alkali labile substitutions on the polysaccharides can also be determined by acetalation/methylation analysis (Lomax *et al.*, 1983).

1.3.3.2 Lignin.

Due to the enormous complexity of bonding patterns in lignin, it is extremely difficult to structurally analyse lignins, by wet chemical means, to the same extent that we can for polysaccharides. For quantitative analysis of total lignin a number of techniques are available such as permanganate oxidation (Erickson *et al.*, 1973), acetyl bromide determination (Morrison, 1972), Klason lignin (Effland, 1977) and Christian lignin (Christian, 1971). It should be noted that each method has its own limitations and difficulties and care should be taken in choosing any particular method. Structural studies are much more

limited and few methods are available. One of the most common is nitrobenzene oxidation (Chang & Allen, 1971) which cleaves ether bonds to yield a variety of aldehydes which can then be used to give us a syringyl, guaiacyl and *p*-coumaryl content. Nitrobenzene oxidation abounds with problems and difficulties, such as low yields of products, phenolic acids are included as their parent aldehydes and the propyl side chain is cleaved (Lapierre *et al.*, 1989). A relatively new technique, thioacidolysis (Lapierre *et al.*, 1985) which specifically cleaves alkyl aryl ether bonds overcomes many of these problems and promises to be an important technique. However it must be remembered that thioacidolysis and nitrobenzene oxidation measure only the uncondensed component of lignin.

1.3.4 Physical Techniques In Structural Analysis.

1.3.4.1 Microscopy.

The microscope has been an invaluable and widely used tool for many years in cell wall studies. It has been used in the study of the breakdown of cell walls in the rumen (Akin, 1986) the associations between the microorganisms and the cell wall (Grenet, 1989), and in morphological studies (Esau, 1977).

The microscope can also be used for the localisation of specific components. A variety of histological stains have been available for many years (Akin, 1989). The use of stains in localising

polysaccharides, however, is rather more difficult. Problems associated with conventional staining techniques can be overcome with two relatively new techniques, both using a similar approach. Perhaps the more common of these methods is the use of gold labelled enzymes. An enzyme specific to the cell wall component of interest is purified and labelled with gold, then applied to a cell wall preparation and studied by transmission electron microscopy (TEM). This has been successfully applied to a variety of polysaccharides and cell types (Ruel & Joseleau, 1984).

The second method is to raise specific antibodies against the material of interest and label the antibodies with gold. This has recently been applied to rhamnogalacturonan I and xyloglucan in maize (Moore & Staehelin, 1988). It should be noted that great care has to be taken in defining the specificity of the antibodies.

1.3.4.2 Infrared Spectroscopy.

When electromagnetic radiation in the infrared (IR) region is passed through a sample, some of the radiation is absorbed, causing the molecules to vibrate. These absorption bands are characteristic for different functional groups. Therefore an IR spectrum can give us a considerable amount of information about the molecular structure of a given sample. To date, two regions of the IR spectrum have been used in cell wall studies, mid (MIR) and near IR (NIR).

MIR is probably the better known technique and has been used as a routine tool in organic chemistry for many years. In general, it covers the region 2.5-50 μm and, in experienced hands, can yield valuable structural imformation. A variation of classical MIR, multiple reflectance MIR, has recently been applied to rumen digested cereal straws to study changes in surface structure (Russell *et al.*, 1988).

NIR is a comparatively lesser known technique and covers the region 1100-2500 nm (Murray, 1987). The spectra from NIR tend to be somewhat smooth and featurless, and precise assignment of absorption bands is somewhat tentative (Himmelsbach, 1989), therefore it is obviously of limited use in structural studies. However it has found a practical use in evaluating feedstuffs and in following trends, by the mathematical manipulation of a large number of samples (Russell *et al.*, 1989).

1.3.4.3 NMR Spectroscopy.

The nucleus of a hydrogen atom acts as a spinning magnet. When an external magnetic field is applied, the nucleus can take up one of two positions with respect to the applied field, aligned either with (low energy), or against (high energy) the applied field. Under the influence of the external magnetic field the nucleus will 'precess' around the axis of the applied field. If a precessing nucleus is exposed to radio frequency energy of the correct frequency, a low energy nucleus can absorb this energy and jump to the higher energy state. The frequency required to make the jump depends on the immediate environment of the nucleus. Therefore when samples are scanned with a range of frequencies a characteristic range of absorbtions can be detected. In practice it is easier to hold at a steady frequency and alter the magnetic field until each nucleus reaches the applied frequency and therefore comes to resonance (Kemp, 1987).

Due to the laws of quantum mechanics only nuclei with a spin quantum number greater than 1 can be used for NMR, the most commonly used being ^{1}H and ^{13}C. NMR has been used to good effect by organic chemists for some time for the elucidation of molecular structures and is now an indispensable tool to the organic chemist.

The technique was quickly taken up by lignin and carbohydrate chemists and has given considerable insight into some of the finer structure of isolated cell wall polysaccharides (Chanzy *et al*, 1987) and helped unravel some of the mysteries of lignin structure (Nimz, 1974). Classical NMR however has at least two major disadvantages, 1)

High complexity of spectra from complex polymers and 2) it could only be used on soluble cell wall fragments.

With the advent of more and more powerful machines and their increased commercial availability and a more routine use of two dimensional techniques (Himmelsbach, 1989), many of the problems of structural complexity can be overcome.

Solid state NMR (Stejskal & Schaefer, 1975) is a relatively new technique which overcomes the problem of only being able to study isolated materials and study them *in situ*. To date, the technique has not been extensively used on cell walls as resolution is poor due to dipolar broadening (Sanders & Hunter, 1987) and precise assignment is a problem, however with future use and developments, many of these problems may be overcome.

1.3.4.4 Pyrolysis.

Pyrolysis (Py) is a technique whereby complex materials such as cell walls, are thermally dissociated into small, volatile, easily analysed fragments, which can then be studied by either gas chromatography (GC) or mass spectrometry (MS).

The key to good pyrolysis is the ability to dissociate the samples in a controlled and reproducible manner (Irwin, 1982). This can be done in a number of ways but two of the most common are Curie point Py and platinum filament Py.

In Curie point Py, a ferromagnetic wire is induced to its Curie point by a high frequency coil. The rapid heating rates and reproducibility of this method make it highly advantageous over most other methods (Boon, 1989). In Py-MS, the high frequency coil can disturb the electromagnetic fields in the ion source, so pyrolysis has to be performed further away thus causing problems with the transfer of higher molecular weight fragments. This can be overcome by using platinum filament Py whereby a platinum filament is resistively heated directly inside the ion source (Genuit & Boon, 1987).

Once the sample has been pyrolysed, the fragments can then be either fed directly into an MS or a GC (with characterisation of the peaks by MS).

For MS analysis, the fragments can be ionised in a variety of ways e.g electron impact (EI), chemical ionisation (CI), photoionisation and field ionisation depending on the materials and components of interest. For cell wall studies more than one method may be used for optimum

analysis, as some types of ions may be fragmented too much by one method and another, softer, method may be more suitable to obtain a molecular ion (Boon, 1989).

Pyrolysis has been applied to a number of cell wall studies, (Hartley & Haverkamp, 1984: Pouwels *et al*, 1987: Saiz-Jimenez *et al.*, 1987: Genuit & Boon, 1987: Faix *et al.*, 1987: Ohinishi & Kato, 1977: Windig *et al.*, 1983).

Identification of many of the ions (especially higher molecular weight ones) by comparison with standards still tends to be somewhat tentative and tandem MS is needed for positive identification. In tandem MS a second spectrometer is attached to the first in such a way that any ion may be passed through to the second spectrometer in order to obtain a fragmentation pattern for that ion.

The advantages of pyrolysis as a routine analytical tool are the rapid and simple sample preparation, small sample size, the ease with which most systems can be automated and the rapid analysis time. Serious disadvantages however do exist, in the high cost of very specialised, complex and delicate equipment and the need for highly trained staff (Meuzelaar *et al.*, 1984).

1.4 CELL WALL STRUCTURE.

1.4.1 Cellulose.

Cellulose is the most abundant cell wall polymer and makes up the microfibrillar component of the cell wall. Cellulose fibrils are composed of long chains of ß 1-4 linked glucosyl residues arranged with a twofold screw axis *i.e* two glucosyl residues per turn of helix. It would appear that primary and secondary cell walls have different types of cellulose fibril. Primary fibrils are thought to be made up of 60-70 glucan chains and have a diameter of 4.5-8.5 nm (Preston, 1974). Secondary cell wall fibrils, however, are much thicker (Muhlethaler, 1967) and may result from the aggregation of primary fibrils (Darvill *et al.*, 1980). The degree of polymerisation (DP) is also different in secondary and primary fibrils. Primary cellulose seems to have a non-uniform DP of either under 500 or between 2,500 and 4,500 (Blaschek *et al.*, 1982). Secondary fibrils, on the other hand, have a high DP of approximately 14,000 (Marx-Figini; 1966 and Marx-Figini and Schulz 1966). X-ray diffraction analysis of fibrils show that secondary fibrils have a high degree of crystallinity and the glucan chains are arranged in a parallel manner (i.e the reducing ends pointing the same direction). Primary fibrils, however, have a much lower degree of crystallinity and it has not been firmly established whether these too have a parallel arrangement although it is generally presumed that they have. ^{13}C NMR studies of cellulose from a variety of sources

(Atalla & Vanderhart, 1984; Vanderhart & Atalla, 1984) have indicated that native cellulose may be a composite of two distinct forms termed cellulose I_α and cellulose I_β.

Purified cellulose preparations from cell walls are always found to contain sugars other than glucose in minor amounts. These may be contaminants or may, possibly, be integral components of the cellulose chain. It is thought that primary cellulose fibres in dicots are completely covered by hydrogen-bonded hemicellulose especially xyloglucans (Valent & Albersheim, 1974), other sugars in cellulose preparations therefore not being integral components of the cellulose. The presence of D-galactose in cellulose preparations, however, could be explained by the work of Hughes & Street (1974). They found that the concentration of galactose, in cellulose preparations, could be increased by the addition of D-galactose to *in vitro* cultured roots. They speculate that D-galactose may cause premature cellulose chain termination thus accounting for the observed phytotoxicity of D-galactose.

1.4.2 Hemicelluloses.

The term hemicellulose was first coined by Schulze in 1891 (see Wilkie, 1979 for original reference) to describe polysaccharides extracted from plant tissues with dilute alkali. This definition was generally adhered to for many years as meaning any 'cell wall or intercellular polysaccharide that can be extracted, by alkali, from higher plant tissues' (Wilkie, 1979). This broad definition was challenged, however, by Bauer *et al.*, (1973) who has redefined the term to include cell wall polysaccharides which are found non-covalently bound to cellulose. This definition has attempted to reclassify the hemicelluloses into a group of polysaccharides with similar chemical properties and possible biological function.

The nature of the hemicelluloses seems to differ considerably between the dicots and the monocots and slightly between species.

1.4.2.1 Xylans.

The xylans are, quantitatively, the dominant hemicellulose in graminaceous cell walls. For this reason they are also the most studied. However the xylans of primary cell walls have not been studied as much as those of secondary walls. The basic structure of the xylans is a ß 1-4 linked xylopyranosyl backbone with a wide variety of side chains. The side chains vary from species to species and from tissue to tissue, however these variations probably reflect variations in proportions rather than the nature of the features (Wilkie, 1979). The most common side chain is a single arabinofuranosyl residue attached to

C-3 of the xylose, they have also been found attached to the C-2 in xylans extracted from barley aleurone layers (McNeil *et al.,* 1975) and husks of sorghum grain (Woolard *et al.*, 1976, 1977).

The second most common side chains are single D-glucuronosyl or 4-*O*-methyl-D-glucuronosyl residues attached to the C-2 of the xylose. Other more complex side chains have been found in monocot xylans e.g Gal-(ß 1-4)-Xyl-(ß 1-2)-Araf, Gal-(ß 1-5)-Araf, GluA-(ß 1-4)-Xyl-(ß 1-4)-Gal and Xyl-(ß 1-2)-Araf. A non-branched homoxylan containing no side chains has also been isolated from esparto grass (Ehrenthal *et al.*, 1954) and tobacco (Eda *et al.*, 1976). An acidic GluA-(ß 1- 4)-Xyl-(ß 1-4)-Gal side chain has been isolated from the cell walls of several members of the Gramineae e.g ryegrass (Morrison, 1974 A), oat plants (Reid & Wilkie, 1969) and barley leaf (Buchala, 1973). Monocot xylans may also possess *O*-acetyl esters at the *O*-5 or *O*-2 of the arabinose, or both, and the *O*-2 or *O*-3 , or both, of the xylose (Morrison, 1974 C).

The degree of acetyl substitution probably affects the xylans ability to aggregate with other molecules (Northcote, 1972, Preston, 1979) and the degree of side chain substitution would appear to affect the solubility of the xylan and its ability to bind to cellulose. The higher the degree of substitution the greater its solubility and the less its ability to bind to cellulose (McNeil *et al.*, 1975). An unsubstituted ß 1-4 xylan chain has a threefold left handed conformation (Marchessault & Setterini, 1964). This structure has a relatively flexible form due to limited intra molecular hydrogen bonding (Setterini & Marchessault, 1965) whereas arabinosyl substituents appear to produce a more extended conformation (Dea *et al.*, 1973) and thus 'stiffen' the xylan.

Other substituents may also affect the xylan in a similar manner (Fincher & Stone, 1986).

Molecular weight determinations of monocot xylans differ greatly depending on the technique used. Sedimentation by ultracentrifugation and osmometry tend to give values between 20 Kd-70 Kd. However gel filtration methods give much higher values of upto 5000 Kd, this is presumably a gross overestimation due to the notorious difficulties in M_{wt} determination of polysaccharides by this method (Fincher & Stone, 1986), such as aggregation in aqueous solutions (Blake & Richards, 1971).

1.4.2.2 ß D-Glucans.

Non endospermic mixed linked ß D-Glucans have, up to date, only been found in cell wall preparations from the Gramineae and would appear to be quite specific to this group of plants. These polysaccharides are composed of chains of mixed ß 1-3 and ß 1-4 linked glucans with the ratio of 3-linked to 4-linked being from 1 to 2.5 (Fincher & Stone, 1986). The ratio of linkages seems to vary with the age of the tissue, the proportion of 4 linked increasing with age. There is no evidence for strictly repeating sequences of linkages. However they would appear not to be totally random in nature as a water soluble ß D-glucan from barley and oats contains sequences of two or three consecutive 1-4 linkages separated by 1-3 linkages (Parrish *et al.*, 1960). It has since been conclusively shown that runs of more than

three consecutive 1-4 linkages are present in a water soluble ß-glucan from barley with blocks of up to fourteen units (Woodward *et al.*, 1983). Blocks of continuous 1-3 linkages have also been widely reported, with blocks of up to four consecutive linkages in oat endosperm (Goldstein *et al.*, 1965), and longer blocks have been reported from rice endosperm (Shibuya & Misaki, 1978).

The DP of the glucan chains in wheat decreased with age from 75 to 28 (Buchala & Wilkie, 1973). The method of DP determination used, however, depends on the retention of a reducing end. Results from other methods indicate that the glucans may aggregate, through chemical bonding, to protein to produce a molecule of M_{wt} 10^4 Kd. Ultracentrifugation and sedimentation studies of oat coleoptile glucans indicated a DP of 1,500 (Wada & Ray, 1978). Again gel filtration methods give much higher results even up to 40,000,000 d (Forrest & Wainright, 1977). The precise role of ß D-glucan would appear to be as a storage polysaccharide as the ß D-glucans of oat coleoptiles are catabolised and disappear when grown in the dark without an energy source (Nevins *et al.*, 1977). However their general abundance and the difficulty involved in removing them from cell wall preparations are more consistent with structural polymers.

1.4.2.3 Xyloglucans.

The basic structure of xyloglucans consist of a backbone of repeating ß 1-4-D-glucosyl units with D-xylose α- linked to the *O*-6 of some of the glucose residues. These xylose side chains may in turn be extended by ß linked D-Gal or α-Fuc-2 α-D-Gal ß linked to the *O*-2 of the xylose. They have been found in several monocots, e.g. rice, bamboo, oats and barley. However they are present in much smaller amounts (approx 2%) than in the dicots (approx 20%). The monocot xyloglucans would appear to be less frequently substituted than dicot xyloglucans and the presence of fucosyl side chains has not been firmly established. Xyloglucans would appear to be structural polymers and it is thought that most, if not all are hydrogen bonded to the cellulose as evidenced by the need for strong alkali treatment to extract xyloglucan from cellulose *in vitro* and its ability to bind rapidly and strongly with cellulose *in vivo* (McNeil *et al.*, 1984). This has further been evidenced by immunogold localisation in red clover where the anti-xyloglucan antibodies were found exclusively associated with the cellulose fibril containing regions (Moore & Staehelin, 1988).

However a role as growth regulators has also, recently, been proposed in dicots (Albersheim *et al.*, 1983). Small amounts of xyloglucan have been found to be released from the cell wall during auxin induced growth. Auxin is also known to induce the secretion of an *endo* ß-1-4-glucanase which will cleave pea xyloglucan. It has also been demonstrated that a xyloglucan fragment will inhibit 2,4-D

stimulated wall elongation in nanomolar quantities (York *et al.*, 1984). Since this initial discovery several biologically active cell wall fragments have been reported (Sharp *et al.*, 1984: Tran Thanh Van *et al.*, 1985: Tong *et al.*, 1986: Bishop & Ryan, 1987: McDougall & Fry, 1988).

1.4.3 Pectic Polysaccharides.

Pectins are a complex mixture of polysaccharides found in the middle lamella of the cell wall, which can be extracted with hot water or aqueous solutions of chelating agents e.g. ethylene-diamine-tetra-acetate (EDTA) (Selvendran, 1985). These extracts can then be divided into neutral (arabinogalactans) and acidic (rhamnogalacturonans and homogalacturonan) fractions.

The rhamnogalacturonans can again be divided into RG I and RG II. Rhamnogalacturonan I (RG I) was first isolated, by extraction with an *endo*-polygalacturonase, from suspension cultured sycamore cells (McNeil *et al.*, 1980). RG I consists of a backbone of alternating 2-linked L-rhamnose and 4-linked D-galacturonic acid residues. Approximately half of the rhamnose units are branched at *O*-4 with either L-arabinose or D-galactose (McNeil *et al.*, 1982). RG I may also contain small amounts of L-fucose (McNeil *et al.*, 1980). The of RG I is thought to be around 2000 daltons (McNeil *et al.*, 1984) and makes up 23% of the total pectin content (7% dry matter) of suspension cultured sycamore cell walls (McNeil *et al.*, 1980).

The galactosyl residues in the backbone may also carry methyl esters, acetyl esters and ferulate esters (Fry, 1982: Rombouts & Thibault, 1986). Enzymic degradation has shown that the side chains tend to be located in specific regions of the backbone causing 'hairy' and 'smooth' regions (DeVries *et al.*, 1982).

Rhamnogalacturonan II is a complex polysaccharide composed of approximately 30 glycosyl residues, containing at least twelve different sugars e.g 2-*O*-methylfucose, 2-*O*-methylxylose, apiose, 3-C-carboxy-5-deoxy-L-xylose (aceric acid), 3-deoxy-D-Manno-2-octulosonic acid and 3-deoxy-*lyxo*-2-heptulosaric acid (Stevenson *et al.*, 1988). Through the study of fragments generated by mild acid hydrolysis, approximately twenty different glycosidic linkages have been found, although the precise structure of the polysaccharide is still uncertain. RG II was originally found in cell walls of suspension cultured sycamore, however it has since been found to be present in all angiosperms tested, one gymnosperm (Thomas *et al.*, 1987) and also in the monocots rice (Thomas *et al.*, 1989) and oats (Darvill *et al.*, 1978).

Homogalacturonans are large molecules consisting solely of α 1-4 linked galacturonic acid residues, which may carry methyl ester groups. The esterified residues appear to occur in blocks. It seems likely that they exist in primary cell walls, although they may be covalently linked to other cell wall polymers (McNeil *et al.*, 1984). It is thought that these chains are bound by aggregating with divalent calcium in an 'egg box' manner (Grant *et al.*, 1973).

Arabinogalactans are, as the name implies, polymers of arabinofuranose and galactose. There would appear to be two types found in cell walls. The most common consisting of a linear ß 1-4 linked galactose with occasional side chains of 1-5 linked arabinose residues at the *O*-3 of galactose (type I) while the other (type II) consists of a mixed ß 1-3, ß 1-4 linked chain of galactose and 5- linked arabinose (Clarke *et al.*, 1979). Arabinogalactans have been demonstrated in a number of monocot secondary cell walls.

Arabinans are highly branched polymers of L-arabinofuranose residues linked in the α configuration. The linkage patterns in the arabinan is highly complex and detailed structures are difficult to determine (Darvill *et al.*, 1980). To date, arabinans have never been found in primary or monocot cell walls.

The precise manner in which these polymers are bound together, in situ, is a much debated topic and a number of schemes have been proposed (DeVries *et al.*, 1982; Jarvis, 1984; Selvendran, 1985).

Most structural work has come from dicot pectins and there has been some doubt expressed as to whether monocots contain pectin at all. Certainly the amounts of uronic acids present in monocots is much lower and are approximately one fifth of the value for dicots (Chesson, personal communication). However pectins similar to rhamnogalacturonans have been discovered in onions (Ishii, 1982) and rice (Shibuya & Iwasaki, 1978). Similarly, methylation analysis of purified primary cell walls from *Lolium multiflorum* and *Lolium perenne* demonstrated the presence of 2- linked rhamnose and a high proportion of 3-*O*-methyl rhamnose which would suggest a large proportion of the

rhamnose carry side chains consistent with the general model of a rhamnogalacturonan (Chesson *et al.*, 1985). It has since been found that this pectin makes up 5-6% of the mesophyll cell wall of ryegrass (Chesson, personal communication).

It may be appropriate at this point to stress the difference between monocot and graminaceous cell walls. In a study of pectin contents in a variety of taxonomic groupings, Jarvis *et al* (1988), discovered that monocots, as a whole, have a high to medium pectin content and that it was members of the *Glumiflorae* (which includes the gramineae) which have relatively little.

1.4.4 Cell Wall Proteins.

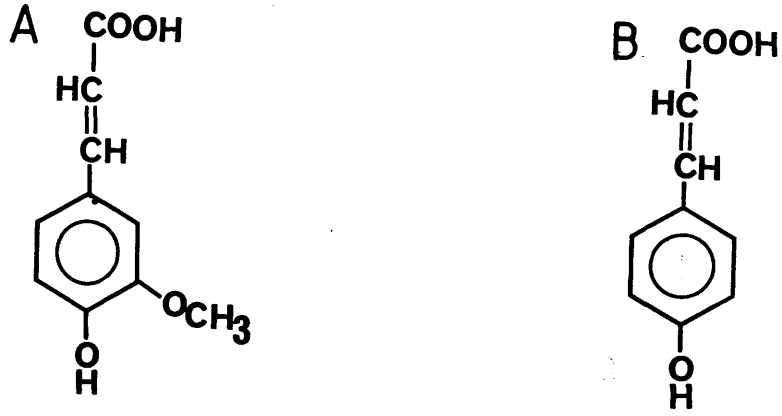
As in most cases, most of what is known about cell wall proteins comes from work with dicots. Original research has shown that dicot cell walls contain appreciable amounts of protein (5-10%). The composition of this protein, however, is somewhat unusual in that it has a very high concentration of hydroxyproline (approx 20%). Difficulties in original attempts to extract this protein suggested that much of it is a structural protein and was called extensin as it was thought to be involved in controlling cell wall extensibility (Lamport, 1965).

When fragments of these proteins are extracted they invariably contain arabinosyl and/or galactosyl residues. Hydrolysis of the peptide linkages in cell walls with 0.2M BaOH releases a mixture of hydroxyproline rich arabinosides. These could be separated into mono-, di-, tri- and tetra- arabinosides, with the tetra predominating in dicots. As previously mentioned the cell wall proteins of monocots have not been as extensively studied. Suspension cultured monocot cell walls have been reported to contain 0.13-0.16% hydroxyproline, compared to dicots which may contain up to 2% (Burke *et al*, 1974). Hydroxyproline-arabinosides have been isolated from four different monocot species (Lamport & Miller, 1971) but only once from a member of the Gramineae (Kieliszewski & Lamport, 1987), although the majority of the protein was unglycosylated. Of the hydroxyproline arabinosides isolated the tri-arabinoside predominates with small amounts of the di-arabinoside. It would thus appear that the cell wall proteins of dicots and monocots differ considerably in structure and possibly in function.

1.4.5 Phenolic Acids.

Phenolic acids have long been known to be found in plant cell walls as ester linked components of lignin (Shimada *et al*, 1971), however they have also been found in alkali hydrolysates of cell walls of the Gramineae suggesting they are linked to hemicellulose by alkali-labile bonds (Hartley, 1972). The predominant acids found in the Gramineae, are ferulic (4-hydroxy, 3-methoxy cinnamic acid) (Figure 1.4.5.1 A) and *p*-coumaric (4-hydroxy cinnamic acid) acids (Figure 1.4.5.1 B), mainly in their *trans* form, but small amounts of sinapic (3,5,-dimethoxy-4-hydroxycinnamic acid), vanillic (4-hydroxy, 3-methoxy benzoic acid) and *p*-hybrid benzoic acids have also been found (Gordon *et al*, 1985).

Figure 1.4.5.1 Structures of A) Ferulic acid and B) *p*-Coumaric acid.



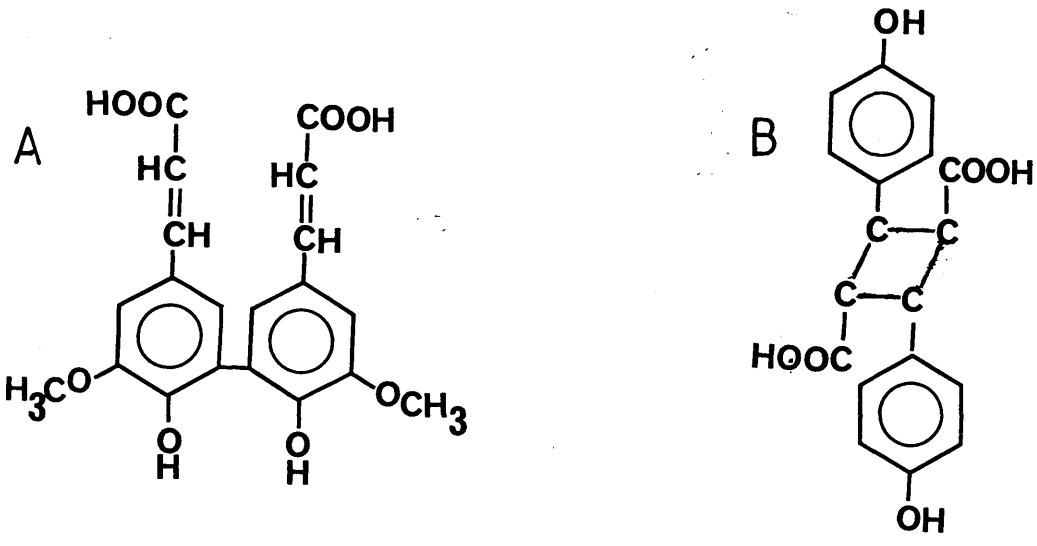
Since their discovery in hydrolysates of alkali-treated feedstuffs, several groups have isolated fragments of polysaccharide containing ester linked ferulic and *p*-coumaric acids. In the monocotyledons these have been linked through the *O*-5 of arabinose (Mueller-Harvey *et al.*, 1986 ; Smith & Hartley, 1983; Kato & Nevins, 1985; Kato *et al.*, 1987) and to the *O*-6 of the galactose and the *O*-3 of the arabinose in the pectic fraction of dicotyledons (Fry, 1982).

The precise role of phenolic acids in the cell wall is uncertain although a number of theories have been proposed. It has been suggested that they play a role in inhibiting enzymic degradation of the hemicellulose (Hartley, 1972), that they provide a reservoir of phenylpropanoid units for lignin synthesis (Gordon, 1975), that they act as U.V receptors and control phototropism (Towers and Abeysekera, 1984) and that they are responsible for limiting cell wall extensibility by cross linking (Fry, 1983).

The role in controlling cell wall extensibility arose from the discovery of dehydrodiferulic acid, a C5-C5 linked dimer of ferulic acid (Figure 1.4.5.2 A) in water insoluble pentosans from *Triticum aestivum* (Markwalder and Neukom, 1976) and alkali hydrolysates from *Lolium multiflorum* (Hartley and Jones, 1976). Fry (1979) proposed that ferulic acid esterified to hemicellulose chains, in the presence of H_2O_2 and the enzyme peroxidase, will cross link the hemicellulose chains thus reducing cell wall extensibility. This proposal is backed up by the discovery that gibberellins, commonly regarded as growth promoters, inhibit peroxidase secretion (Fry, 1983).

This theory, of phenolic acids playing a major role in controlling cell wall extensibility, has recently been expanded by the discovery of 4,4'-dihydrotruxillic acid (Hartley *et al.*, 1988) (Figure 1.4.5.2 B) in alkali extracts of *Lolium multiflorum*. Truxillic acid is a cyclodimer of -coumaric acid through the unsaturated propyl side chains. It was later discovered that a variety of these dimers exist (Ford & Hartley, 1988) between *p*-coumaric acid and ferulic acid i.e *p*-CA-*p*-CA, *p*-CA-FA and FA-FA. These cyclodimers may also exist in a number of isomeric forms i.e head to head (Truxillic acid) or head to tail (Truxinic acid) (Ford & Hartley, 1989). It has been suggested that they are products of photodimerisation in the cell wall.

Figure 1.4.5.2 Structures of A) Diferulic acid and B) Truxillic acid.



The proposal of Towers and Abeysekera (Towers & Abeysekera, 1984) states that the U.V mediated isomerisation of hemicellulose bound phenolic acids also effects cell wall extensibility and could be responsible for phototropic responses. However their model system

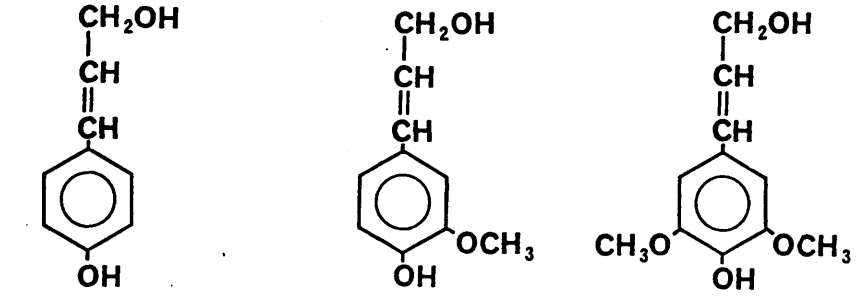
depends on the presence of another photoreceptor which will absorb in the blue region (400-450 nm) and transfer its excitation energy (Towers and Abeysekera, 1984). To date, however, no such receptor has been demonstrated.

The light mediated synthesis of truxillic acid offers an intriguing alternative to Towers & Abeysekera's, light controlled, model for the role of phenolic acids in controlling cell wall extensibility.

1.4.6 Lignin.

Lignin is a complex polymer, formed by the dehydrogenative polymerisation of *p*-hydroxycinnamyl alcohols, which impregnates the cell wall thereby imparting rigidity and helping resist attack by microorganisms. After cellulose it is the second most abundant natural organic product (Nimz, 1974). It is unhydrolysable with acid, readily oxidised, partially soluble in alkali and readily condenses with alcoholic and phenolic compounds (Pearl, 1967). There are three major groups, each based on their monomer units. 1) Gymnosperm (softwood) lignin or guaiacyl lignin, a dehydrogenation polymer of coniferyl alcohol (Figure 1.4.6.1 A), 2) Angiosperm (hardwood) lignin or guaiacyl-syringyl lignin, a mixed polymer of coniferyl and sinapyl alcohols (Figure 1.4.6.1 B) and 3) Grass lignin or guaiacyl-syringyl-*p*-hydroxyphenyl type lignin, a mixed polymer of coniferyl-sinapyl and *p*-hydroxyphenyl alcohols (Figure 1.4.6.1 C) (Higuchi, 1980). In addition, grass lignins are thought to have *p*-coumaric acid esterified to the terminal hydroxyl groups of the lignin side chains (Higuchi, 1981) and *p*-coumaric and ferulic acids attached by ether linkages (Scalbert *et al*, 1985). These definitions are very generalised, as some grasses are thought to be similar to hardwood lignins (Harkin, 1973). Unfortunately much of the structural studies carried out on lignin has been performed on softwood lignin due to its importance in the pulp and paper industries.

Figure 1.4.6.1 Structures of lignin monomers.



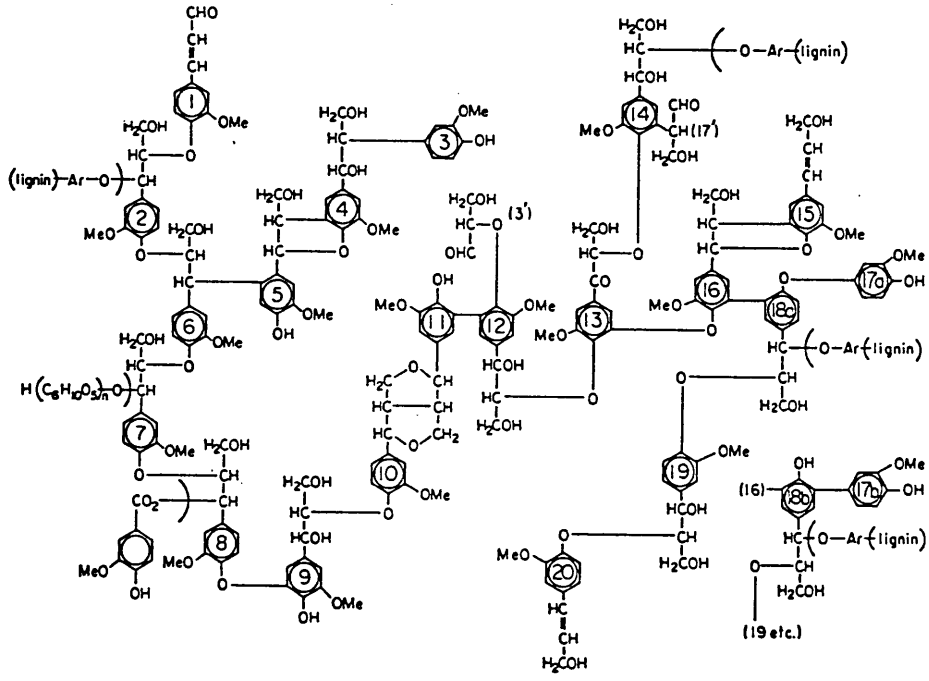
As mentioned earlier, due to the complex nature of lignin, its structural elucidation has proved to be a long and difficult process. The aromatic nature of lignin was established early on. The arylpropane skeleton was first conceived by Freudenberg in the 1930's (Adler, 1977), principally based on the elemental composition of spruce lignin preparations.

Degradation studies using a wide variety of oxidative techniques e.g permanganate oxidation, nitrobenzene oxidation, ethanolysis and acidolysis strongly suggested certain bond types. The structures proposed were further backed up by the study of dehydrogenation polymers (DHP's) using ferric chloride or oxidative enzymes to polymerise simple phenolic monomers.

With the aid of these chemical data and the use of physical techniques such as IR and NMR a number of different lignin models have been proposed (Nimz, 1974: Adler, 1977: Freudenberg & Neish,

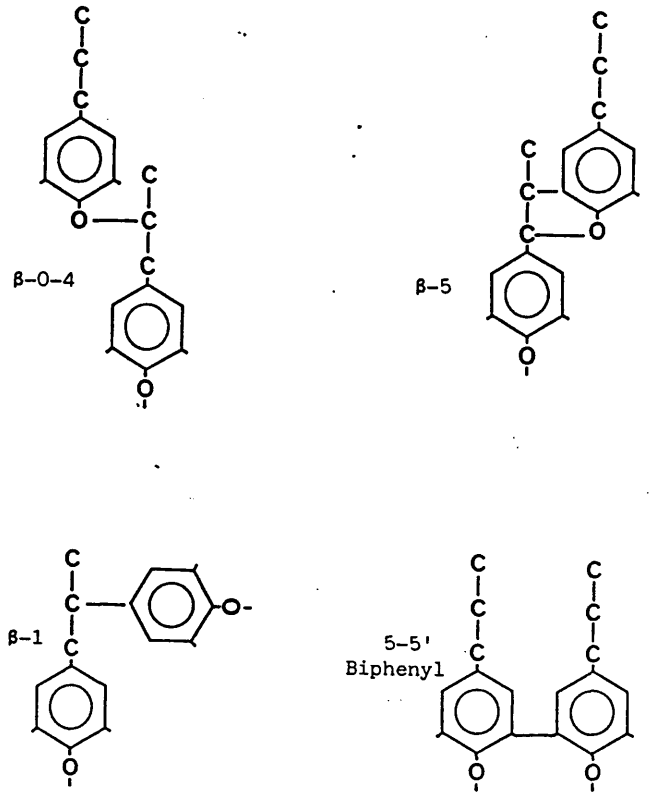
1968) (Figure 1.4.6.2). These schemes, however, generally agree as to the number and types of linkages involved and differ only the occurrence and frequent of monomer units and linkages.

Figure 1.4.6.2 Structure of lignin.



The most common linkage is a aryl glycerol-ß-aryl ether structure (Figure 1.4.6.3 A) which accounts for 48% of the linkages in spruce lignin (Adler, 1977) and is generally regarded as a sign of true lignin. Other linkages include noncyclic benzyl aryl ether (Figure 1.4.6.3 B), phenylcoumarin structures (Figure 1.4.6.3 C) and biphenyl structures (Figure 1.4.6.3 D), in all there are thought to be over twenty different types of linkage involved in the lignin structure.

Figure 1.4.6.3 Structure of some common lignin bonds.



As stated earlier, the synthesis of lignin is mediated by the dehydrogenative polymerisation of *p*-hydroxy cinnamyl alcohols. This is thought to be achieved by the action of the enzyme peroxidase. It has been proposed that the alcohols are dehydrogenated by cell wall bound peroxidase via a superoxide radical which is suggested to be formed by the reduction of O_2 by NAD (Elstner & Heupel, 1976). The coniferyl alcohol radicals so produced then spontaneously couple to form dimers, trimers and higher oligomers. Further dehydrogenation of these dimers etc results in the formation of lignin (Higuchi, 1980). These proposals have been supported up by the discovery that the enzyme lignin peroxidase produced by the white rot fungus *Phanerochaete*

chryosporium (Tien & Kirk, 1983; Glenn *et al.*, 1983) will degrade lignin by radical formation via H_2O_2 (Schoemaker *et al.*, 1985 A & B) these radicals will then also polymerise via radical formation (Haemmerli *et al.*, 1986).

1.5 LIGNIN-CARBOHYDRATE LINKAGES.

Lignin-carbohydrate linkages have been a much debated topic ever since the discovery of lignin as a cell wall component (Pearl, 1967). Original doubts were over the existence of such a linkage, however, although there is still no direct evidence for such a linkage, the wealth of circumstantial evidence makes it a difficult proposal to reject (Conchie *et al.*, 1988), and modern debates are over their nature, distribution and significance.

The existence of lignin-carbohydrate linkages was first postulated by the early wood chemists. Again, therefore much of our knowledge of this subject derives from wood. Although a linkage was suspected, its precise nature was unknown and a variety of types e.g ester, ether, glycosidic, acetal, hemiacetal, ketal and hydrogen bonds have at some time been proposed (Brownell, 1971).

One of the original methods of studying lignin-carbohydrate linkages was through the study of milled wood lignins (MWL), usually isolated by the Björkman procedure (Björkman, 1956). Early work by Brownell (1971) on MWL of black spruce (*Picea mariana*) suggested that the lignin-carbohydrate bond was inconsistent with an acetal, hemiacetal, ketal, ester or glycosidic bond but may be an ether. Subsequent research on chemical extracts from eucalypt wood (Stewart, 1973) indicated that uronic acids are either ether linked to lignin and ester linked to the xylose in the glucuronoxylan or ester linked to the lignin and glycosidically linked to the carbohydrate, the possibility of

glycosidic linkages to the lignin is not ruled out. He also suggests that in eucalypt, and possibly other hardwoods, virtually all lignin is linked through the uronic acid residues.

Eriksson & Lindgren (1977), working on Björkman MWL of Norway spruce (*Picea abies*) postulated that the lignin was attached to the galactoglucomannan through galactose residues onto the mannose backbone and to the xylan through arabinose side chains. Further research by the same group (Eriksson *et al.*, 1980) on MWL of black spruce indicated that the lignin is ether linked to the arabinose either through the *O*-2 or *O*-3 positions, linkages through the *O*-4 could not be excluded. Linkages directly to the xylose were also proposed. They further go on to suggest that lignin is linked to the *O*-3 position of the galactose in the galactoglucomannan, and possibly, at other positions, and that lignin is ester linked to 4-*O*-methylglucuronic acid. Although they had no direct evidence they suggested a linkage between cellulose and lignin, as cellulosic material could not be removed through several purification steps. To summarise, they suggested that lignin is attached to all types of sugar units in the hemicellulose and possibly cellulose, thus supporting Freudenberg's view that "lignin-carbohydrate linkages are formed by the non-enzymic addition of hydroxyl and carboxyl groups in the carbohydrates to quinone methide intermediates in lignin biosynthesis" (Freudenberg & Neish, 1968).

The work discussed so far has concentrated purely on wood. Studies of the linkages in the monocots, in general, and the Gramineae in

particular are less extensive. Over the past few years, however, more and more work is being published, in this field.

Morrison (1973) was one of the first researchers to address the question in graminaceous cell walls when he isolated a lignin-carbohydrate complex (LCC) from *Lolium multiflorum* and *Lolium perenne* (Morrison, 1974 B) with dimethyl sulphoxide (DMSO). In all, Morrison (1974, C), proposed the presence of at least three types of linkage between the lignin and carbohydrate, one cleaved on borohydride reduction, another cleaved by alkali and one resistant to alkali. He goes on to suggest that phenolic acids act as cross linking agents between lignin and carbohydrate. The carbohydrate component consisted of ß 1-4 linked xylose and a significant amount of ß 1-4 D-glucose which may have originated from a cellulose like polymer (probably xyloglucan). He further postulates that the LCC is a unit of the cell wall structure.

An interesting approach was used by Gaillard & Richards (1975) when they recovered solubilised LCC's from bovine rumen liquor. Their results agreed with the presence of ester linkages between the lignin and the carbohydrate, although only some of the linkages were broken by alkali. This original idea was followed up by Neilson & Richards (1978) who postulated a structure for the complex and the existence of glycosidic bonds between D-glucose, D-xylose and L-rhamnose to the lignin. This approach has since been used to study the solubilised LCC'S from ryegrass in the ovine rumen (Conchie *et al.*, 1988). The complexes isolated tended to have either a high (>100 Kd) or low (<4 Kd) molecular weight, the high M_{wt} containing the higher carbohydrate

concentration. Reducing sugars (principally arabinose and xylose) were found in the low M_{wt} fraction thus suggesting ether linkages to the phenolics. The presence of rhamnose in the LCC'S suggest it could play an important role in cross linking. All LCC'S contained protein which could not be removed by any of the purification steps. Amino acid analysis indicated very little hydroxyproline which has been implicated in lignin-protein binding (Dill *et al.*, 1984).

Protein was also found in the LCC'S prepared by enzymic degradation of pangola grass (Ford, 1986) which was low in hydroxyproline content. The structural relationship between the components of these LCC'S was very complex and involved monomeric and oligomeric pentose units.

^{13}C NMR studies of wheat straw lignin preparations (Scalbert *et al.*, 1986) suggested the participation of ferulic acid in the alkali labile cross links between lignin and carbohydrate and further suggested that all lignin is attached to the hemicellulose. Further work by the same group (Scalbert & Monties, 1986) indicates that graminaceous cell walls may have a higher concentration of alkali labile bonds between the lignin and carbohydrate, or within the lignin network than either softwood or hardwood.

In order to study the nature and distribution of alkali labile i.e ester bonds in cell walls of ryegrass, cereal straws and clover, Chesson *et al.*, (1983) and Lomax *et al.*, (1983), adapted the acetalation-methylation technique of DeBelder & Norman (1968), for the

localisation of alkali labile bonds, for use on whole cell walls. In general they found a considerable degree of substitution to xylose (through *O*-2, *O*-3 or both) and arabinose (through *O*-5 or *O*-5 and *O*-2). Analysis of acetyl and phenolic acid ester content could only explain approximately two thirds of the alkali labile bonds (in clover over half remained unexplained) and it was postulated that most, if not all, of the unaccounted for bonds were to the lignin component.

1.6 CELL WALL DIGESTION.

When a plant feed stuff e.g cereal straw enters the rumen it is attacked by a variety of protozoa, anaerobic fungi and bacteria. These microorganisms attack the cell walls by releasing a wide battery of hydrolytic enzymes at the surface of the cell wall (Chesson & Forsberg, 1988).

Microscopic examination of plant material undergoing digestion in the rumen clearly demonstrates that some cell walls are much more susceptible than others (Gordon *et al.*, 1985). Mesophyll and phloem are most susceptible followed by epidermis with vascular tissue being most resistant. The obvious difference would appear to be that primary cell walls are more easily degraded than secondary cell walls.

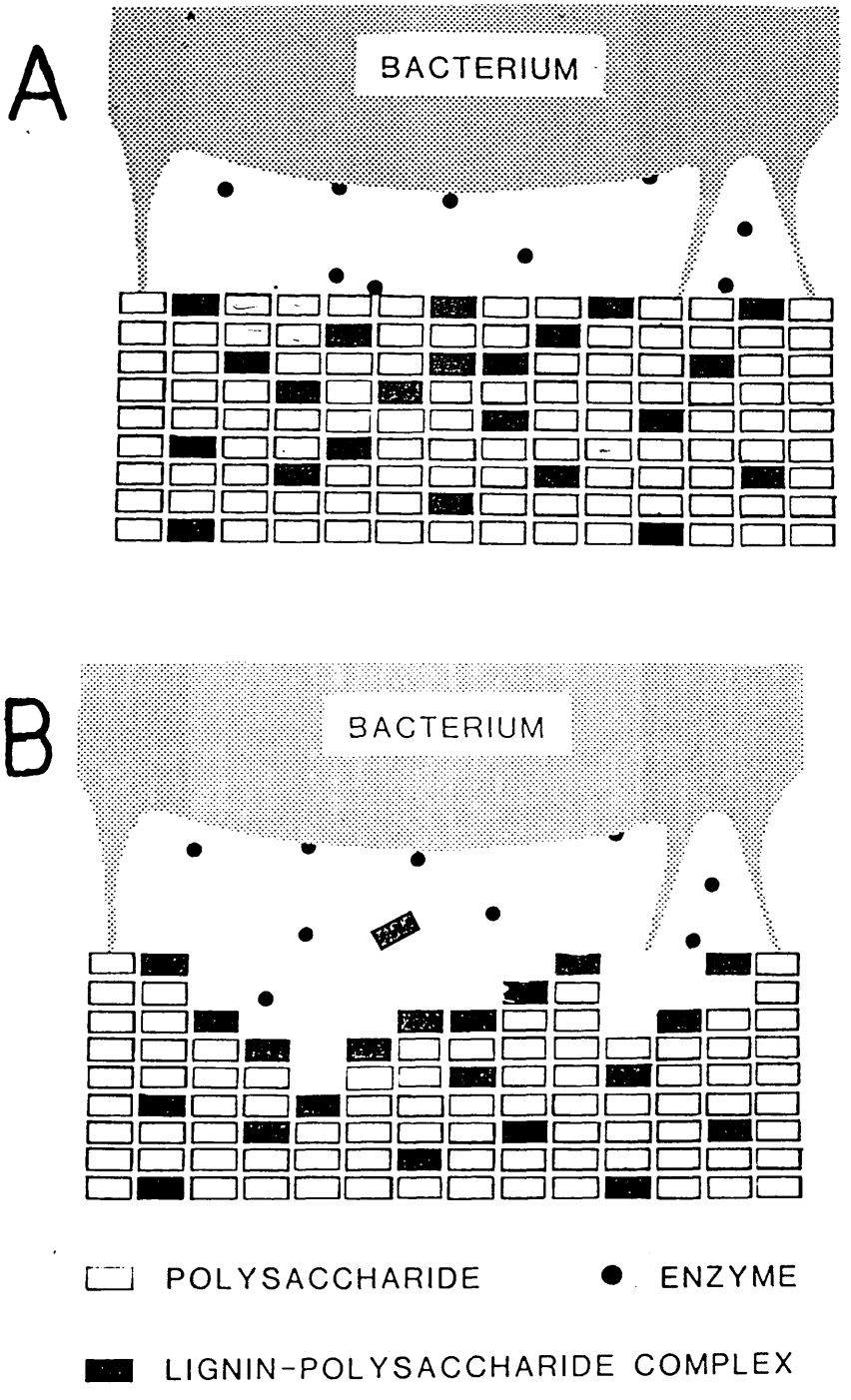
Original thoughts revolved around the idea that some cell wall component, possibly lignin, inhibited enzymic and or microbial degradation, therefore more heavily lignified materials are more resistant than non-lignified materials. While this observation is generally true chemical analysis does not agree. In the case of cereal straws approximately half of the straw is solubilised in the rumen. The residue is highly resistant to further degradation, If one component of the cell wall is inhibitory to degradation one would therefore expect an increase in concentration after rumen incubation. On analysis it is found that carbohydrate content remains essentially the same, the only difference being that total phenolics tend to slightly accumulate, although lignin is still solubilised at much the same rate as other cell wall components. As mentioned previously, the effects of alkali on

digestibility and lignin content strongly suggest that lignin plays a major role in limiting digestibility.

The proposed model of cell wall digestibility is demonstrated in Figure 1.6.1. The cell wall is built up of carbohydrate and carbohydrate-lignin blocks. The carbohydrate-lignin blocks are resistant to degradation so as degradation proceeds and the carbohydrate blocks are broken down, a 'wall' of lignin-carbohydrate blocks cover the surface, thus preventing further degradation. This would account for the observed lack of change in carbohydrate content and the small increase in lignin content. Chesson *et al.*, (1983) demonstrated the accumulation of alkali labile bonds to the *O*-5 of the arabinose, also fitting in with this model.

A study of the surfaces of rumen incubated straw (Russell *et al.*, 1988) by multiple reflectance infrared spectroscopy, clearly demonstrated that the inner layers of the straw were degraded and the outer layer were virtually untouched. The inner surface also showed the build up of lignin, and acetyl esters.

It was also suggested that alkali treatment only affects the outer layers and dissolves silica, acetyl esters and lignin. It would also appear to open up cracks and pores in the surface thus opening it up for enzymic attack.


A
BACTERIUM
B
BACTERIUM
POLYSACCHARIDE
ENZYME
LIGNIN-POLYSACCHARIDE COMPLEX

2.0 EXPERIMENTAL.

2.1 SAMPLE PREPARATION.

2.1.1 Starting materials.

Perennial ryegrass (*Lolium perenne* c.v Perma) was harvested in June 1986, from an established field and stored at -40°C until used. The frozen grass was repeatedly blended in a Waring blender with iced distilled water and sieved on a 300 µm nylon mesh until all green material had been washed out. The 'non-mesophyll' ryegrass was then freeze dried and stored at room temperature.

Barley straw (*Hordeum vulgare* c.v Golden Promise) was harvested in August 1984 at the Rowett Research Institute. The straw was dried and milled to pass a 1mm sieve then stored at room temperature.

2.1.2 Mild acid hydrolysis.

Five gram samples were refluxed with 500 ml of 30 mM oxalic acid for three hours. The insoluble residue was then filtered off, on a grade 1 glass sintered filter, and thoroughly washed with distilled water. The oxalic acid was then neutralised with an equimolar amount of calcium

acetate. The calcium oxalate precipitate was then filtered off on a grade 4 sintered glass filter. The acetic acid was then evaporated from the hydrolysate under vacuum at 40°C, and the hydrolysate and residue were freeze dried. The freeze dried hydrolysate was then extracted with 5 x 10 ml of methanol. The methanol soluble and methanol insoluble fractions were retained and freeze dried.

2.1.3 Purification of FA-ARA.

100 mg Samples of ryegrass methanol soluble fraction was dissolved in water and passed through a C18 Bond Elut cartridge (Analytichem International). The retained material was then eluted with 1 ml of methanol, filtered and FA-ARA was separated by reverse phase HPLC. Solvent A was water and solvent B was 50% methanol in acetonitrile ($^{v}/_{v}$). The flow rate was 1 ml/min and the gradient ran from, 0% B to 7% B in 5 mins then up to 22% B in another 120 mins then up to 100% B in a further 10 mins. The appropriate fractions were collected and freeze dried. The collected material was then passed through a 1m x 1.7 cm column of Sephadex G-10 (Pharmacia), the major U.V absorbing peak being collected and freeze dried. Purity was established by TLC (solvent 1).

2.1.4 Dimethyl sulphoxide extraction.

A four gram sample of oxalate hydrolysed material was freezer milled under liquid nitrogen for three minutes in a SPEX 6700 freezer mill

(Spex Industries). The samples were then extracted with 250 ml of dimethyl sulphoxide (DMSO) in an ultrasonic bath for three hours, ensuring the temperature did not exceed 40°C. The DMSO soluble material was then dialysed against distilled water, in the dark, for four days at room temperature with constant agitation and freeze dried. The DMSO soluble fractions were then purified on a 1m x 1.7cm column of Sephadex G-100 (Pharmacia).

2.1.5 Driselase hydrolysis.

Driselase (a commercial cellulase preparation, Sigma) was partially purified according to the method of Fry, (1982). The final enzyme preparation was dissolved in 50 ml of 100 mM sodium acetate buffer, pH 5.0, dispensed into 1 ml aliquots and stored at -20°C.

Samples of oxalate hydrolysed, DMSO extracted cell walls (100 mgs) were added to 10 ml acetate buffer plus 1 ml of enzyme and incubated at 45°C for ninety six hours under toluene. The hydrolysed material was then filtered off and washed with distilled water, the soluble and insoluble fractions were collected and freeze dried.

At all stages of sample preparation and handling, great care was taken to exclude light in order to minimise *cis-trans* isomerisation of phenolics (Hartley & Jones, 1975) and possible dimerisation (Cohen *et al.*, 1963).

2.2 GENERAL ANALYSIS.

2.2.1 Total carbohydrates.

Total carbohydrate was determined by the phenol sulphuric method of Dubois *et al.*, (1956) using glucose as a standard.

2.2.2 Total phenolics.

Total phenolics were determined by the acetyl bromide method of Morrison, (1972) using ferulic acid as a standard.

2.2.3 Uronic acids.

Uronic acids were determined by the method of Blumenkrantz & Asboe-Hanson, (1973) using glucuronic acid as a standard.

2.2.4 Monosaccharides.

Monosaccharides were determined as their alditol acetates according to the method of Blakeney *et al.*, (1983). Approximately 10 mg samples were accurately weighed into soviral tubes and 0.25 ml of 72% ($^{v}/_{v}$) H_2SO_4 was added, along with a short glass rod with rounded ends. These were vortexed intermittently for one hour, when 2.75 ml of water was added, the tubes were flushed with nitrogen, sealed and heated at 100°C for three hours. The cooled tubes were then neutralised with 0.64 ml of concentrated NH_4OH solution and 0.1 ml of a 20 mg/ml aqueous solution of inositol (internal standard) was added. A sample of hydrolysate (200 µl) was then transferred to a glass B14 tube and 1 ml of a 2% solution ($^{w}/_{v}$) of sodium borohydride in DMSO was added and heated at 40°C for ninety minutes. These were then cooled, 0.1 ml of glacial acetic acid added and mixed well. Acetic anhydride (2 ml) and 1-methyl imadazole (200 µl) were then added and left to stand at room

temperature for 15 mins. The excess acetic anhydride was then neutralised with 5 ml of water, the tubes mixed and cooled in ice. Dichloromethane (2 ml) was then added, mixed and the aqueous layer removed. This was repeated three times and the dichloromethane was dried with anhydrous Na_2SO_4. The dry dichloromethane was then transferred to a 2 ml vial, dried and redissolved in 1 ml of dichloromethane.

The alditol acetates were then analysed on a Carlo-Erba HRGC 5300 MEGA GC with a 3 m x 3 mm column of 3% SP 2340 on SUPELCOPORT 100/200, isothermally at 225°C. The injector being set at 230°C and the detector at 240°C, with nitrogen as the carrier gas at 160 KPa.

2.2.5 Phenolic acids.

Phenolic acids were determined by reverse phase HPLC. The solvents were:

Solvent A; 1% acetic acid ($^v/_v$) in 0.01M tetra methyl ammonium hydroxide (Sigma) in water.

Solvent B; 1% acetic acid ($^v/_v$) in 0.01M tetra methyl ammonium hydroxide in methanol.

The flow rate was 1.5 ml/min and a gradient was run from 17% B to 26% B in 10 mins then up to 80% B in a further 10 mins.

Samples were prepared by accurately weighing out approximately 20-30 mgs of dried sample into soviral tubes and adding 1 ml of 1M NaOH, purged with nitrogen and left at room temperature overnight in the

dark. Anisic acid (0.1 ml of a 1 mg/ml solution in methanol, internal standard) was then added and the samples acidified to < pH 1 with 6M HCl and filtered on a sintered glass filter (grade 1). The hydrolysates were then extracted 3 x 5 ml of ethyl acetate and the extracts were dried down under vacuum in a 50 ml round bottomed (RB) flask. A small amount of ethyl acetate was then used to transfer the extracts back to the soviral tube and dried down again. The samples were then redissolved in 1 ml of solvent B, and filtered on a 2 µm membrane filter prior to injection.

Standard ferulic and *p*-coumaric acids were purchased from Fluka and anisic acid from Sigma. Diferulic acid was synthesised according to the methods of Richtenzain, (1949) and Baumgartner & Neukom, (1972). The structure of the final product was confirmed by mass spectrometry (major ions (*M/Z* (Molecular ion; relative intensity)) 298 (M+ -88; 100) 324 (M+ -62; 65) 342 (M+ -44; 57)). Purity was established by GC-MS of TMS derivatives. One major peak, retention time (RT) 44.65 mins was found. Its major ions were 73 (M+ -574; 100), 558 (M+ -89; 52), 381 (M+ -266; 37), 559 (M+ -88; 21), 207 (M+ -440; 19), 281 (M+ -366; 14), 75 (M+ -576; 12), 560 (M+ -87; 11), 382 (M+ -265; 11) and 366 (M+ -281; 10). This structure is suggestive of the *cis-cis* form of diferulic acid (Hartley & Jones, 1976).

Truxillic acid was synthesised by the method of Cohen *et al.*, (1963). The purity and structure of the final product was determined by GC-MS of TMS derivatives. The RT for truxillic acid was found to be 60.57 min and the major ions found were, 73 (M+ -543; 100), 308 (M+ -308; 56), 293 (M+ -323; 38), 219 (M+ -397; 29), 45 (M+ -571: 16), 75 (M+ -541;

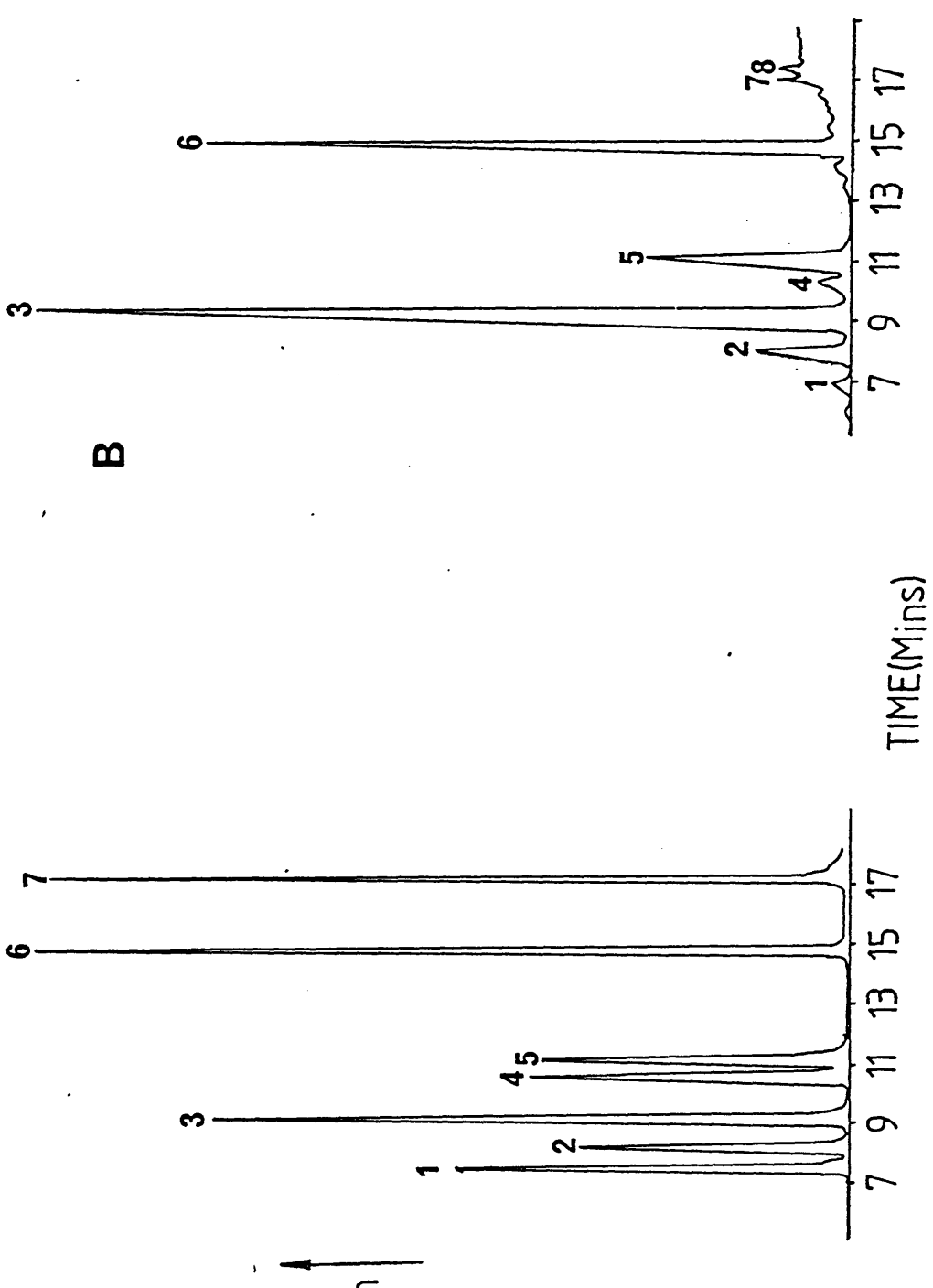
14) 249 (M+ -367; 10) and 179 (M+ -437; 9) thus agreeing closely with published data (Hartley *et al.*, 1988). As this is essentially the same spectra observed for TMS *p*-coumaric acid, the RT of TMS *p*-coumaric acid was checked and found to be 32.7 min.

Response factors were calculated by carrying standard solutions through the procedure described. Figure 2.2.1 (A) shows the separation obtained with standard compounds and (B) shows the separation achieved with an actual sample. The identities of the peaks can be seen in Table 2.2.1 along with their respective, calculated response factors (rf), retention times (RT) and relative retention times (RRT).

TABLE 2.2.1. Identities and response factors of peaks from HPLC analysis of phenolic acids (A) standards, B) cell wall hydrolysate).

PEAK	IDENTITY	RT		RRT		rf
		A	B	A	B	
1	TRUXILLIC ACID	7.50	6.90	0.49	0.46	4.893
2	*cis*-COUMARIC ACID	8.27	8.00	0.55	0.53	0.311
3	*trans*-COUMARIC ACID	9.27	9.25	0.61	0.61	0.311
4	*cis*-FERULIC ACID	10.72	10.42	0.71	0.69	0.546
5	*trans*-FERULIC ACID	11.32	11.22	0.75	0.74	0.546
6	ANISIC ACID (I.S)	15.13	15.10	1.0	1.0	1.0
7	DIFERULIC ACID	17.62	17.47	1.16	1.16	0.823
8	DIFERULIC ACID *		17.83		1.18	0.823

* isomeric form not present in synthesised sample.


A
O.D
280nm
1
2
3
4
5
6
7
7 9 11 13 15 17
B
1
2
3
4
5
6
7
8
7 9 11 13 15 17
TIME(Mins)

2.3 CHROMATOGRAPHY.

2.3.1 HPLC.

HPLC was performed on a 10 cm x 4.6 mm (i.d) column of 5 µm Spherisorb ODS2 with an LKB BROMMA system comprising a 2150 pump, a 2152 LC controller, a high pressure mixing valve and a 2151 UV/VIS variable wavelength monitor. Injection was performed via a Rheodyne valve with a 20 µl loop. All reagents were of HPLC grade (May & Baker) and water was distilled and deionised before use. All solvents were filtered on a 2 µm acetate filter and thoroughly degassed before use. All samples were also filtered on 2 µm filters. Phenolics were detected by U.V absorption at 280 nm.

2.3.2 Gas chromatography-Mass spectrometry.

G.C was performed on a Carlo-Erba (HRGC 5160 MEGA) gas chromatograph with on column injection onto a fused silica bonded-phase column (50m x 0.33mm (i.d)), 0.5 µm film of BP-1 (S.G.E) with helium as a carrier gas (130 KPa). The gradient used was 200°C for 5 mins then up to 300°C at 4°C/min.

M.S was performed on a Hewlett Packard 5970 mass spectrometer. Trimethyl silyl derivatives were produced by the method of Smith & Hartley, (1983).

2.3.3 Molecular sieve chromatography.

All molecular sieve chromatography was performed on 1 m x 1.7 cm columns, and eluted with water at room temperature. Five mg samples were applied. Void volumes were determined by the use of blue dextran (Pharmacia) and inclusion volumes by Na_2SO_4 (detected by BaOH).

2.3.4 Thin Layer Chromatography (TLC) & Paper chromatography (PC).

TLC was performed on silica gel plates (Merck 5748). Phenolics were detected by fluorescence under UV light and diazotised sulphonilamide reagent (Stahl, 1969). Carbohydrates were detected by α-napthol reagent (Jarvis *et al.*, 1977) on TLC and by aniline pthalate reagent on PC (Wilson, 1959).

Solvent system 1; Chloroform: Methanol: Acetic acid: Water (50:50:15:5).

Solvent system 2; Toluene: Acetone: Acetic acid (9:1:1).

Paper chromatography was performed on Whatman no 1 paper with a solvent system of Butanol: Acetic acid: Water (60:20:20).

2.4 PYROLYSIS.

I would like to thank Professor J.J.Boon of the FOM Institute, Amsterdam, Netherlands, and his group for welcoming me to his laboratory and performing the pyrolysis analysis.

2.4.1 Py-GC.

The pyrolysis unit used (Figure 2.4 A) has been described previously (Boon *et al.*, 1987). The sample was applied via a particulate suspension or solution in water onto a ferromagnetic wire which was flushed with argon, then placed in a glass liner. The liner was placed in the pyrolysis unit resting on a Kalrez interface directly above the G.C column. The liner was surrounded by a ceramic tube maintained at 160°C which is in turn surrounded by a high frequency coil. The unit was flushed with helium for 20 secs followed by hydrogen (carrier gas) during which time the coil was heated to its Curie point within 0.1s (510°C). The pyrolysed sample was then flushed onto the beginning of the column. G.C was performed on a Carlo Erba (4200) G.C with a Quadrex 007 series bonded OV-1701 column (100 m x 0.32 mm 1 μm film).

2.4.2 Curie point Py-MS.

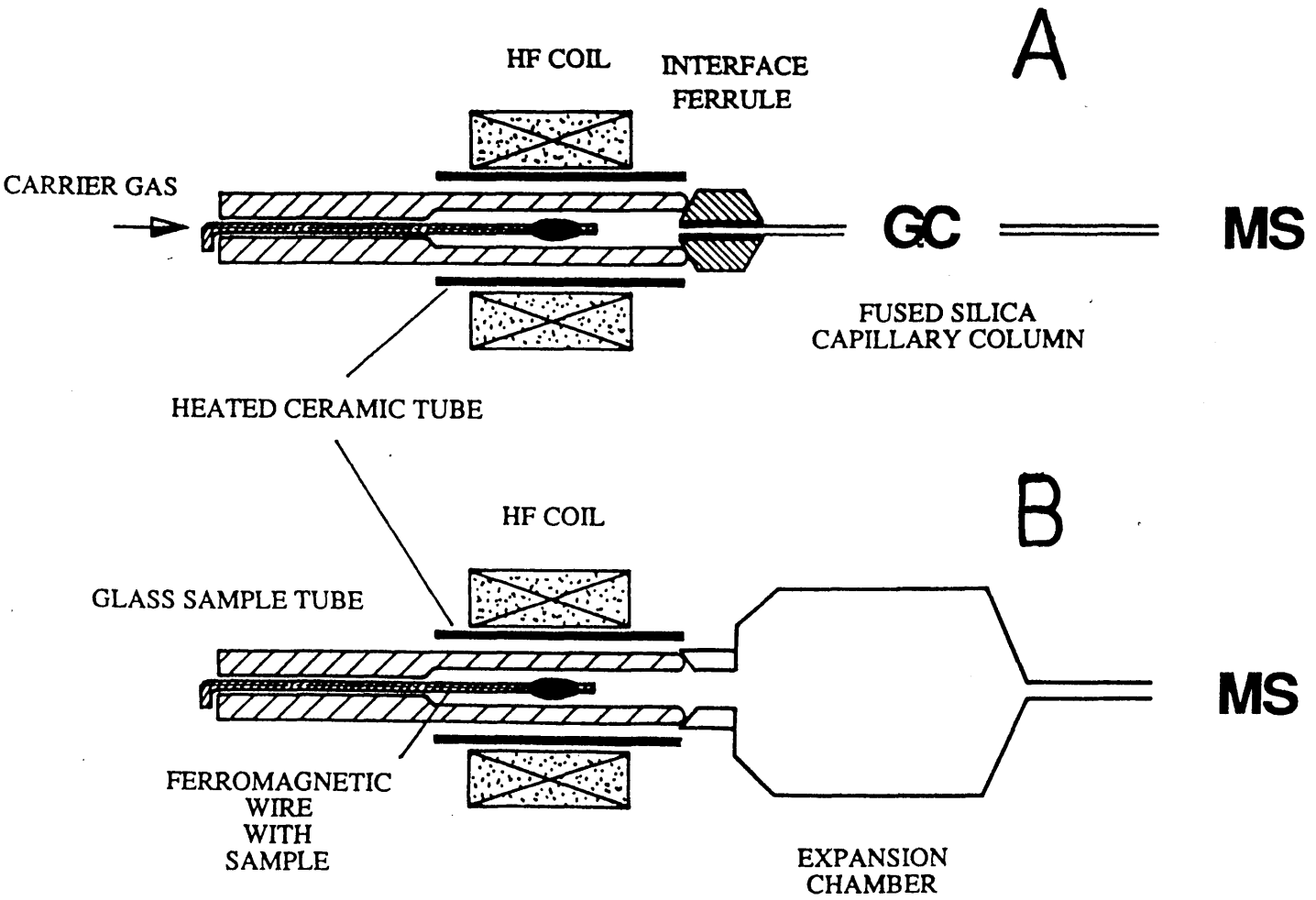
The pyrolysis unit used (FOM-3LX) (Figure 2.4 B) has been described previously (Boon *et al.*, 1984). The pyrolysis is performed by the inductive heating of the sample on a ferromagnetic wire. The

volatile fragments enter the ion source of the mass spectrometer (Balzers QMA 150/QMG 511) through a heated expansion chamber and ionised by electron impact (17eV). The mass range 20-220 a.m.u were scanned at 10 scans/second (average 200).

2.4.3 Platinum filament pyrolysis.

Mass spectrometry was performed on a Jeol DX-303 double focussing mass spectrometer with a Jeol DCI unit and a Jeol DA-5000 data system.

Samples were dried, from particulate suspension or solution, onto the platinum sample loop of the MS probe. The wire was heated, resistively at a rate of 790°C/min upto 830°C. The evaporised sample was then ionised by either electron impact (17 eV) or chemical ionisation with ammonia at 20 Pa. The accelerating voltage was 3 KV, the scan speed one second and the dynamic resolution was 1200.


A
HF COIL
INTERFACE
FERRULE
CARRIER GAS
GC
MS
FUSED SILICA
CAPILLARY COLUMN
HEATED CERAMIC TUBE
B
HF COIL
GLASS SAMPLE TUBE
MS
FERROMAGNETIC
WIRE
WITH
SAMPLE
EXPANSION
CHAMBER

2.5 METHYLATION ANALYSIS.

The method used was essentially that of Hakomori, (1964) and Lomax *et al.*, (1983; 1985). Samples were freezer milled as described previously and dried overnight, under vacuum, over P_2O_5. Approximately 10 mg samples were then accurately weighed out into soviral tubes and sonicated with 1ml of dry DMSO containing 0.151 mg methyl allose.

Methyl sulphinyl sodium was then prepared by adding 600 mg of petroleum ether washed sodium hydride to 12 ml of dry DMSO and sonicated under N_2 at 60°C for 1.5 hours. One ml of the methyl sulphinyl sodium was then added to the samples under N_2, sonicated for 30 mins and left at room temperature overnight.

Methyl iodide (1 ml) was then added to frozen samples in aliquots of 0.1ml x 3, 0.2 ml x 1 and 0.5 ml x 1 allowing 15 mins between each addition. The samples were then left for 2 hours after which methylation should have been complete.

The samples were then added to 20 ml of 5.5 M NaCl, the tubes being washed out with a further 5 ml. The pH was then adjusted to < 1.5 using 2N HCl. The solution was then extracted x 3 with chloroform, the chloroform extracts were then extracted x 1 with 0.05 M HCl and x 4 with water.

The chloroform extracts were then dried in 50 ml RB flasks and transferred back to soviral tubes containing 0.5 mg of quebrachitol. The chloroform was then evaporated off and 1 ml of 90% formic acid was added, under N2, sonicated and heated at 100°C for 5 hours. The

formic acid was then evaporated off and 1 ml of 0.25 M H_2SO_4 was added under N_2, sonicated and left overnight at 100°C.

The acid was then removed by adding an excess of Amberlite IRA 400 (14-52 mesh) in the bicarbonate form and left for 30 mins, filtered into 50 ml RB flasks and evaporated. The samples were then redissolved in 1 ml of water and reduced by adding a small amount of sodium borodeuteride and left for 3 hrs. Excess borodeuteride was destroyed by adding 50 % aqueous acetic acid and evaporated to dryness x 4 with acid methanol and x 1 with methanol.

Acetic anhydride (1 ml) was added, sonicated and heated at 100°C overnight. Excess acetic anhydride was co-distilled off with toluene and the samples were then transferred to soviral tubes with chloroform and water. The aqueous layer was then removed and the chloroform layer washed a further three times with water then dried. The samples were then redissolved in 0.5 mls acetone for injection.

The permethylated sugars were then analysed by the method of Lomax *et al.*, (1985). Samples were run on 50 m x 0.33 mm (i.d) wall coated fused silica columns of SP-1000, CP-SIL 88 (both, 0.22 µm film, Chrompak) and a fused silica bonded phase column of BP-1 (0.5 µm film, S.G.E). Analysis was performed on Carlo-Erba 4160 and 5160 GC's with on column injection and flame ionisation detection. GC-MS was used for identification of peaks on BP-1. The gradients used were, 45°C up to 210°C at 39.9°C/min for CP-SIL 88 at 130 Pa, 50°C up to 220°C at 99.9°C/min for SP-1000 at 140 Pa and 50°C up to 190°C at 99.9°C/min for BP-1 at 130 Pa. The results were integrated from retention coefficients calculated from the internal standards (allose and

quebrachitol) by a Spectra Physics SP4270 integrator linked to a Prime 550 computer via an RS232 interface (Lomax & Conchie, 1982).

2.6 ACETALATION/METHYLATION ANALYSIS.

The method used was essentially that of DeBelder & Norman (1968) and Lomax *et al.*, (1983). Samples were treated and weighed as for methylation analysis. Two ml of dry DMSO was added with 20 mg of *p*-toluene sulphonic acid. Approximately 2 ml of methyl vinyl ether was then condensed into the tubes. The tubes were sealed, mixed and kept at 15°C for 3.5 hours. The tubes were then centrifuged and the supernatant removed and the residues were washed x 3 with dry acetone, then dried overnight over P_2O_5.
The samples were then treated as for methylation analysis.

2.7 THIOACIDOLYSIS.

I would like to thank Dr C Lapierre (INRA, Grignon, France) for performing the thioacidolysis analysis.

The method used was that of Lapierre *et al.*, (1983; 1985) and pre-methylation of the samples was performed as described by Lapierre & Rolando, (1988).

Approximately 1 mg samples were pre-methylated with diazomethane

which was prepared by reacting 2 g of N-methyl-nitroso-*p*-toluenesulphonamide, in 20 mls of 2-methoxyethanol/diethylether mixture (1/1, $^{v}/_{v}$) with 10 ml of 60 % ($^{w}/_{w}$) KOH (aq). One ml of this was then added to the samples and was allowed to stand for 24 hours at room temperature, this was repeated three times. The solvent was then evaporated off and the methylated samples were freeze dried. The freeze dried samples were then dissolved in 10 mls of dioxane/ethanethiol mixture (9/1, $^{v}/_{v}$) in 0.2M BF3 etherate. This was allowed to stand for four hours at 100°C. The cooled reaction mixture was then adjusted to pH 3.4 with 0.4M $NaHCO_3$ (aq) and extracted with dichloromethane. Hexacosane (0.107 mg) in dichloromethane was added (internal standard) and the extracts were dried over Na_2SO_4, evaporated and redissolved in 1 ml of dichloromethane. The samples were then silylated by adding 10 µl of sample to 50 µl of BSTFA and 5 µl pyridine and left at room temperature for 24 hours.

GC and GC-MS were performed on a Girdel model 30 GC fitted with a moving needle type injector, a fused silica capillary column (50 m x 0.32 mm (i.d) 1 µm film thickness, CPSIL 5CB, Chrompak) and combined with either a flame ionisation detector or with a Nermag R 10-10B quadropole spectrometer operating in electron impact mode (70eV). The carrier gas was helium (1.5 bar inlet pressure) and the temperature was programmed from 180°C-280°C (+5°C/min) with the injector at 260°C and the detector at 280°C. Each analysis was performed on 5 µl of silylated sample. Integration was performed by a Shimadzu integrator.

2.8 SPECTROSCOPY.

2.8.1 InfraRed spectroscopy (IR).

I would like to thank Dr J.D.Russel and Mr A.R.Fraser of the Macaulay Land Use Research Institute, Aberdeen for performing the IR analysis of the LCC's.

All infrared spectra, except those of ryegrass and barley straw LCC's were recorded from 2.5 mg 13 mm KBr disc on a Pye Unicam SP3-200 IR spectrophotometer.

The spectra for DMSO soluble fractions were recorded from 0.8 mg 13mm KBr disc on a Perkin Elmer 580B spectrophotometer.

2.8.2 Ultra Violet Spectroscopy (U.V).

LCC's were dissolved in 50% aqueous DMSO ($^{v}/v$) and their U.V spectra recorded on a Perkin Elmer 124 double beam spectrophotometer.

2.8.3 Nuclear magnetic resonance spectroscopy (NMR).

I would like to thank Mr D.Rycroft (Dept of Chemistry, University of Glasgow, Glasgow) for performing the solution state NMR and the SERC solid state NMR service (Durham University, Durham) for the solid state NMR analysis.

^{13}C NMR spectroscopy of the lignin carbohydrate complexes were recorded in D6-DMSO solution on a Bruker 200 MHz FT spectrometer operating at 50 MHz. Chemical shifts are expressed with respect to tetramethylsilane and set from the central resonance of DMSO at 39.5 ppm. The ^{1}H NMR spectra of FA-ARA was recorded at 200 MHz in CD3OD solution. Two dimensional spectra were obtained by the COSY procedure. Solid state CP/MAS NMR spectra were obtained at the SERC solid-state NMR service, Durham, on a Varian VXR-300 spectrophotometer operating at 75.4 MHz at room temperature for ^{13}C. Contact time was normally 1 ms, aquisition time 9.6 or 19.2 ms and relaxation delay 2 s, but contact times were varied up to 10 ms to check whether heterogeneous relaxation times distorted the relative signal intensities

2.8.4 Electron spin resonance spectroscopy (ESR).

I would like to thank Mr.D.MacPhail and Dr B.Goodman of the Macaulay Land Use Research Institute, Aberdeen, for performing the ESR analysis.

ESR spectra were recorded on a Varian E104 spectrometer operating at approximately 9.5 GHz (x-band) frequency.

The spectra were recorded from solid samples at room temperature with a microwave power of 1 mW, a modulation amplitude of 2.5 G and a receiver gain of 6.3×10^{11} for ryegrass LCC and 2×10^{4} for the barley LCC.

3.0. RESULTS/DISCUSSION.

3.1 MILD ACID HYDROLYSIS.

3.1.1 Introduction.

Barley straw (*Hordeum vulgare* c.v Golden Promise) and perennial ryegrass (*Lolium perenne* c.v Perma) were chosen as the starting materials for this research as it was thought that the poorly lignified ryegrass and, relatively, highly lignified barley straw may provide an interesting contrast in the nature and distribution of their phenolic components and in their linkage patterns to the carbohydrates. Straw is a natural source of relatively pure secondary cell walls whereas ryegrass contains a mixture of primary and secondary cell walls. In order to facilitate direct comparison of the two and to increase the phenolic content of the starting material, a non mesophyll-fraction of ryegrass was used.

As mentioned in the introduction, arabinose has been implicated as being of particular importance in phenolic-carbohydrate linking (Chesson *et al.*, 1983) and bridging the hemicellulose to the lignin (Chesson, 1988). It was therefore thought that a useful starting point for this investigation would be the selective removal of arabinose.

Glycosidic linkages vary greatly in their response to acid hydrolysis (Lindberg *et al.*, 1975). Some, such as the Glc ß 1-4 Glc linkages found in cellulose, are very stable and need extensive and harsh treatment for complete hydrolysis. Others, such as those between the xylose units

in a ß 1-4 xylan, are less stable and need less extensive, or harsh, treatment. Amongst the weakest linkages known in cell walls are those involving arabinofuranose, especially those between arabinofuranosyl residues and arabinofuranose-xylopyranose residues. It should therefore be possible to selectively cleave these units by using very mildly acidic conditions (Fry, 1982).

3.1.2 Oxalate hydrolysis.

Hydrolysis with oxalic acid brought about a 26.3% weight loss in barley straw and a 36.4% weight loss in ryegrass. Table 3.1.2.1 shows the chemical compositions of barley straw and ryegrass, before and after oxalate hydrolysis. Mild acid treatment solubilised a considerable proportion of the arabinose with, 68.41% and 83.06%, of the original, being solubilised in barley and ryegrass respectively. The two starting materials showed a considerable difference in response with regards to phenolics. In barley straw, relatively little of the phenolic material was solubilised, (47.82% of the phenolic acids and 19.01% of the lignin), whereas in ryegrass 70.91% of the phenolic acids and 60.31% of the lignin was released. This could suggest a higher phenolic substitution of the arabinose in the ryegrass compared to barley straw or may reflect the higher solubilisation of arabinose in the ryegrass. It can also be seen that ferulic acid and the dimeric acids were more readily solubilised than *p*-coumaric acid, which seems to have been preferentially retained in both the ryegrass and barley straw.

TABLE 3.1.2.1. Chemical composition of barley straw (BS) and the non-mesophyll fraction of ryegrass (RG) and their residues (BS & RG HYD) after oxalate hydrolysis.

Results expressed as % dry matter (% of original).

	BS	BS HYD (73.70)	RG	RG HYD(63.60)
RHAMNOSE	0.20	0.05 (18.43)	0.14	0.15 (68.14)
FUCOSE	0.11	0.05 (33.50)	0.27	0.28 (65.95)
ARABINOSE	2.24	0.96 (31.59)	4.73	1.26 (16.94)
XYLOSE	20.49	22.73 (81.75)	21.98	23.98 (69.39)
MANNOSE	0.37	0.24 (47.81)	1.27	0.23 (11.52)
GALACTOSE	0.78	0.17 (16.06)	5.78	0.28 (3.08)
GLUCOSE	28.39	27.22 (70.66)	27.38	38.27 (88.89)
URONIC ACIDS	16.06	15.99 (73.38)	14.47	10.26 (45.09)
TOTAL CARBOHYDRATE	**47.63**	**51.92**	**61.55**	**64.45**
TRUXILLIC ACID	0.20	0.11 (40.54)	0.11	0.04 (23.13)
cis-COUMARIC ACID	0.03	0.04 (98.27)	0.05	0.03 (38.16)
trans-COUMARIC ACID	0.26	0.30 (85.04)	0.23	0.15 (41.48)
cis-FERULIC ACID	0.02	0.02 (73.70)	0.17	0.06 (22.45)
trans-FERULIC ACID	0.20	0.14 (51.59)	0.51	0.16 (19.95)
DIFERULIC ACID	0.06	0.04 (49.13)	0.08	0.02 (15.90)
TOTAL PHENOLIC ACIDS	0.78	0.64 (52.18)	1.14	0.45 (29.09)
LIGNIN	9.50	10.44 (80.99)	4.71	2.94 (39.69)
TOTAL PHENOLICS	**10.28**	**11.08**	**5.85**	**3.39**
TOTAL	**57.13**	**63.00**	**66.99**	**77.39**

(Lignin = acetyl bromide phenolics - total phenolic acids).

Methylation analysis of the starting materials (Table 3.1.2.2) shows a general consistency with previously published data (Lomax *et al.*, 1983; Gordon *et al.*, 1985), although both barley straw and ryegrass showed a lower recovery of 2,3,6-*O-Me* glucose. Comparison of the methylated sugar analysis with alditol acetate analysis shows several discrepancies. This demonstrates one of the major problems associated with alditol acetate analysis due to the wide range of labilities of glycosidic linkages in polysaccharides compared to those between permethylated sugars (Lomax *et al.*, 1983).

Acetalation/methylation analysis of the starting materials and oxalate hydrolysed barley straw residue (Table 3.1.2.3) clearly showed that, in barley straw, at least, the di-substituted sugars would seem to be most resistant to oxalate hydrolysis with 59.34% of the original 2,5-arabinose and 148.17% of the 2,3-xylose retained after extraction. The high retention of phenolic material would then suggest that they are mainly di-substituted especially to xylose, and that di-substitutions stabilise these, usually weak, glycosidic bonds. This is unlikely to be due to electron delocalisation (Jarvis, personal communication), but could possibly be due to steric hindrance.

Table 3.1.2.2. Methylation analysis of barley straw and non-mesophyll ryegrass.

Results expressed as % dry matter.

	Barley	**Ryegrass**
2,3,4-*O-Me*-ARA	0.053	0.126
2,3,5-*O-Me*-ARA	1.747	3.556
2,3-*O-Me*-ARA	0.299	0.519
2,5-*O-Me*-ARA	0.391	0.559
3,5-*O-Me*-ARA	0.265	0.570
UNME-ARA	0.091	-
TOTAL-ARA	**2.846**	**5.330**
2,3,4-*O-Me*-XYL	0.320	0.721
2,3-*O-Me*-XYL	14.366	10.776
2,4-*O-Me*-XYL	0.177	0.495
2-*O-Me*-XYL	2.179	3.821
3-*O-Me*-XYL	1.337	1.507
UNME-XYL	0.784	1.984
TOTAL-XYL	**19.163**	**19.304**
2,3,4-*O-Me*-RHA	0.127	0.126
3-*O-Me*-RHA	0.135	0.103
4-*O-Me*-RHA	0.076	-
TOTAL-RHA	**0.338**	**0.229**
2,3,4-*O-Me*-FUC	+	-
2,4-*O-Me*-FUC	0.216	-
2-*O-Me*-FUC	0.316	-
TOTAL-FUC	**0.532**	-
2,3,4,6-*O-Me*-GLC	0.939	0.219
2,3,4-*O-Me*-GLC	0.129	1.149
2,3,6-*O-Me*-GLC	26.908	21.883
2,4,6-*O-Me*-GLC	0.913	2.371
2,3-*O-Me*-GLC	1.277	0.727
2,6-*O-Me*-GLC	1.094	1.949
3,6-*O-Me*-GLC	0.605	0.365
2-*O-Me*-GLC	0.209	0.226
6-*O-Me*-GLC	0.185	-
UNME-GLC	0.318	0.355
TOTAL-GLC	**32.577**	**29.244**
2,3,4,6-*O-Me*-GAL	0.551	0.872
2,3,4-*O-Me*-GAL	0.138	0.272
2,3,6-*O-Me*-GAL	0.126	0.222
2,4,6-*O-Me*-GAL	0.519	+

4,6-*O*-*Me*-GAL	0.116	0.114
TOTAL-GAL	**1.450**	**1.480**
2,3,6-*O*-*Me*-MAN	0.349	0.077
3,4,6-*O*-*Me*-MAN	+	-
2,6-*O*-*Me*-MAN	+	+
TOTAL-MAN	**0.349**	**0.077**
TOTAL CARBOHYDRATE	**57.255**	**55.664**

+ = less than 0.05%.

TABLE 3.1.2.3. Acetalation/Methylation analysis of starting materials and oxalate hydrolysed barley straw.

Results expressed as % dry matter (% of original).

ALKALI LABILE SUBSTITUTIONS	Barley	Barley oxalate residue	Ryegrass
2,5-*O*-*Me*-ARA	0.154	0.124 (59.34)	0.129
5-*O*-*Me*-ARA	0.369	0.054 (10.79)	0.598
2,3-*O*-*Me*-XYL	1.047	2.105 (148.17)	0.880
2-*O*-*Me*-XYL	1.932	0.847 (32.31)	2.040
3-*O*-*Me*-XYL	1.611	0.816 (37.33)	1.154

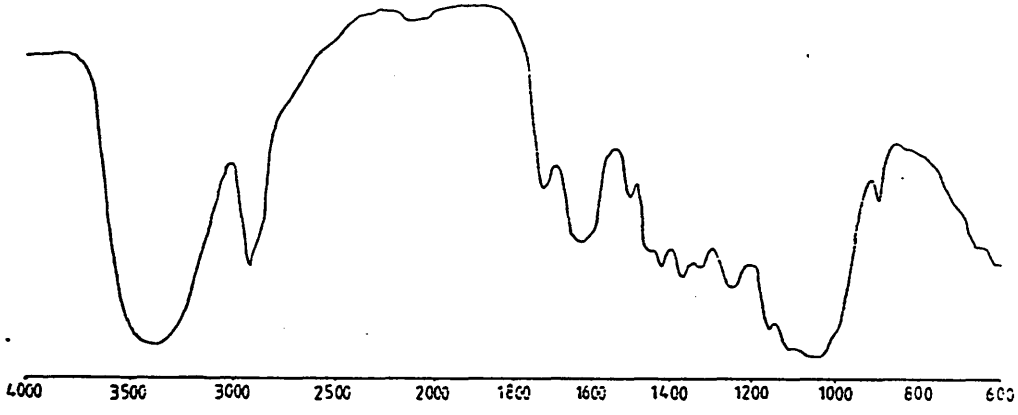
Infrared (IR) spectroscopy of the starting materials (Figures 3.1.2.1 A & 3.1.2.1 B) supports the chemical data i.e lignin (1420, 1450 (not in ryegrass) and 1500), strong pyranose ß linked C1 (896) and high acetyl (1730 and 1250). It is interesting to note that the C=O stretch (1730) is more intense in ryegrass than barley straw, whereas the acetyl band at 1210 is similar in both. Interestingly, compared with the results of Russell et al., (1988), no trace of silica (800) could be seen in the barley straw. Comparison of the spectra for the original materials and oxalate hydrolysed materials (Figures 3.1.2.2 A and 3.1.2.2 B) show no apparent change in gross composition.

The solubilised fractions from oxalate hydrolysis were found, by thin layer chromatography (TLC, solvent system 1), to be highly complex mixtures of monomeric and oligomeric carbohydrate and unresolved phenolic material. The oxalate soluble fractions were freeze dried and extracted with methanol to produce methanol soluble and methanol insoluble fractions (water soluble). This fractionation brought about the solubilisation, in methanol, of 65.9% for barley straw and 54.13% for ryegrass, representing 17.33% and 19.70% of the starting materials respectively.

As can be seen from their chemical compositions (Table 3.1.2.4), the bulk of the arabinose and phenolic material would seem to be soluble in methanol with approximately 50% of the barley straw phenolic acids and over 60% of the ryegrass phenolic acids going into the methanol fraction. Further analysis showed that methanol soluble phenolics were predominantly ferulic acid.

Figure 3.1.2.1 Infrared spectra of A) Barley straw and B) Ryegrass.

A


4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
WAVENUMBER (Cm⁻¹)

B

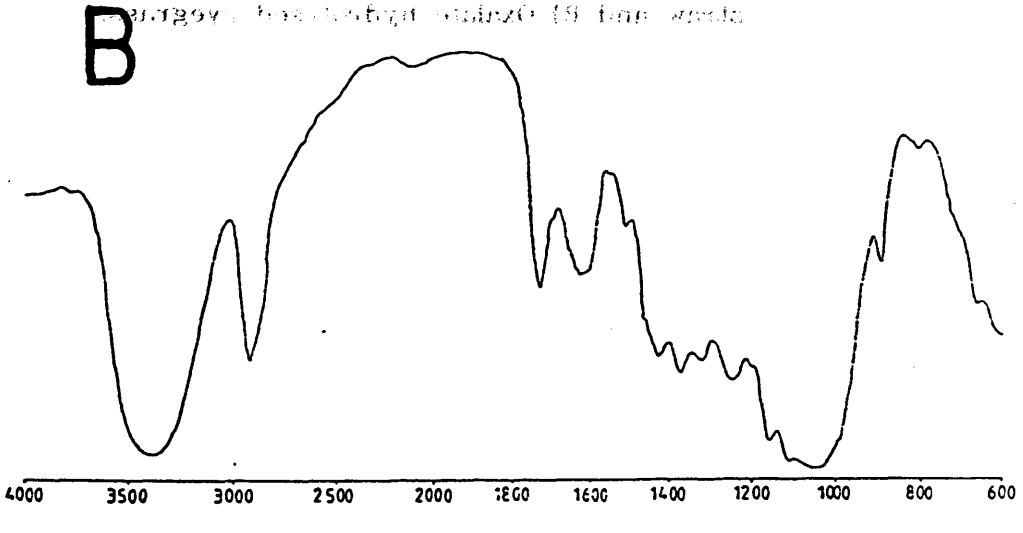
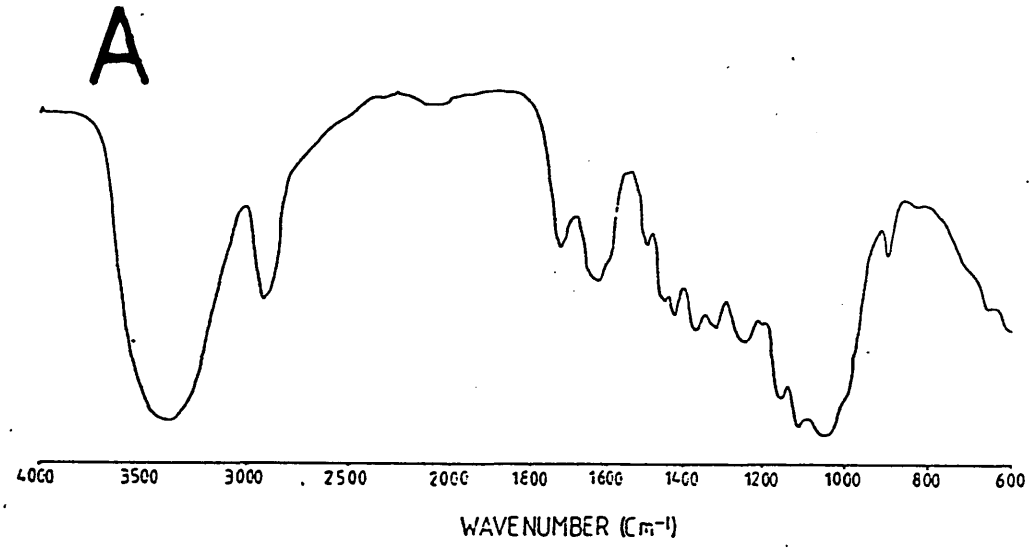

4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
WAVENUMBER (Cm⁻¹)

Figure 3.1.2.2 Infrared spectra of A) Oxalate hydrolysed barley straw and B) Oxalate hydrolysed ryegrass.


A
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
WAVENUMBER (Cm⁻¹)

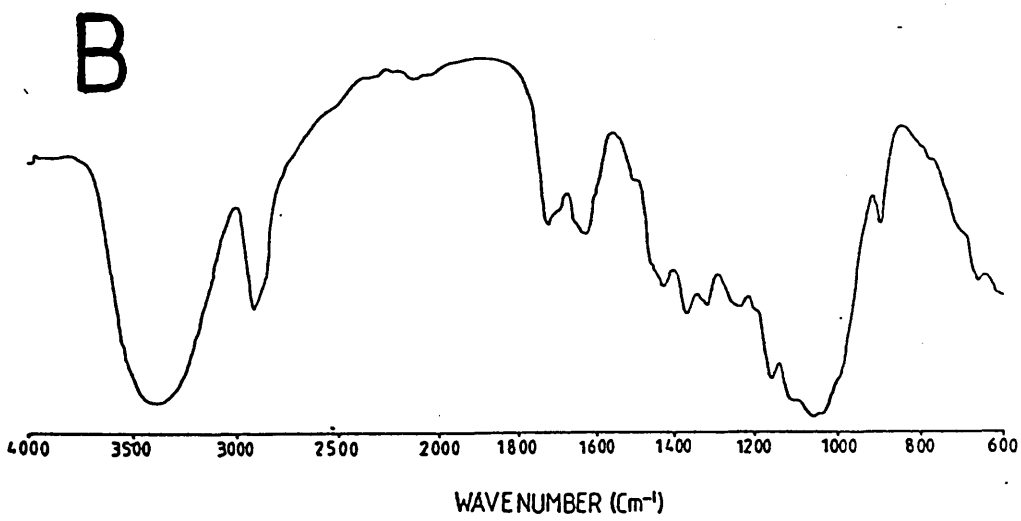

B
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
WAVENUMBER (Cm⁻¹)

TABLE 3.1.2.4. Chemical composition of hydrolysate fractions (Barley straw (BS) and ryegrass (RG) water (H_2O) and methanol (MeOH) soluble.

Results expressed as % dry matter (% of original in starting materials).

	BS H2O(8.97)	BS MeOH(17.33)	RG H2O(16.7)	RG MEOH(19.7)
RHAMNOSE	-	0.17 (14.73)	1.03 (122.86)	-
FUCOSE	0.50 (40.77)	0.25 (39.39)	0.45 (27.83)	-
ARABINOSE	1.61 (6.45)	14.38 (111.25)	4.49 (15.85)	23.09 (96.17)
XYLOSE	36.76 (16.09)	38.86 (41.98)	20.61 (15.66)	34.80(31.19)
MANNOSE	0.66 (16.00)	0.96 (69.32)	1.92 (25.25)	0.32 (51.49)
GALACTOSE	2.97 (34.16)	4.12 (91.54)	2.47 (7.14)	4.61 (15.71)
GLUCOSE	13.26 (1.32)	5.22 (3.19)	34.32 (20.93)	2.14 (1.54)
URONIC ACIDS	6.02 (3.36)	9.87 (10.65)	11.00 (12.69)	10.97 (14.93)
TOTAL CARBOHYDRATE	55.76	63.96	65.29	67.9
TRUXILLIC ACID	0.00 -	0.34 (29.46)	-	0.14 (25.07)
cis-COUMARIC ACID	0.01 (0.12)	0.07 (40.44)	-	0.08 (31.52)
trans-COUMARIC ACID	0.08 (23.92)	0.41 (27.33)	-	0.59 (50.53)
cis-FERULIC ACID	0.04 (17.94)	0.13 (112.65)	-	0.38 (44.03)
trans-FERULIC ACID	0.17 (7.62)	1.08 (93.58)	-	2.20 (84.98)
DIFERULIC ACID	0.08 (11.96)	0.21 (60.66)	-	0.20 (49.25)
TOTAL PHENOLIC ACIDS	0.38 (4.37)	2.25 (49.99)	-	3.54 (61.17)
LIGNIN	4.46 (4.21)	11.96 (21.82)	-	7.87 (32.92)
TOTAL PHENOLICS	4.84	14.21	3.46	11.41
TOTAL	66.62	78.17	79.74	0.34

Infrared analysis of the barley straw and ryegrass MeOH fractions (Figures 3.1.2.3 A & 3.1.2.3 B) clearly show high acetyl and ester contents, little can be ascertained from the lignin regions (1400-1650) and little detail can be determined from the carbohydrate region. Comparison of the infrared spectra for the water soluble fractions (Figures 3.1.2.4 A & 3.1.2.4 B) show some distinct differences, the large absorptions in barley straw fraction at 1400-1450 and 1500-1700 araise from carboxyl groups but not from oxalate (Russell, personnal communication). The ryegrass fraction is more similar to the MeOH fractions.

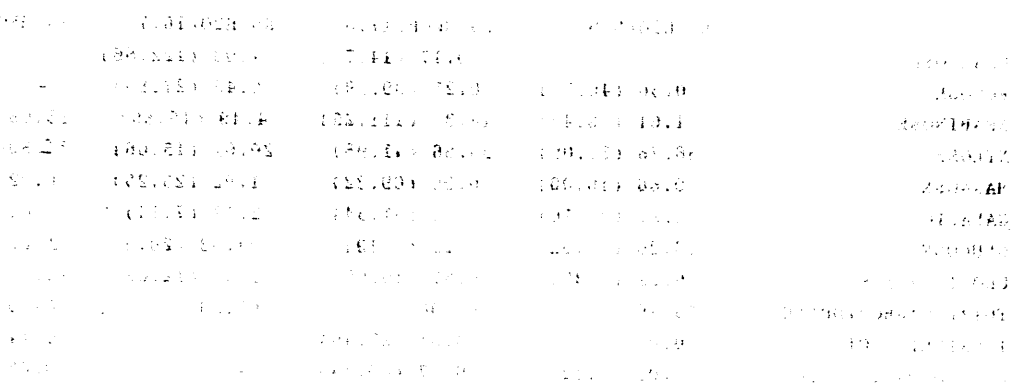
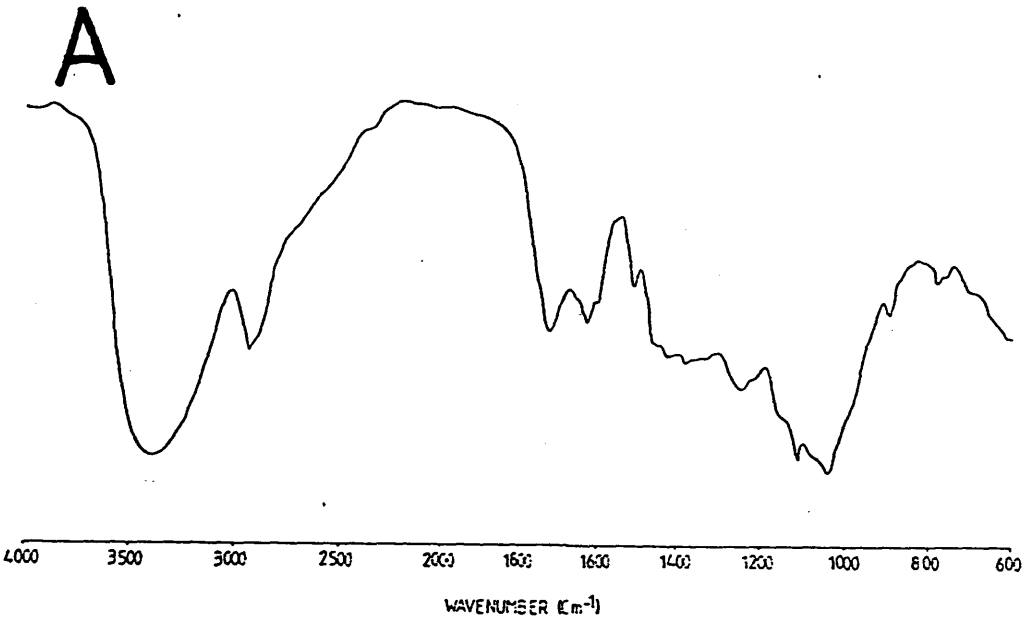


Figure 3.1.2.4 Infrared spectra of A) Barley MeOH and B) Ryegrass MeOH.


A
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
WAVENUMBER (Cm⁻¹)

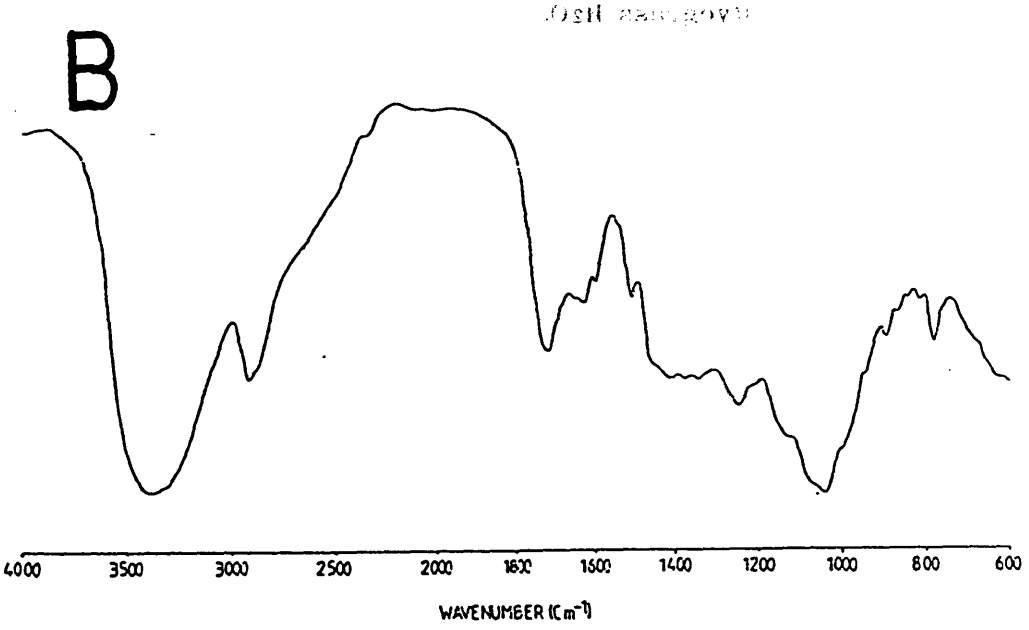
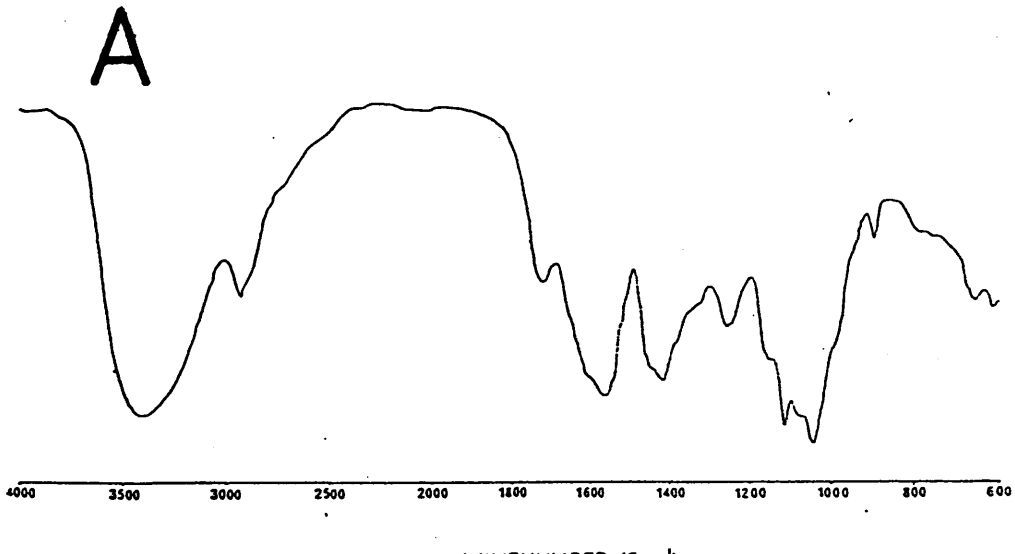
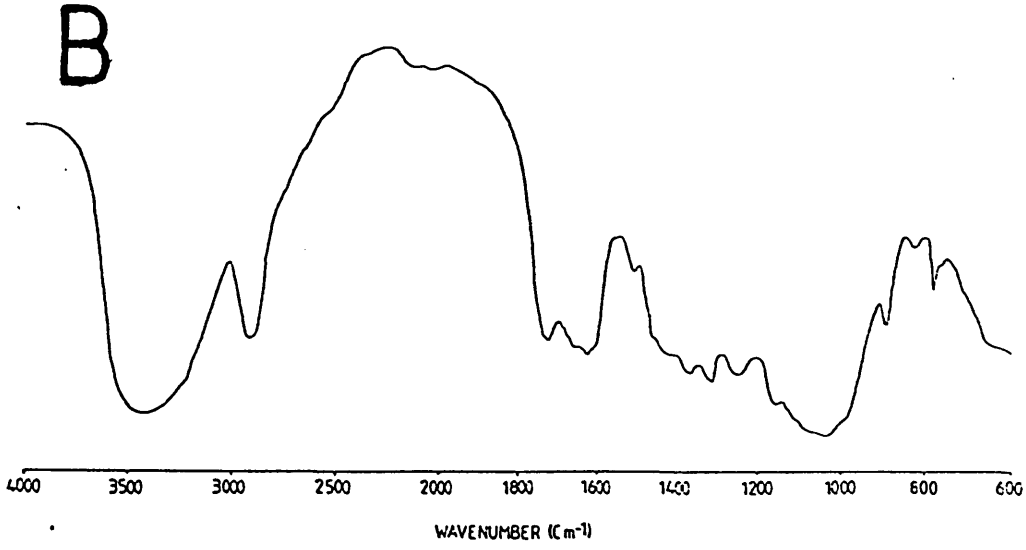

B
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
WAVENUMBER (Cm⁻¹)

Figure 3.1.2.4 Infrared spectra of A) Barley H_2O and B) Ryegrass H_2O.


A
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
WAVENUMBER (Cm⁻¹)


B
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
600
600
WAVENUMBER (Cm⁻¹)

3.1.3 Isolation and characterisation of FA-ARA.

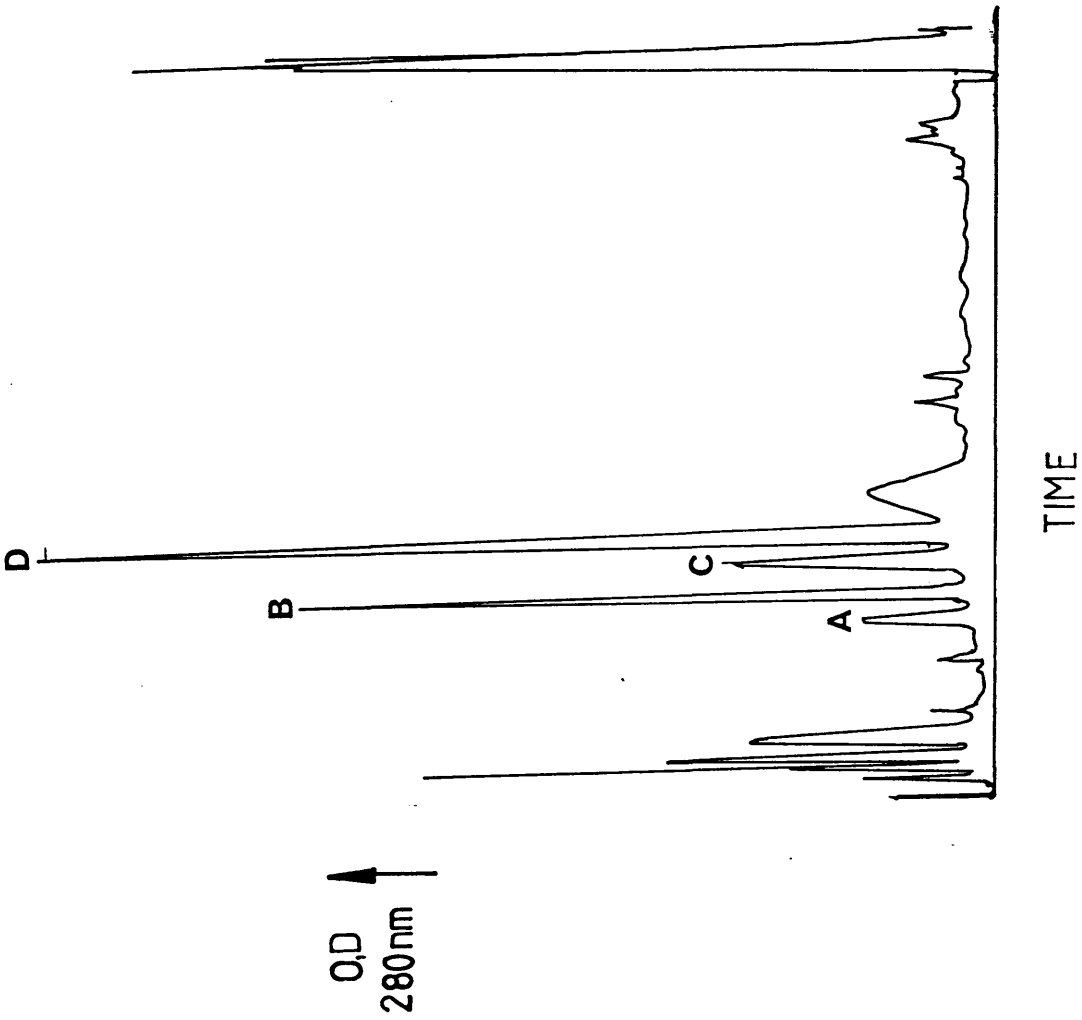
TLC (solvent system 1) of ryegrass and barley straw methanol soluble fractions clearly showed the presence of arabinose, xylose and a series of carbohydrate oligomers (probably xylan fragments). Location of phenolic compounds (by examination under U.V light) revealed two, strongly fluorescing spots at (r.f's) 0.87 and 0.80. On exposure to NH_3 the spot at 0.87 turned bright yellow/green and the spot at 0.80 turned deep blue, suggestive of ferulic acid and *p*-coumaric acid esters respectively (Fry, 1988). Both spots were found to consistently co-chromatograph with carbohydrate spots, even when run on paper chromatography. Preliminary attempts at purification by preparative TLC, and subsequent alkaline hydrolysis, suggested that the spot at 0.87 was ferulic acid, ester linked to arabinose (FA-ARA) and the spot at 0.80 was *p*-coumaric acid also ester linked to arabinose. Ferulic acid and *p*-coumaric acid (*p*-CA-ARA) were identified by TLC (solvent system 2) and arabinose by GC analysis as its alditol acetate.

In order to increase yields of these components, preparative HPLC was used as a rapid purification method. As can be seen (Figure 3.1.3.1) a number of U.V absorbing components were separated, the four major peaks designated A, B, C and D were each isolated. Analysis of the separated peaks by TLC showed that peaks A and B both ran as a single phenolic-carbohydrate spot with the same r.f as that found for the proposed *p*-coumaric acid-arabinose (0.80), thus

suggesting that the HPLC peaks A & B represented the *cis* and *trans* isomers of the *p*-coumaric acid respectively. The same was also found to be true for peaks C and D which ran with the same r.f as that for the proposed ferulic acid-arabinose spot (0.87). TLC also revealed some oligomeric carbohydrate as a contaminant. Unfortunately, yields of the peaks were very low and only peak D could be collected in significant quantities.

Once collected, peak D was further purified by molecular sieve chromatography on Sephadex G-10 to yield a bright yellow powder which, on alkali hydrolysis produced only ferulic acid (identified by TLC) and arabinose (identified as its alditol acetate by GC).

Figure 3.1.3.1 Separation of FA-ARA by reverse phase HPLC.


D
B
C
A
TIME
O.D
280nm

The infrared (IR) spectrum for FA-ARA (Figure 3.1.3.2) shows major absorption bands at 3680-3020 (O-H stretch), 2930 (C-H stretch), 1720-1670 (C=O stretch), 1630 (aliphatic C=C stretch), 1595 (aromatic C=C stretch), 1510 (aromatic C=C stretch), 1430 (CH_3-O), 1300-1240 (acetyl ester C=O stretch), 1200-1145 (unassigned), 1040 (carbohydrate C-O), 850 and 822 (possibly trisubstituted aromatic pattern, 822 not present in standard spectrum of ferulic acid). This agrees closely with the spectrum observed for 2-*O*-[5-*O*-(trans-feruloyl)-α-L-arabinofuranosyl]-D-xylopyranose (Smith & Hartley, 1983) and *O*-ß-D-xylopyranosyl-(1-4)-*O*-[5-*O*-*trans*-feruloyl)-α-L-arabinofuranosyl-(1 3)]-*O*-ß-D-xylopyranosyl-(1-4)-D-xylopyranose (Kato *et al.*, 1987). The spectrum for HPLC peak B (Figure 3.1.3.3) was, essentially, very similar to that of FA-ARA. The unassigned band at 1430, in FA-ARA, was missing, as it is in the standard spectrum for *p*-coumaric acid. The proposed tri-substitution pattern in FA-ARA (850 and 822) was, as expected, not present and have been replaced by a single band at 835 similar to a *para* substitution pattern.

Figure 3.1.3.2 Infrared spectrum of FA-ARA.

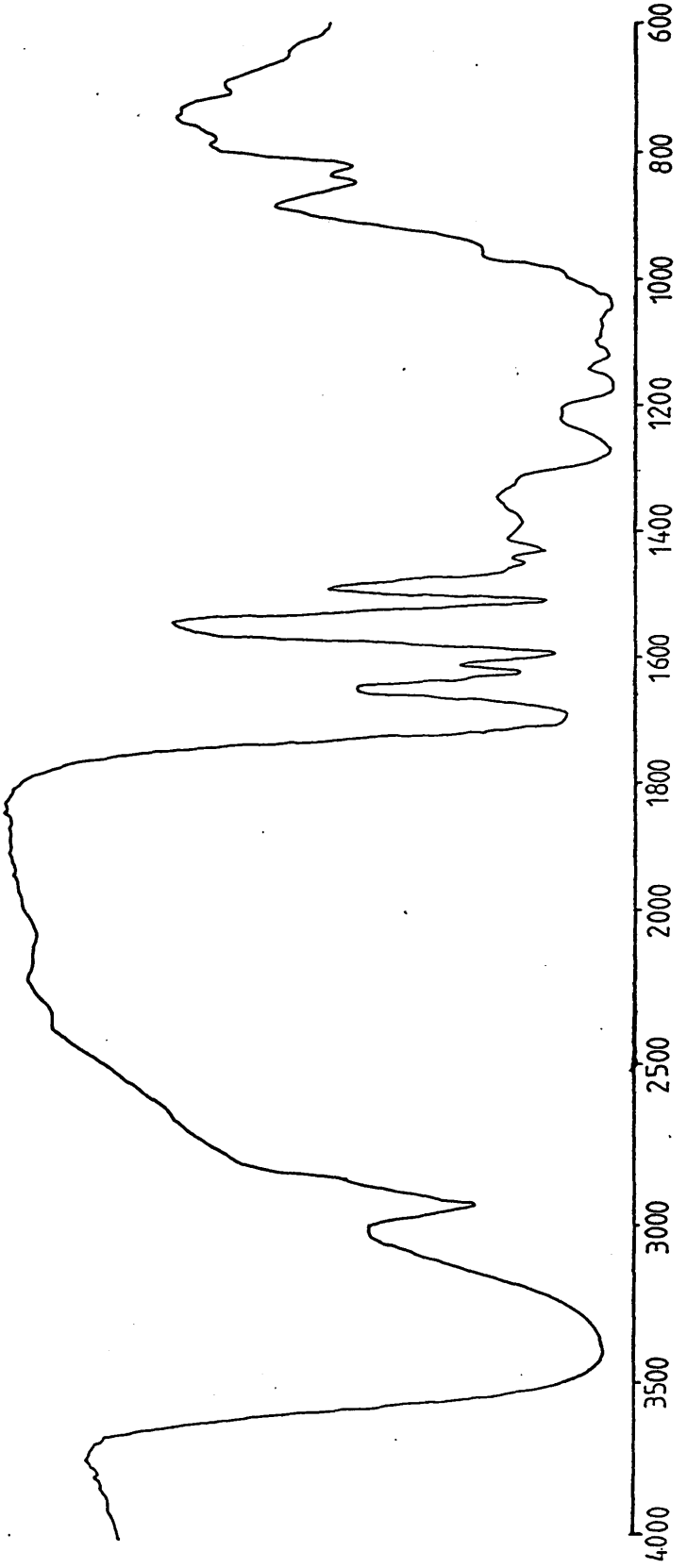

4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
WAVENUMBER (Cm⁻¹)

Figure 3.1.3.3. Infrared spectra of *p*-CA–ARA.

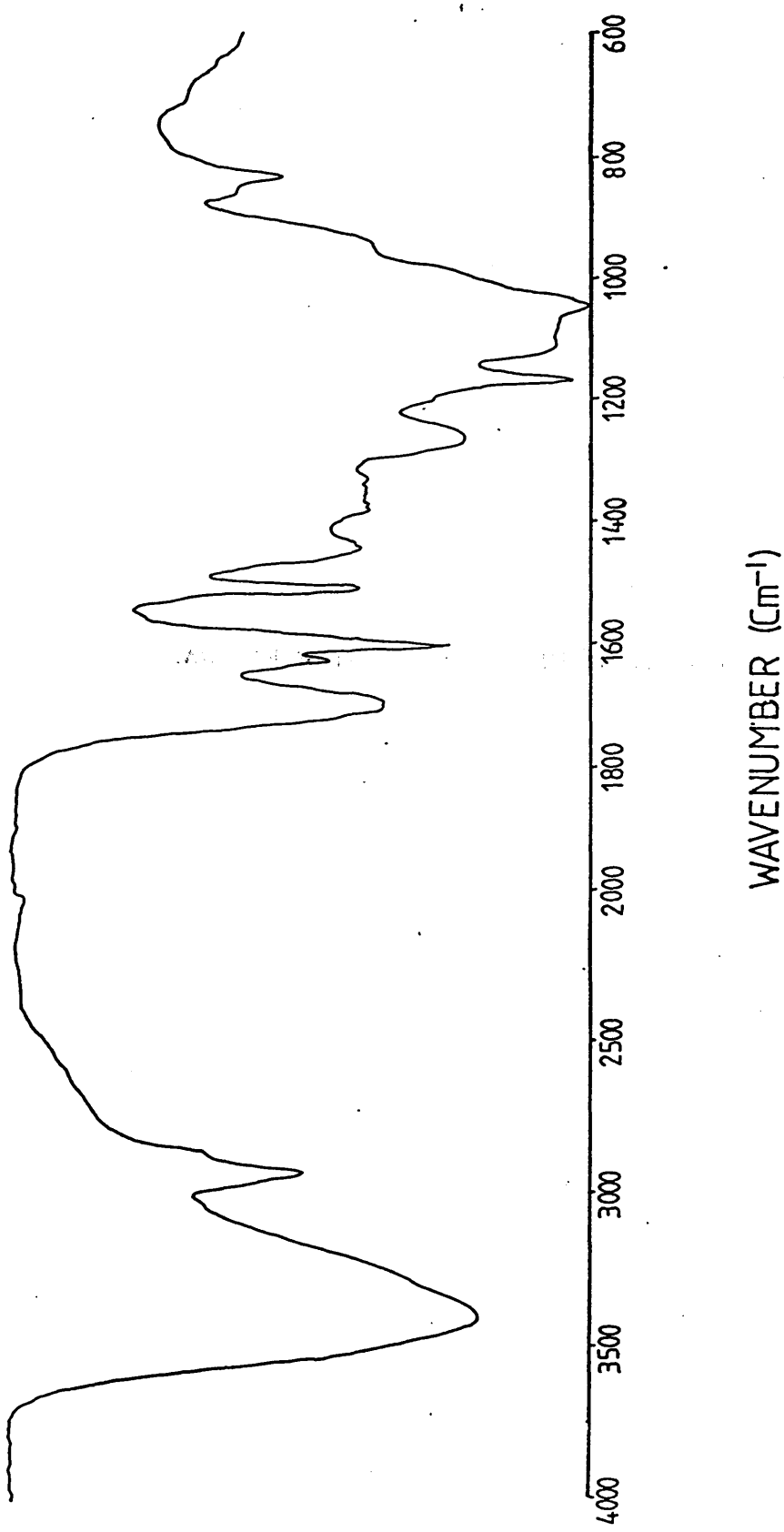
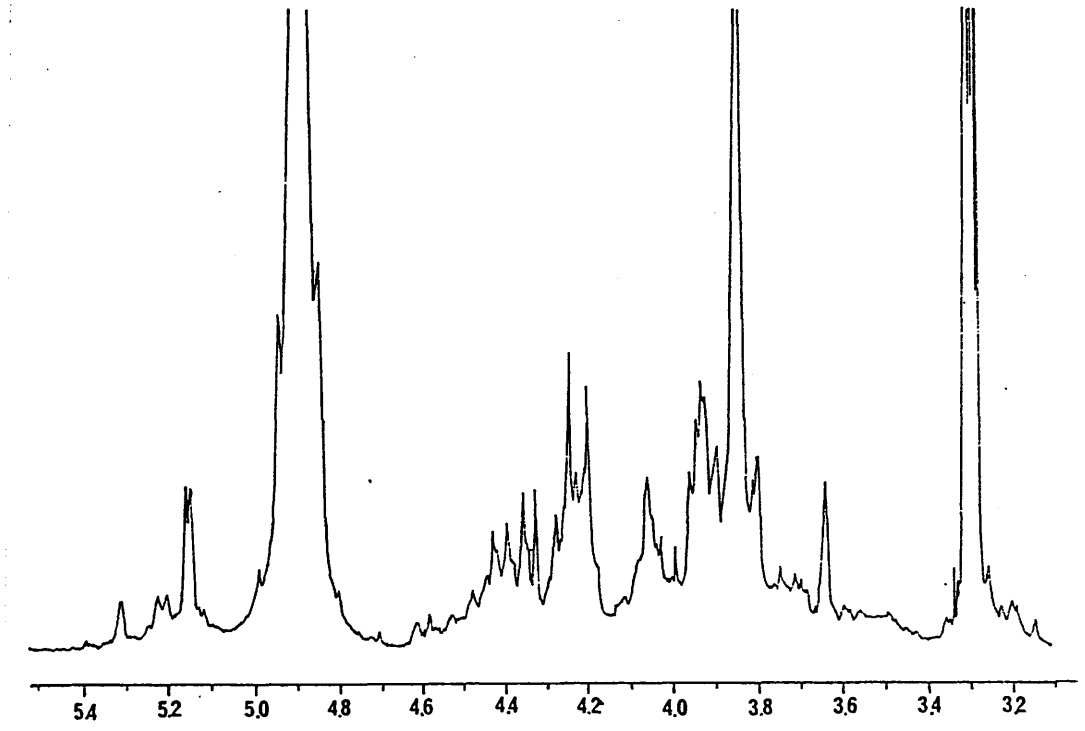
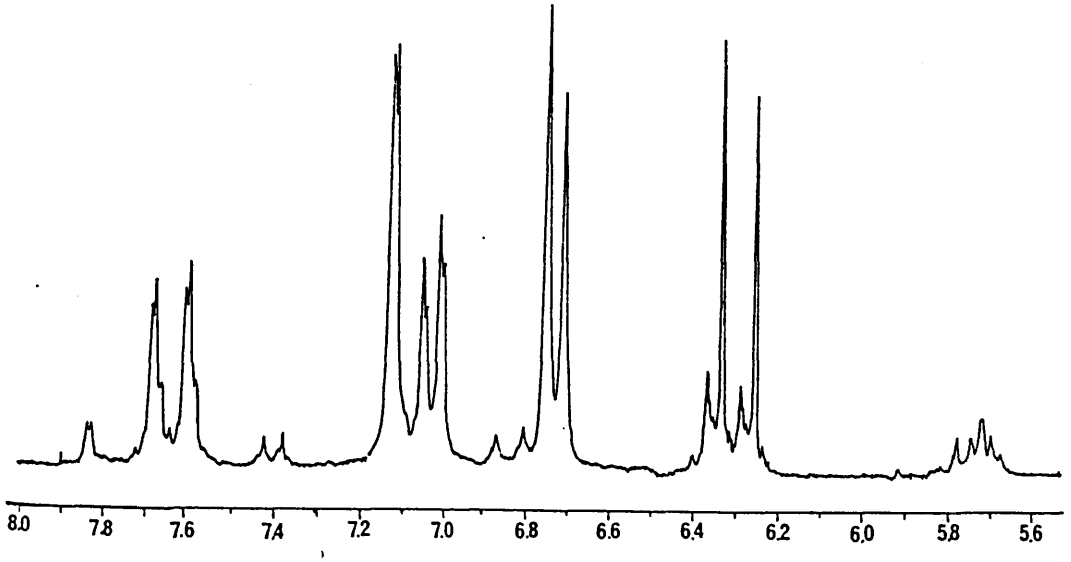

4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
WAVENUMBER (Cm⁻¹)

Figure 3.1.3.4 ^{1}H NMR spectrum of FA-ARA.


5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2


8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
6.4
6.2
6.0
5.8
5.6

^1H NMR analysis of FA-ARA gave the spectrum shown in figure 3.1.3.4 A & B, the NMR signals were assigned, as shown in table 3.1.3.1, these assignments were based upon comparison with ^1H NMR spectra for authentic α-L-arabinofuranose and *trans*-ferulic acid (Table 3.1.3.2) and comparison with published data (Smith & Hartley, 1983 ; Meuller Harvey *et al.*, 1986). Figure 3.1.3.5 demonstrates the numbering system used for designating the protons in ferulic acid and arabinose.

Figure 3.1.3.5 Numbering system for designation of protons.

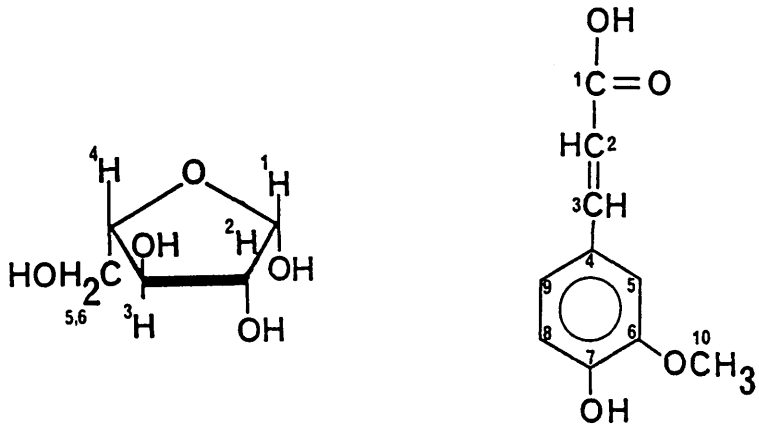


TABLE 3.1.3.1. Assignment of ^1H NMR signals for FA-ARA.

δ	J	ASSIGNMENT
7.64	16/2	FER H-2
7.11	2	FER H-5
7.02	8/2	FER H-9
6.73	8	FER H-8
6.30	16	FER H-3
5.15-5.17		ARA H-1
4.18-4.40		ARA H-5/6
3.90		ARA H-4
3.85-4.05		ARA H-2
3.85	SINGLET	FER H-10
3.50-3.70		ARA H-3

TABLE 3.1.3.2. Assignments of ^1H NMR signals for standard α-L-Arabinose and *trans*-Ferulic acid.

α-L-ARABINOFURANOSE			*TRANS*-FERULIC ACID		
δ	J	ASSIGNMENT	δ	J	ASSIGNMENT
5.08	3	H-1	7.58	16	H-2
4.85	SINGLET	METHANOL-OH	7.15	2	H-5
3.97	12/1.5	H-3	7.05	8/2	H-9
3.85	3/2	H-4	6.80	8	H-8
3.75	10/3	H-5/6	6.29	16	H-3
3.55	12/3	H-2	3.88	SINGLET	H-10
3.30	MULTIPLET	METHANOL-OCH_3			

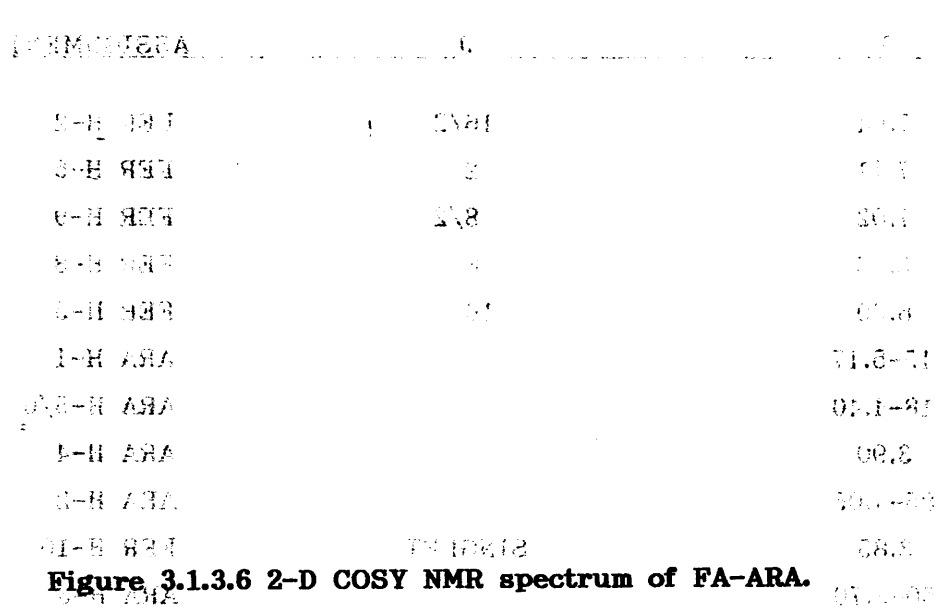
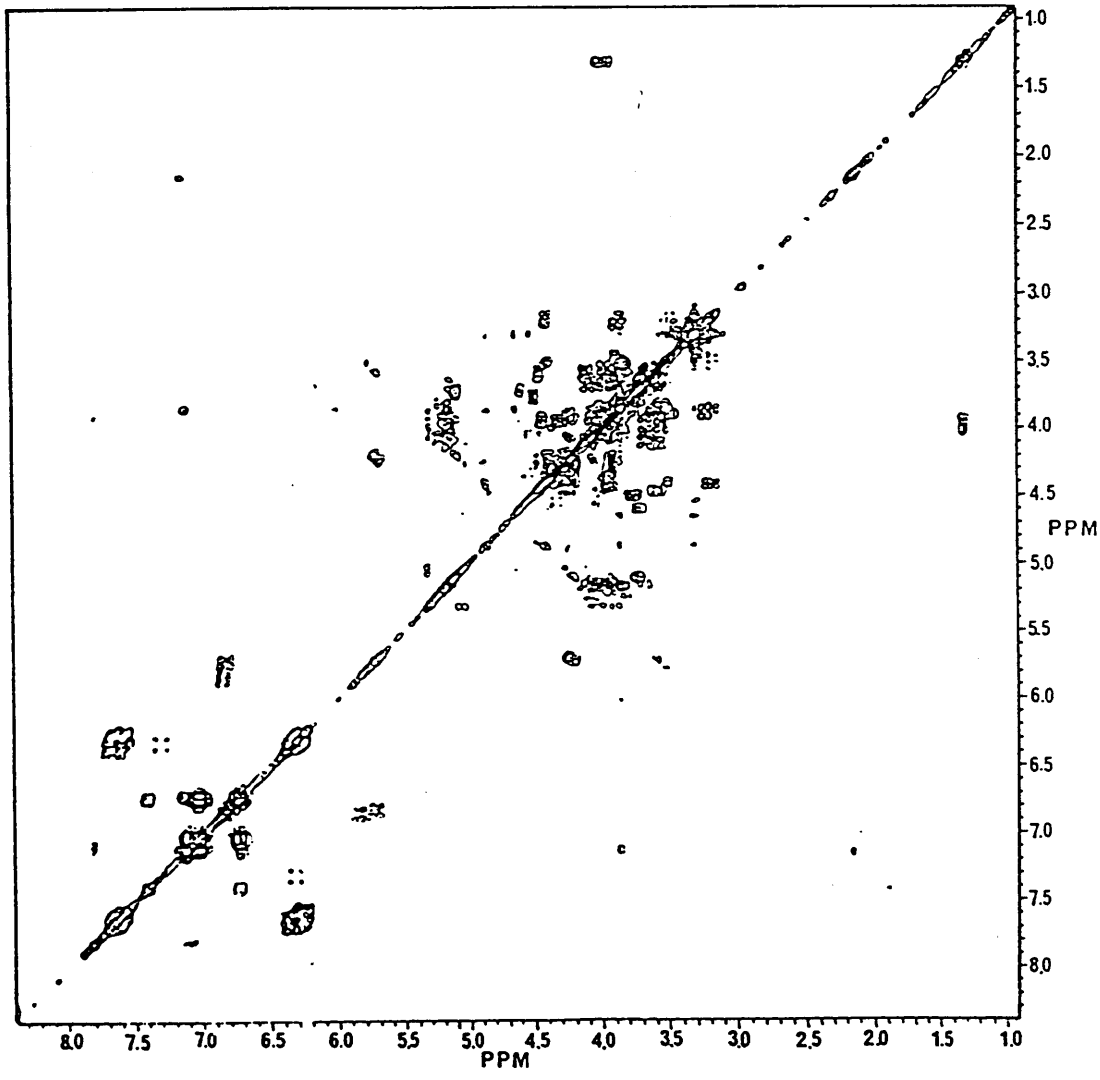


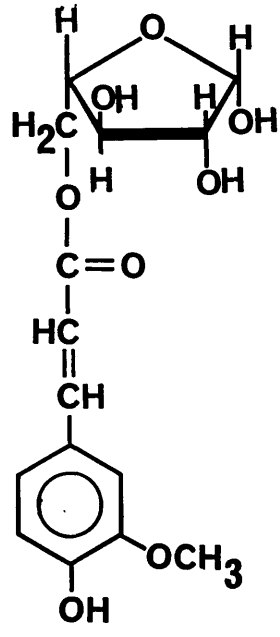
Figure 3.1.3.6 2-D COSY NMR spectrum of FA-ARA.


1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
PPM
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0
PPM

The downfield shift of ARA H 5 & 6 is indicitive of an ester linkage at the *O*-5 position.

Figure 3.1.3.7 shows the proposed structure of FA-ARA *i.e trans*-ferulic acid ester linked through the *O*-5 of α-L-arabinofuranose or 5-*O*-(*trans*-feruloyl)-α-L-arabinofuranose.

Figure 3.1.3.7 Structure of 5-*O*-(*trans*-feruloyl)-α-L-arabinfuranose.



3.2 DMSO EXTRACTION.

3.2.1 Introduction.

In the Gramineae, at least, the only sugar to have been conclusively shown to be covalently bound to phenolic material is arabinose. It was thought likely that any lignin-carbohydrate linkages in oxalate insoluble residues of graminaceous walls would, in all probability, be to sugars other than arabinose. In order to study some of these linkages, the oxalate hydrolysed material was extracted with DMSO to yield a lignin-carbohydrate complex (LCC). DMSO is a very powerful organic solvent, which cleaves hydrogen bonds, and has previously been used for the extraction of LCC's and hemicellulose (Morrison, 1974 B: Bouveng & Lindberg, 1965: Joseleau & Gancet, 1981).

3.2.2 DMSO extraction.

DMSO extraction resulted in a 12.1% weight loss in oxalate hydrolysed barley straw and a 18.6% weight loss in oxalate hydrolysed ryegrass.

Table 3.2.1.1 shows the chemical composition of the DMSO extracted materials.

TABLE 3.2.2.1. Chemical composition of DMSO residues.

Results expressed as % dry matter (% of original).

	Barley DMSO residue (64.78)	Ryegrass DMSO residue (51.77)
RHAMNOSE	0.11 (35.62)	0.00 -
FUCOSE	0.37 (217.89)	0.31 (59.43)
ARABINOSE	0.88 (25.45)	1.29 (14.12)
XYLOSE	21.79 (68.89)	19.68 (46.35)
MANNOSE	0.10 (17.51)	0.27 (11.01)
GALACTOSE	0.08 (6.64)	0.08 (0.72)
GLUCOSE	34.29 (78.24)	54.41 (102.88)
URONIC ACIDS	- -	18.46 (66.17)
TOTAL CARBOHYDRATE	**57.62**	**76.04**
TRUXILLIC ACID	0.00 -	0.12 (56.48)
cis-COUMARIC ACID	0.03 (64.78)	0.04 (41.42)
trans-COUMARIC ACID	0.24 (59.79)	0.18 (40.52)
cis-FERULIC ACID	0.00 -	0.08 (24.36)
trans-FERULIC ACID	0.09 (29.15)	0.21 (21.32)
DIFERULIC ACID	0.02 (21.59)	0.02 (12.94)
TOTAL PHENOLIC ACID	0.38 (31.56)	0.64 (29.06)
LIGNIN	12.73 (86.80)	- -
TOTAL PHENOLICS	**13.11**	-
TOTAL	**70.35**	**77.33**

As can be seen from Table 3.2.2.1, the significant difference from the oxalate hydrolysed samples is a loss of xylose. Interestingly, DMSO seems to have extracted approximately 20% of the original phenolic acids in barley straw but very little in the ryegrass.

Table 3.2.2.2 shows the results of methylation analysis of the DMSO extracted residues. It is interesting to note that the remaining arabinose is mainly terminal, and in barley in the pyranose form.

Taking 2,3,4-*O*-methyl xylose as terminal residues, it would appear that the average chain length is higher than in the starting materials.

TABLE 3.2.2.2. Methylation analysis of DMSO residues.

Results expressed as % dry matter (% of original).

	Barley DMSO residue	Ryegrass DMSO residue
2,3,5-*O-Me*-ARA	1.017	0.723
2,3-*O-Me*-ARA	0.081	0.275
2,5-*O-Me*-ARA	+	+
3,4-*O-Me*-ARA	0.883	0.112
3,5-*O-Me*-ARA	0.112	0.229
5-*O-Me*-ARA	-	+
UNME-ARA	+	-
TOTAL ARABINOSE	**2.093**	**1.339**
2,3,4-*O-Me*-XYL	0.382	0.568
2,3-*O-Me*-XYL	20.843	14.954
2,4-*O-Me*-XYL	+	0.169
2-*O-Me*-XYL	1.587	1.458
3-*O-Me*-XYL	1.469	0.819
UNME-XYL	0.171	0.559
TOTAL XYLOSE	**24.452**	**18.527**
2,3,4-*O-Me*-RHA	+	0.115
2,4-*O-Me*-RHA	+	-
3,4-*O-Me*-RHA	+	-
4-*O-Me*-RHA	+	-
TOTAL RHAMNOSE	-	**0.115**
3,4-*O-Me*-FUC	+	-
2-*O-Me*-FUC	+	-
TOTAL FUCOSE	-	-
2,3,4,6-*O-Me*-GLC	0.399	0.469
2,3,4-*O-Me*-GLC	0.570	0.206
2,3,6-*O-Me*-GLC	49.129	49.944
2,4,6-*O-Me*-GLC	0.828	0.303
2,3-*O-Me*-GLC	2.352	2.345
2,4-*O-Me*-GLC	+	-
2,6-*O-Me*-GLC	1.742	1.053
3,6-*O-Me*-GLC	1.128	0.840
4,6-*O-Me*-GLC	+	+
2-*O-Me*-GLC	0.257	0.281
3-*O-Me*-GLC	0.087	+
6-*O-Me*-GLC	0.241	0.161
UNME-GLC	0.158	0.104
TOTAL GLUCOSE	**56.891**	**55.706**
2,3,4,6-*O-Me*-GAL	0.168	0.348
2,3,4-*O-Me*-GAL	+	+

2,3,6-*O-Me*-GAL	-	0.488
2,4,6-*O-Me*-GAL	0.092	0.084
2,3-*O-Me*-GAL	+	0.099
2,6-*O-Me*-GAL	+	-
3,6-*O-Me*-GAL	-	+
4,6-*O-Me*-GAL	0.620	-
TOTAL GALACTOSE	**0.880**	**1.019**
2,3,4,6-*O-Me*-MAN	-	+
2,3,4-*O-Me*-MAN	+	0.390
2,3,6-*O-Me*-MAN	0.123	0.585
3,4,6-*O-Me*-MAN	-	+
2,6-*O-Me*-MAN	-	0.067
TOTAL MANNOSE	**0.123**	**1.042**
TOTAL CARBOHYDRATE	**84.439**	**77.748**

+ = less than 0.05%.

Acetalation/Methylation analysis (Table 3.2.2.3) again shows a significant retention of the disubstituted xylose, whereas the disubstituted arabinose content has been reduced.

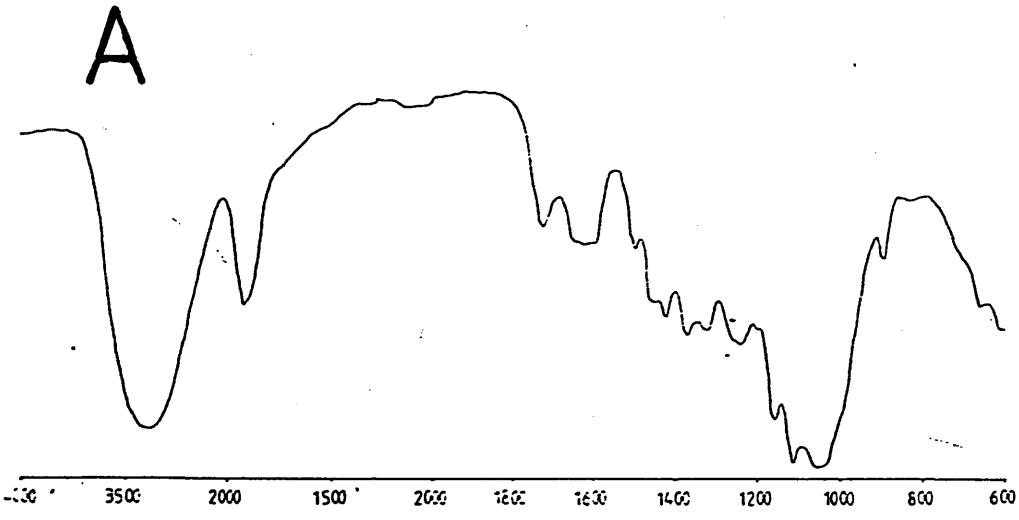
TABLE 3.2.2.3. Acetalation/Methylation analysis of DMSO residues.

Results expressed as % dry matter (% of original).

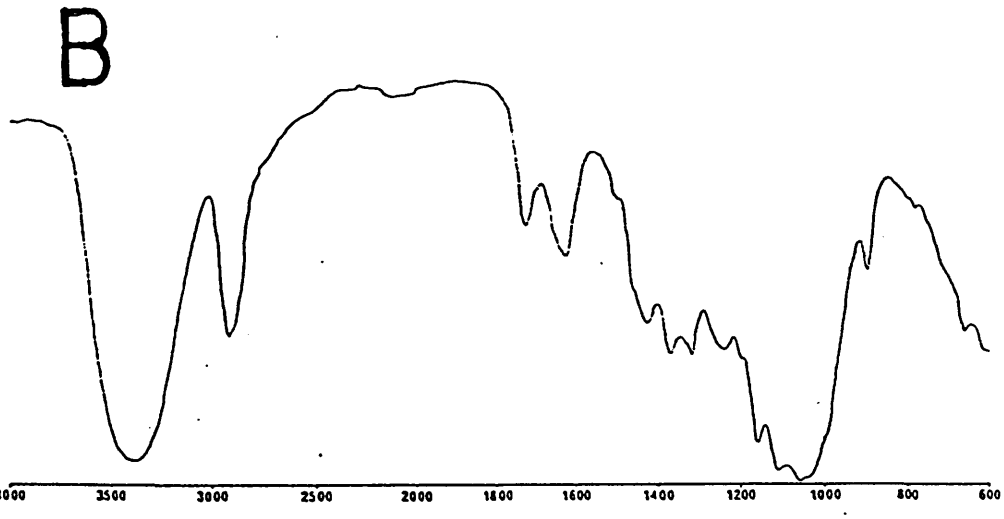
ALKALI LABILE SUBSTITUTIONS	Barley DMSO residue	Ryegrass DMSO residue
2,5-*O*-*Me*-ARA	0.044 (18.51)	0.043 (17.26)
5-*O*-*Me*-ARA	0.038 (6.67)	0.493 (42.68)
2,3-*O*-*Me*-XYL	1.330 (82.29)	1.958 (115.19)
2-*O*-*Me*-XYL	0.531 (17.80)	1.998 (50.70)
3-*O*-*Me*-XYL	0.456 (18.34)	1.801 (80.76)

Comparison of the IR spectra of barley DMSO residue (Figure 3.2.2.1 A) and barley oxalate residue (Figure 3.1.2.2 A) shows no significant structural changes except for a possible, apparent increase in carbohydrate and ester content (1740). Comparison of the equivalent ryegrass fractions (Figures 3.2.2.1 B & 3.1.2.2 B) again shows no structural differences except for an apparent increase in carbohydrate content. Comparison of the barley and ryegrass DMSO residues samples show no difference except for, as would be expected, the major lignin bands at 1500 and 1455, which are more obvious in the barley fraction.

Figure 3.2.2.1 Infrared spectra of A) DMSO extracted, oxalate hydrolysed barley straw and B) DMSO extracted, oxalate hydrolysed ryegrass.



WAVENUMBER (Cm^{-1})



WAVENUMBER (Cm^{-1})

3.2.3 Purification and characterisation of Lignin-Carbohydrate complexes.

To assess the homogeneity of the solubilised lignin-carbohydrate complexes (LCC's), attempts were made to fractionate them by molecular sieve chromatography with a range of gels. As can be seen (Figure 3.2.3.1 A) Sephadex G-25 failed to fractionate the ryegrass complex. Sephadex G-100 (Figure 3.2.3.1 B), on the other hand, separated a phenolic-carbohydrate peak at the void volume, from extraneous phenolic and carbohydrate material which did not appear to be linked. The 'purified' ryegrass LCC from G-100 was then run on Sephadex G-200 (Figure 3.2.3.1 C) and Sepharose CL-6B (Figure 3.2.3.2 A) running, in each case, as a single phenolic-carbohydrate peak with the void volume. Very similar results were obtained for the barley straw LCC, both of them representing approximatly 26% of the solubilised material (3% of original barley straw and 4.5% of original ryegrass). The operating range of Sepharose CL-6B is up to $6x10^6$ daltons, therefore suggesting a very high molecular weight complex. As mentioned in the introduction, liquid chromatography in aqueous eluants, may cause aggregation of the LCC's (Conners et al., 1980). Similar DMSO soluble LCC's from perennial ryegrass (Morrison, 1974 B) were found to be included well inside Sephadex G-200, suggesting a M_{wt} of approximately 150 Kd. These complexes, however, were run with 10% DMSO which may be necessary to prevent aggregation, or the ball milling used by Morrison may have depolymerised the complexes.

In an attempt to assess the degree of alkali-labile linking between

the phenolic and carbohydrate components, the purified LCC's were hydrolysed with alkali and rerun on Sepharose CL-6B. As can be seen there was a considerable difference in response between barley straw (Figure 3.2.3.3 B) and ryegrass (Figure 3.2.3.2 B) LCC's. The barley straw LCC has retained its original elution profile at the void volume but alkali hydrolysed approximately 50% of the phenolic material and a little carbohydrate. The ryegrass LCC, on the other hand, was considerably altered. Although the hydrolysed material was very similar to that obtained from barley straw, the elution profile of the high molecular weight LCC was very much different, with a downward shift of the carbohydrate component and very much less phenolic material being retained at the void volume.

TABLE 3.2.3.1. Chemical compositions of LCC'S.

Results expressed as % dry matter.

	Barley LCC	**Ryegrass LCC**
RHAMNOSE	0.00	0.00
FUCOSE	0.31	0.00
ARABINOSE	0.86	1.62
XYLOSE	30.55	36.90
MANNOSE	0.08	0.00
GALACTOSE	0.40	1.07
GLUCOSE	4.85	4.69
URONIC ACIDS	-	-
TOTAL CARBOHYDRATE	**37.05**	**44.28**
TRUXILLIC ACID	0.00	0.09
cis-COUMARIC ACID	0.09	0.10
trans-COUMARIC ACID	0.52	0.50
cis-FERULIC ACID	0.00	0.30
trans-FERULIC ACID	0.23	0.76
DIFERULIC ACID	0.14	0.11
TOTAL PHENOLIC ACIDS	0.97	1.86
LIGNIN	28.58	7.67
TOTAL PHENOLICS	**29.55**	**9.53**
TOTAL	**66.60**	**53.81**

Figure 3.2.3.1 Ryegrass LCC on A) G-25, B) G-100 and C) G-200.

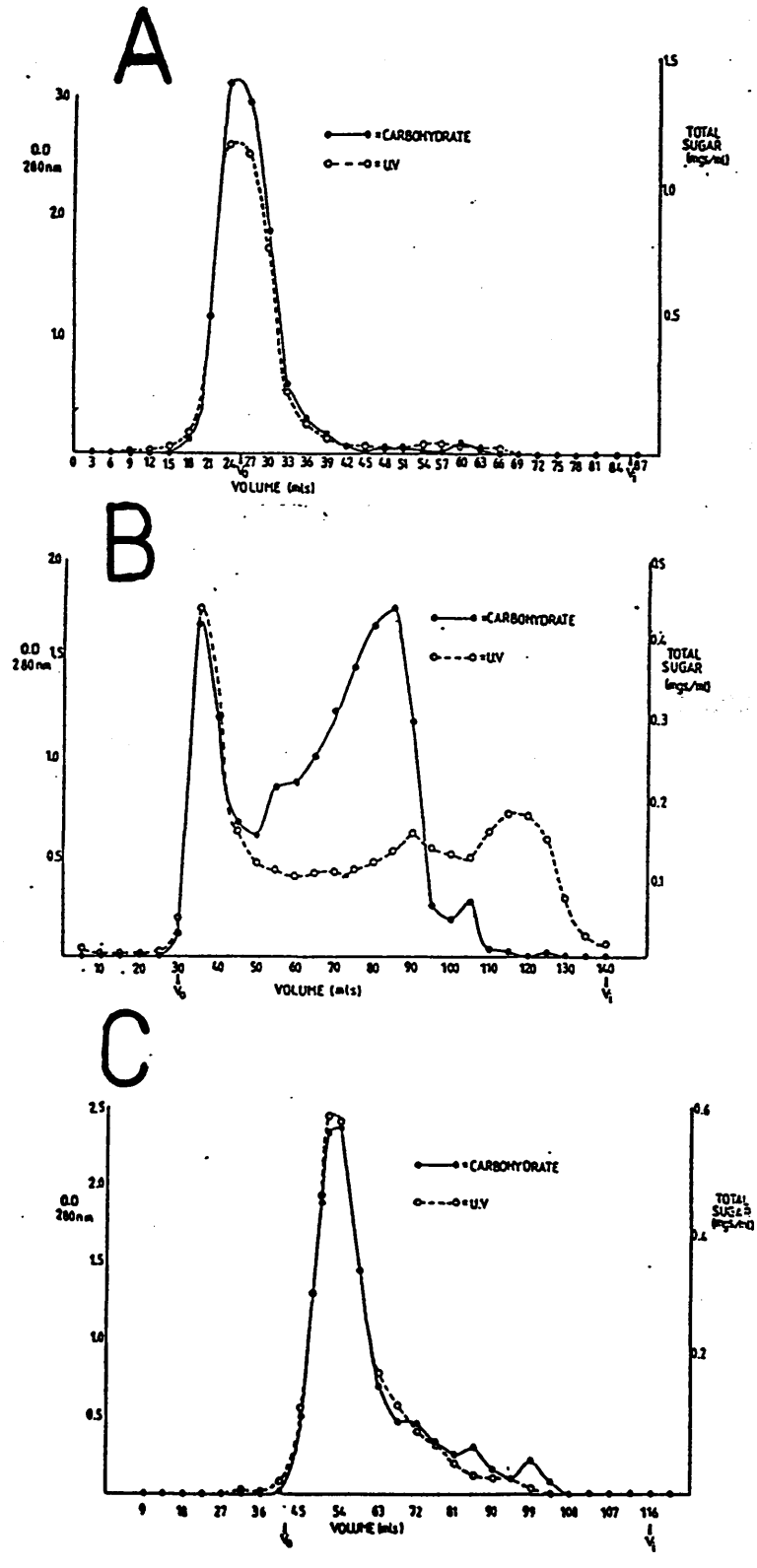

A
CARBOHYDRATE
UV
TOTAL SUGAR
VOLUME (mls)
B
CARBOHYDRATE
UV
TOTAL SUGAR
VOLUME (mls)
C
CARBOHYDRATE
UV
VOLUME (mls)

Figure 3.2.3.2 A) Ryegrass LCC on CL-6B and B) Alkali hydrolysed ryegrass LCC on CL-6B.

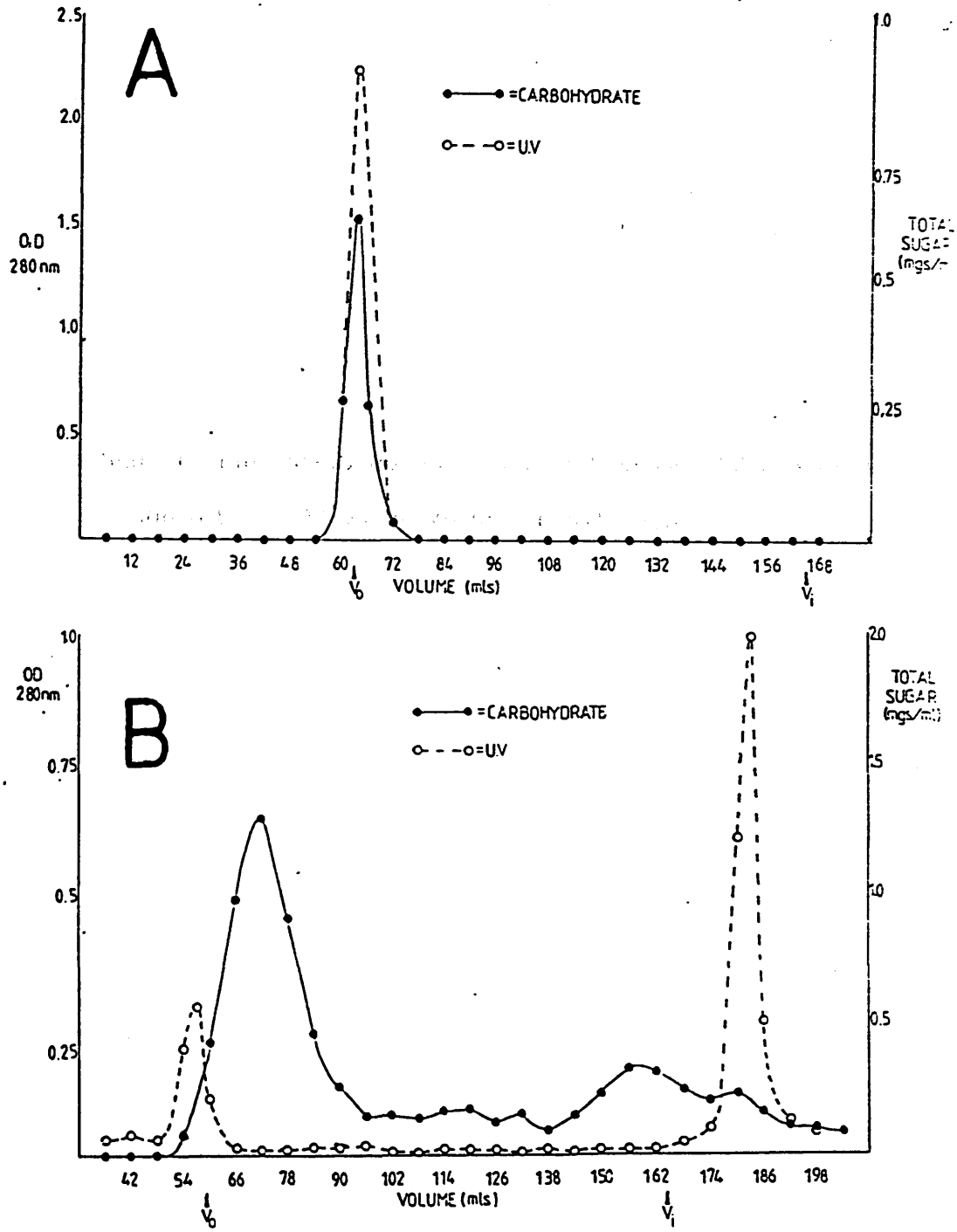
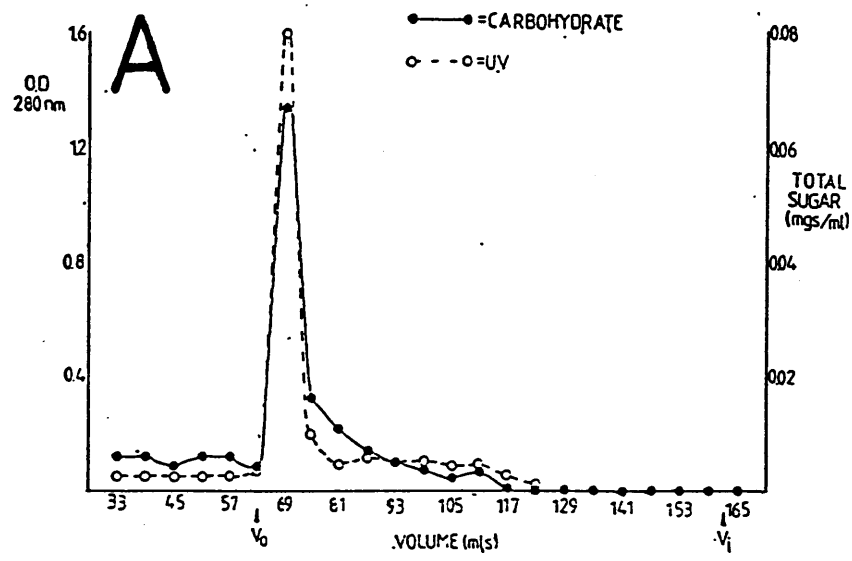
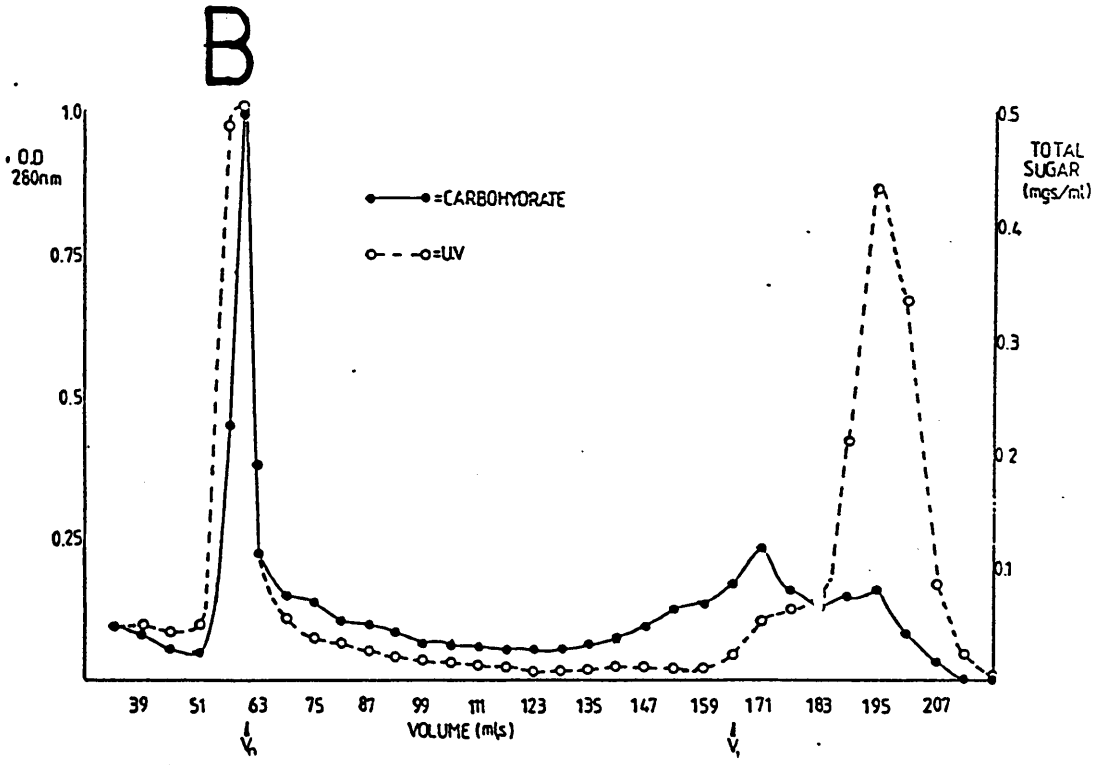

A
=CARBOHYDRATE
=U.V
O.D 280nm
TOTAL SUGAR (mgs/-
2.5
2.0
1.5
1.0
0.5
1.0
0.75
0.5
0.25
12 24 36 48 60 72 84 96 108 120 132 144 156 168
V_o
VOLUME (mls)
V_i
B
=CARBOHYDRATE
=U.V
OD 280nm
TOTAL SUGAR (mgs/ml)
1.0
0.75
0.5
0.25
2.0
1.5
1.0
0.5
42 54 66 78 90 102 114 126 138 150 162 174 186 198
V_o
VOLUME (mls)
V_i

Figure 3.2.3.3 A) Barley straw LCC on CL-6B and B) Alkali hydrolysed Barley straw LCC on CL-6B.


A
=CARBOHYDRATE
=UV
O.D 280 nm
TOTAL SUGAR (mgs/ml)
1.6
1.2
0.8
0.4
0.08
0.06
0.04
0.02
33 45 57 69 81 93 105 117 129 141 153 165
V_0
VOLUME (mls)
V_t


B
O.D 280nm
=CARBOHYDRATE
=UV
TOTAL SUGAR (mgs/ml)
1.0
0.75
0.5
0.25
0.5
0.4
0.3
0.2
0.1
39 51 63 75 87 99 111 123 135 147 159 171 183 195 207
V_0
VOLUME (mls)
V_t

Although NaOH hydrolysis is not conclusive evidence of ester bonds between the phenolics and the carbohydrate it seems likely that this is the case and that in barley straw LCC, approximately half of the phenolics would appear to be linked by alkali labile linkages, whereas in ryegrass LCC a much higher proportion of the phenolics are bound by alkali labile linkages.

The U.V spectra for the LCC's (Figure 3.2.3.4 A & B) both show major absorption at 280 nm (barley λ max) suggestive of the presence of lignin. Both also show significant absorption around 320 nm (ryegrass λ max) which may suggest the presence of cinnamyl type ester linkages (Morrison, 1973). On the addition of sodium acetate, a noticable hypsochromic shift occurs in the barley straw LCC and a shift of max to 280 nm in ryegrass. It is thought that the absorption around 320 nm may arise from carboxyl groups. either free or as esters. If the carboxyls are free the addition of sodium acetate should produce this hypsochromic shift and, if they are present as esters, have no effect, or produce a bathochromic shift (Jurd, 1957). These results may indicate that a large proportion of the phenolic acids in barley straw LCC are present as ethers.

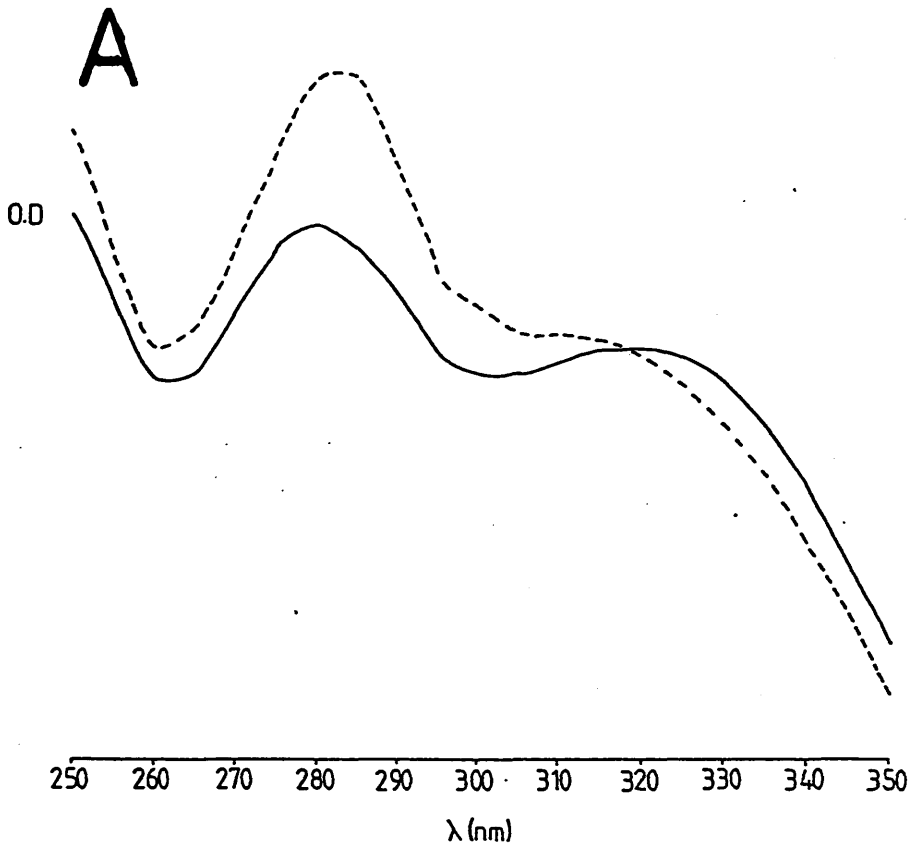
Unfortunately, identification of alkali labile linkages by acetalation/methylation analysis was not possible due to the difficulties involved in this analysis on soluble materials.

As can be seen from Table 3.2.3.1, and methylation analysis of the complexes (Table 3.2.3.2) the carbohydrate component would appear to predominantly consist of a ß 1-4 xylan. Unlike the DMSO solubilised complexes of Morrison (1974 B) there would appear to be a significant

amount of mixed linked glucan present. In barley straw the high 2,4,6 *O-Me*-Glucose content is similar to the 2,3,6 *O-Me*-Glucose content, thus suggesting that most, if not all, the 1-4 linked glucose comes from mixed linked glucan. Ryegrass LCC shows a much higher concentration of 1-4 linked to 1-3 linked glucose therefore suggesting the presence of some xyloglucan, which is more in line with the findings of Morrison (1974 B). The methylation analysis of Morrisons LCC also showed branching on the xylan chain at the *O*-3 position (side chains involving arabinose and galactose). Both, the ryegrass and barley straw LCC's however also suggest the presence of side chains at the *O*-2 position, strangely enough the ryegrass showing more *O*-2 branches than O-3.

The high concentration of xylose and low concentration of arabinose would suggest that the most likely sugar to be involved in phenolic-carbohydrate linkages is xylose. It has been suggested (Chesson et al., 1983) that alkali labile bonds between lignin and xylose do exist. As can be seen from Table 3.2.3.1, phenolic acids account for only 7% of the phenolics present, thus suggesting the presence of true lignin. It is interesting to note that ferulic acid is dominant in the ryegrass LCC whereas *p*-coumaric acid is predominant in the barley LCC, consistent with the theory of *p*-coumaric acid being more closely associated with lignin (Shimada *et al.*, 1971: Scalbert *et al.*, 1985).

Figure 3.2.3.4 U.V Spectra of A) Barley straw LCC and B) Ryegrass LCC. (_____), (- - - - - plus NaOAC).


A
O.D
250
260
270
280
290
300
310
320
330
340
350
λ (nm)

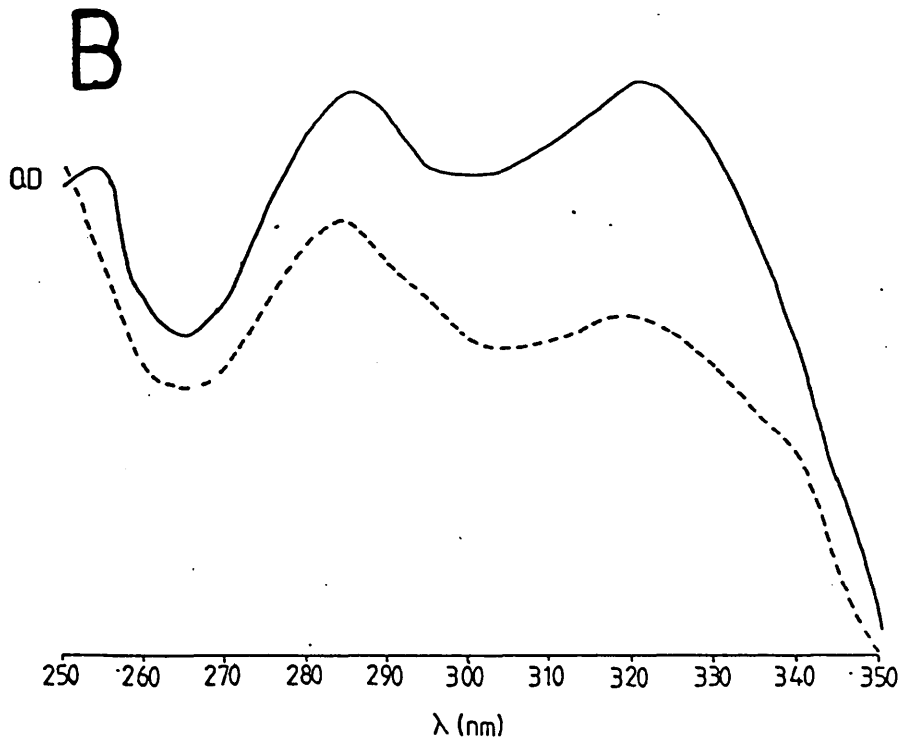

B
O.D
250
260
270
280
290
300
310
320
330
340
350
λ (nm)

Table 3.2.3.2. Methylation analysis of LCC'S.

Results expressed as % dry matter.

	Barley LCC	Ryegrass LCC
2,3,4-*O-Me*-ARA	-	0.415
2,3,5-*O-Me*-ARA	1.513	1.151
2,3-*O-Me*-ARA	0.120	0.207
2,5-*O-Me*-ARA	+	+
3,4-*O-Me*-ARA	+	+
3,5-*O-Me*-ARA	0.181	0.903
2-*O-Me*-ARA	-	+
5-*O-Me*-ARA	-	+
TOTAL-ARA	**1.814**	**2.676**
2,3,4-*O-Me*-XYL	0.865	1.631
2,3-*O-Me*-XYL	52.768	55.602
2,4-*O-Me*-XYL	0.158	0.282
2-*O-Me*-XYL	2.243	4.306
3-*O-Me*-XYL	1.186	4.624
UNME-XYL	+	0.200
TOTAL-XYL	**57.220**	**66.645**
2,3,4-*O-Me*-RHA	-	+
3-*O-Me*-RHA	-	0.469
TOTAL-RHA	-	**0.469**
2,3,4,6-*O-Me*-GLC	1.260	0.238
2,3,4-*O-Me*-GLC	0.117	+
2,3,6-*O-Me*-GLC	3.437	5.254
2,4,6-*O-Me*-GLC	3.877	1.608
2,3-*O-Me*-GLC	0.365	0.220
2,4-*O-Me*-GLC	0.362	+
2,6-*O-Me*-GLC	-	+
3,6-*O-Me*-GLC	0.052	-
UNME-GLC	-	0.071
TOTAL-GLC	**9.470**	**7.391**
2,3,4,6-*O-Me*-GAL	0.266	0.542
2,3,4-*O-Me*-GAL	0.103	0.135
2,3,6-*O-Me*-GAL	+	0.036
TOTAL-GAL	**0.369**	**0.713**
2,3,4,6-*O-Me*-MAN	0.424	+
2,3,4-*O-Me*-MAN	+	+
TOTAL-MAN	0.424	+
TOTAL CARBOHYDRATE	69.297	77.894

+ = less than 0.05%.

The Infrared spectrum for barley LCC (Figure 3.2.3.5 A) demonstrates the presence of substantial acetyl, with characteristic absorptions at 1250 and C=O at 1733 Cm^{-1}. The lignin region, 1400-1650 suggests the presence of syringyl/vanillyl groups (Russell, personal communication). A surprising amount of, what is proposed to be, inorganic sulphate 1120 and 630 is also present. The major carbohydrate bands (990, hemicellulose C-O 1030-1040, 1070-1090 and 1150-1180) are typical of xylan/glucan, but cannot be placed any further.

The spectra for the ryegrass LCC (Figure 3.2.3.5 B) is very similar to that for the barley straw, but its aromatic 'fingerprint', which not surprisingly has an apparently lower content.

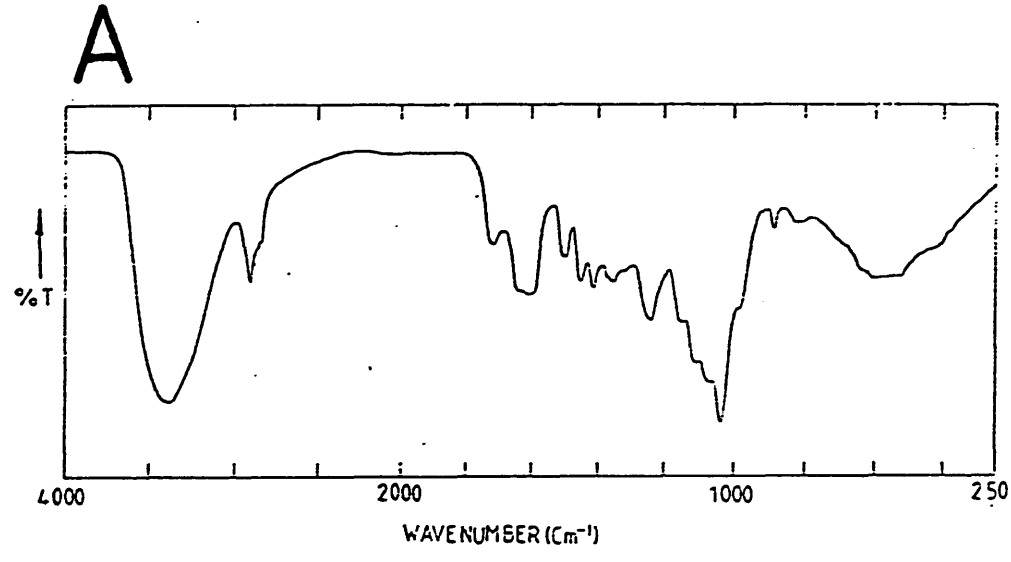
Elemental analysis of the soluble complexes table 3.2.3.3 did not show any significant nitrogen content, unlike many other studies of LCC'S (Ford, 1986; Nordkvist, 1987; Conchie *et al.*, 1988). It is thought unlikely that mild acid hydrolysis could have been responsible for the removal of any N containing compounds.

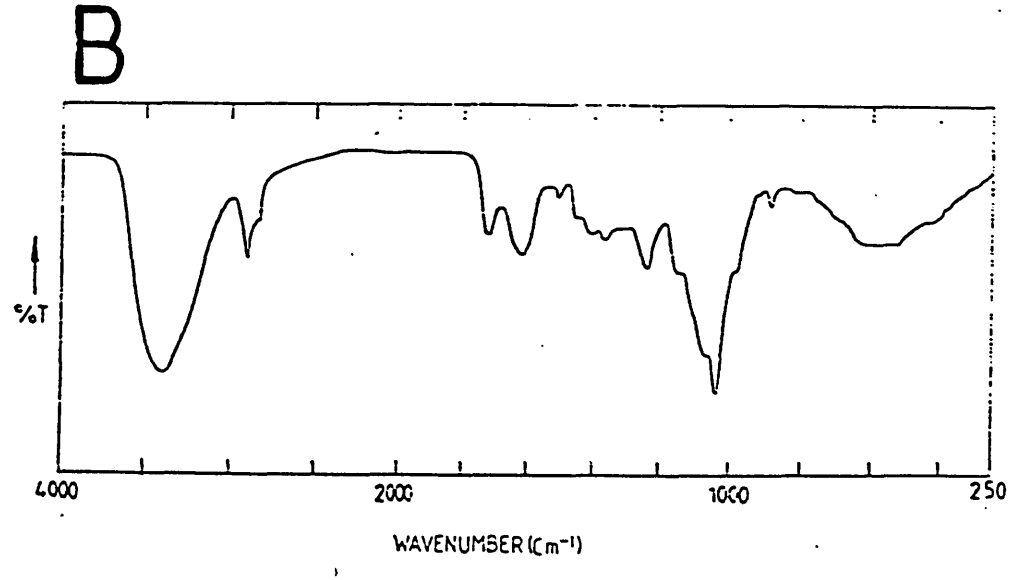
Table 3.2.3.3 Elemental composition of LCC's.

ELEMENT	Barley LCC	Ryegrass LCC
C	46.90	43.50
H	5.62	5.84
N	0.01	0.17

Results expressed as % dry matter.

Figure 3.2.3.5 Infrared spectra of A) Barley LCC and B) Ryegrass LCC.


A
%T
4000
2000
1000
250
WAVENUMBER (Cm⁻¹)


B
%T
4000
2000
1000
250
WAVENUMBER (Cm⁻¹)

In order to produce smaller phenolic-carbohydrate fragments, the barley straw LCC was incubated with driselase (a general cellulase preparation). The hydrolysed material was then passed through a column of Sephadex G-10 (Figure 3.2.3.6). A driselase blank showed that the U.V absorbing component at the void volume was from the enzyme preparation and did not contribute to any other U.V absorbing peak.

The structure of the lignin component in the barley straw LCC was further examined by ^{13}C NMR (Figure 3.2.3.7). The assignments (Table 3.2.3.4) were based on comparison with published data (Himmelsbach & Barton, 1980: Nimz et al, 1981) and the assignment of the ß 1-4 xylan were confirmed by comparison with a standard spectrum. The spectrum closely agrees with the methylation results i.e mainly ß 1-4 xylan with some mixed linked ß D-glucan. Unfortunately, very little can be seen in the aromatic region except, possibly some coumarate (the predominant phenolic acid present) and some coniferyl type structures. The presence of a significant methoxyl signal (signal 18) would, however, suggest a reasonable lignin content, or possibly a high syringyl content. Signal 19 (hydrocarbon $-CH_2-$) would indicate the presence of some 'lipid like' material.

Figure 3.2.3.6 Driselase hydrolysed Barley LCC on Sephadex G-10.

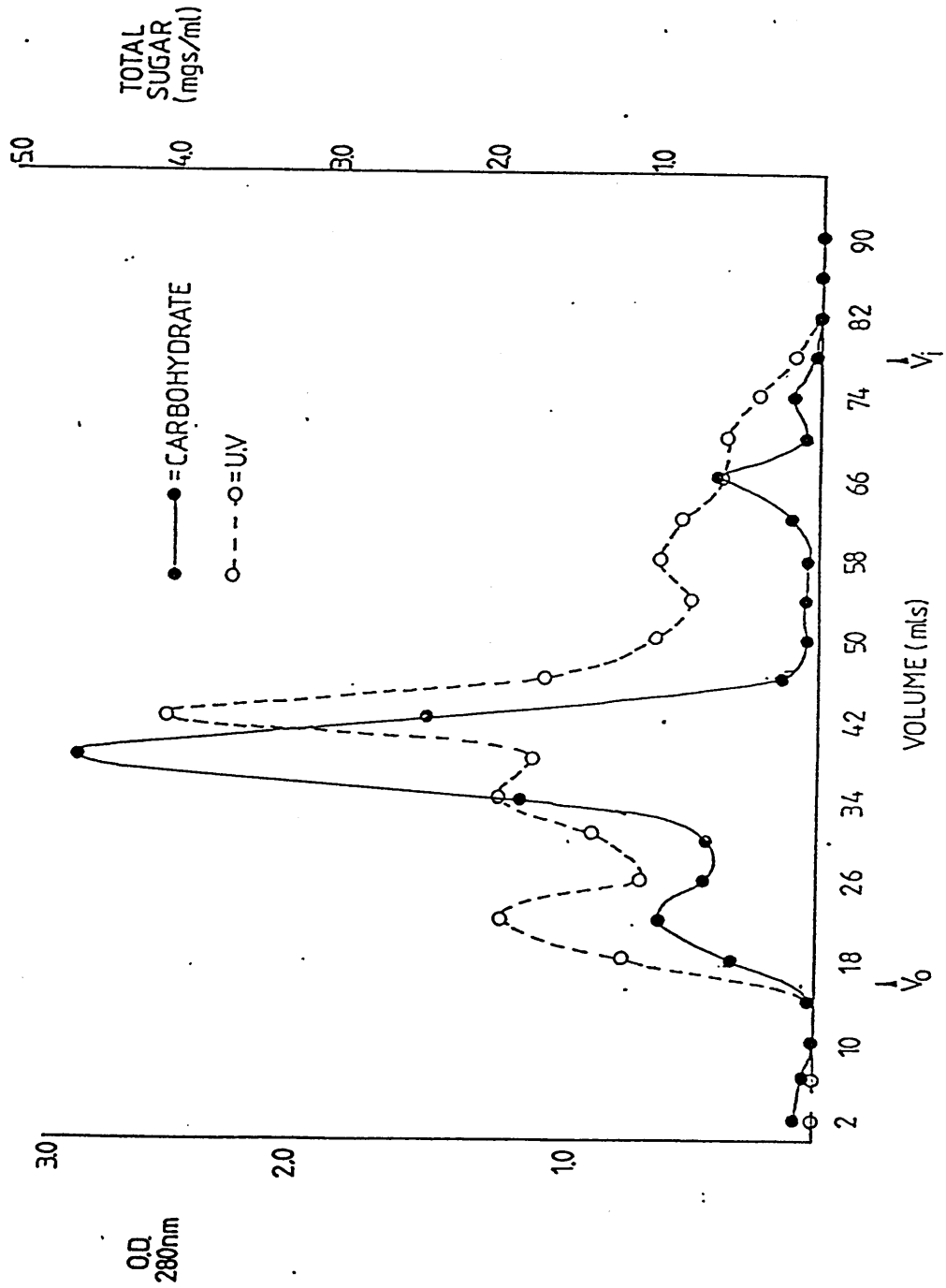

TOTAL SUGAR (mgs/ml)
5.0
4.0
3.0
2.0
1.0
●=CARBOHYDRATE
o - - - o=U.V
O.D 280nm
3.0
2.0
1.0
2
10
18
26
34
42
50
58
66
74
82
90
VOLUME (mls)
V_o
V_i

Figure 3.2.3.7 ^{13}C NMR spectrum of Barley LCC.

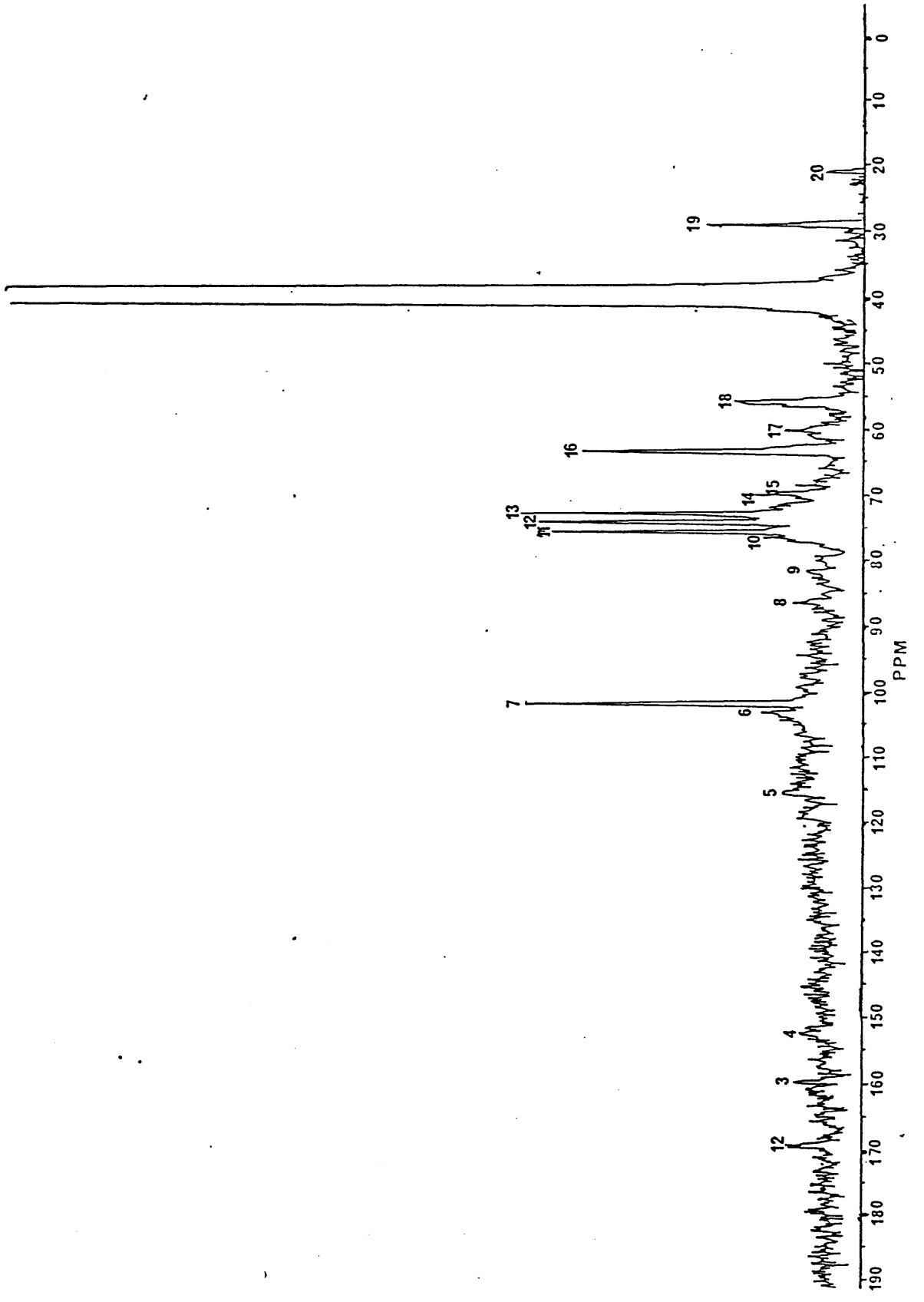
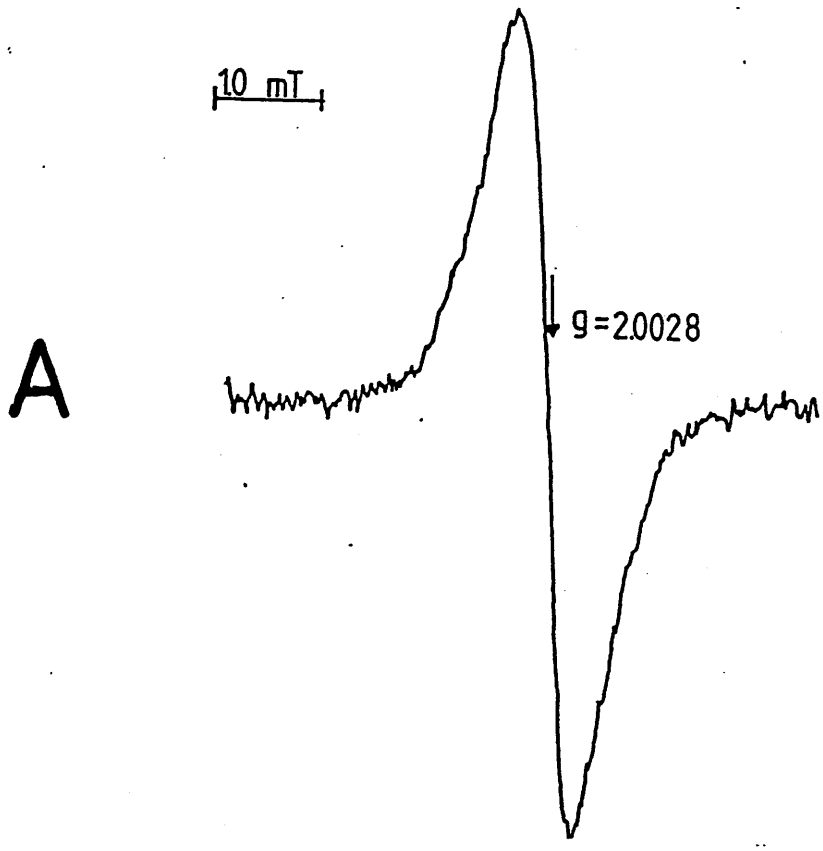

PPM
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
12

Table 3.2.3.4 Assignments of signals from ^{13}C NMR of Barley LCC.

SIGNAL No.	δ.	ASSIGNEMENT.
1	169.1	Acetyl CO2
2	169.0	C-ɣ in coniferyl alcohol
3	159.5	C-4 in *p*-coumarate
4	152.5	C-4 in coniferyl alcohol
5	115.5	C-ß in coniferyl alcohol
6	103.1	C-1 in ß (1-3, 1-4) glucan
7	101.7	C-1 in ß (1-4) xylan
8	86.1	C-3 in ß (1-3, 1-4) glucan
9	82.5	C-4 in ß (1-3, 1-4) glucan
10	76.2	C-2, C-5 in ß (1-3, 1-4) glucan
11	75.5	C-4 in ß (1-4) xylan
12	73.9	C-3 in ß (1-4) xylan
13	72.6	C-2 in ß (1-4) xylan
14	70.0	unassigned
15	69.7	C-ɣ coniferyl & ß-Ar or α-OAr
16	63.2	C-5 in ß (1-4) xylan
17	60.1	C-6 in ß (1-3, 1-4) glucan
18	55.7	Methoxyl (aromatic)
19	28.9	Hydrocarbon $-CH_2-$
20	20.8	Acetyl CH_3

The reasons for the lack of a signal in the aromatic region was, at first, thought to be due to the presence of free radicals which were thought to affect NMR signals (Nordkvist *et al.*, 1988). Electron spin resonance spectroscopy (ESR) was then performed to determine the presence of free radicals in the LCC'S. As can be seen, free radicals do exist in these fractions (Figure 3.2.2.8 A and B), but little can be determined through them. Comparison of the signal intensities shows that the signal from barley LCC was approximately eight fold greater than that from ryegrass LCC. Figure 3.2.3.9 also shows the presence of various copper species demonstrating axial symmetry. The poorly resolved Cu II components may also show a mixed O and N coordination environment (MacPhail, personnal communication). High spin ferric was also found at g = 4.3. It was then suggested that free radicals may not be the reason for the lack of NMR signals but the nature of lignin may result in increased relaxation times (Goodman, personnal communication; Ludemann, personnal communication).

Figure 3.2.3.8 ESR spectra of A) Barley LCC free radical and B) Ryegrass LCC free radical.


A
10 mT
g=2.0028

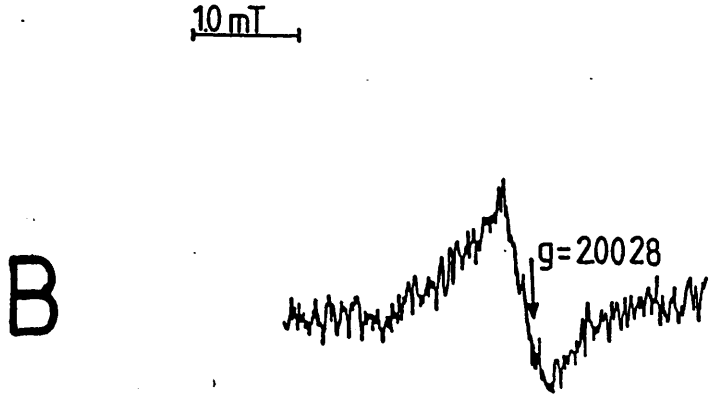
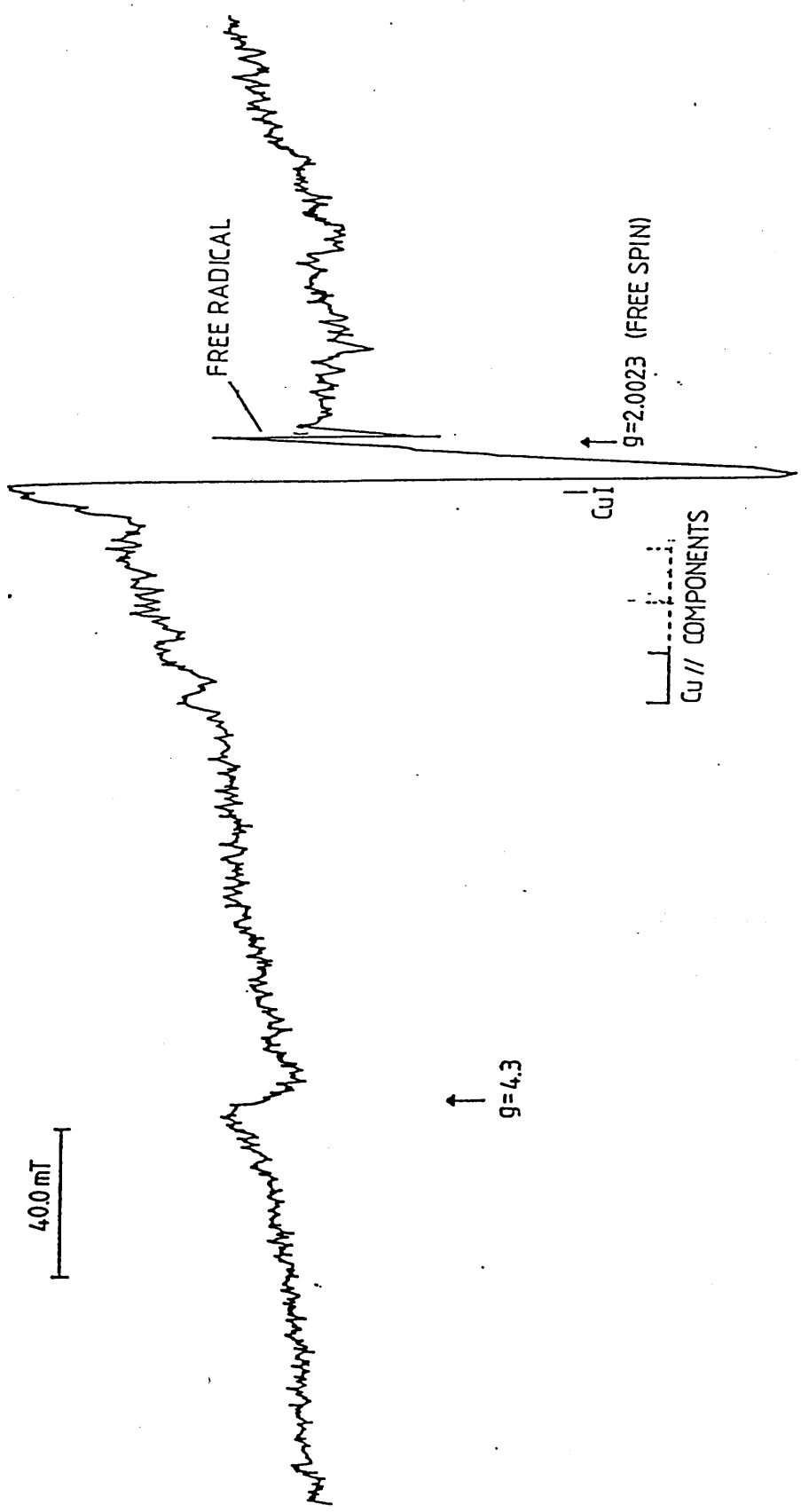

B
10 mT
g=2.0028

Figure 3.2.3.9 ESR spectrum of Barley LCC.


FREE RADICAL
g=2.0023 (FREE SPIN)
Cu⊥
Cu// COMPONENTS
g=4.3
40.0 mT

3.3. ENZYMIC HYDROLYSIS.

3.3.1 Introduction.

In order to enrich the lignin content of the DMSO insoluble material by removing polysaccharide, and possibly release additional phenolic-carbohydrate fragments, an enzymic digestion was added into the fractionation scheme. To maximise hydrolysis of the carbohydrate fraction, an enzyme preparation containing a wide range of polysaccharidase activities was sought.

Driselase, a "cellulase" preparation from the Basidiomycete *Irpex lacteus*, was chosen because its use in cell wall digests is well documented (Fry, 1982: English *et al.*, 1972), it contains a wide range of polysaccharidase activities (Fry, 1988) and most importantly, has been shown to lack non-specific esterases (Fry, 1982).

3.3.2 Driselase extraction.

The enzymic hydrolysis brought about a 36.4% weight loss in DMSO extracted barley and a 76.1% weight loss in DMSO extracted ryegrass. Surprisingly, as can be seen in table 3.3.1.1, driselase treatment did not significantly remove arabinose.

Results of methylation analysis for ryegrass and barley driselase residues are shown in Table 3.3.1.2. The samples were also analysed

by acetalation/methylation to determine the positions of alkali labile linkages in the hemicellulose (Table 3.3.1.3). The most obvious difference here, is the large reduction in disubstituted sugars, with a dramatic reduction in disubstituted xylose in the ryegrass. It is known that acetylated ß-xylose is resistant to driselase treatment (Fry, 1988) which may indicate a large release of alkali labile linked phenolics.

TABLE 3.3.1.1 Chemical composition of driselase residues.

Results expressed as % dry matter (% of original).

	Barley driselase residue (40.20)	Ryegrass driselase residue (12.38)
RHAMNOSE	0.00 -	0.00 -
FUCOSE	0.35 (127.91)	0.00 -
ARABINOSE	0.60 (10.77)	1.17 (3.06)
XYLOSE	19.83 (38.91)	11.84 (6.67)
MANNOSE	0.00 -	0.00 -
GALACTOSE	0.00 -	0.31 (0.66)
GLUCOSE	31.48 (44.58)	36.54 (16.52)
URONIC ACIDS	- -	- -
TOTAL CARBOHYDRATE	**52.26**	**49.86**
TRUXILLIC ACID	-	0.28 (31.52)
cis-COUMARIC ACID	-	0.11 (27.24)
trans-COUMARIC ACID	-	0.49 (26.37)
cis-FERULIC ACID	-	0.04 (2.93)
trans-FERULIC ACID	-	0.11 (2.67)
DIFERULIC ACID	-	0.08 (12.05)
TOTAL PHENOLIC ACIDS	-	1.11 (12.05)
LIGNIN	-	5.95 (15.65)
TOTAL PHENOLICS	**14.96 (63.31)**	**7.06**
TOTAL	**67.19**	**56.92**

TABLE 3.3.1.2. Methylation analysis of driselase residues.

Results expressed as % dry matter.

	Barley driselase residue	Ryegrass driselase residue
2,3,5-*O-Me*-ARA	1.101	1.185
2,3-*O-Me*-ARA	0.062	0.408
2,5-*O-Me*-ARA	0.039	-
3,4-*O-Me*-ARA	0.191	0.316
3,5-*O-Me*-ARA	0.055	0.344
UNME-ARA	+	+
TOTAL ARABINOSE	**1.448**	**2.253**
2,3,4-*O-Me*-XYL	0.405	0.519
2,3-*O-Me*-XYL	16.849	8.509
2,4-*O-Me*-XYL	0.247	0.272
2-*O-Me*-XYL	1.205	1.455
3-*O-Me*-XYL	1.529	0.626
UNME-XYL	0.111	0.746
TOTAL XYLOSE	**20.346**	**12.127**
2,3,4-*O-Me*-RHA	0.078	-
TOTAL RHAMNOSE	**0.078**	-
3,4-*O-Me*-FUC	0.201	-
TOTAL FUCOSE	**0.201**	-
2,3,4,6-*O-Me*-GLC	0.356	0.775
2,3,4-*O-Me*-GLC	0.064	0.453
2,3,6-*O-Me*-GLC	35.047	36.214
2,4,6-*O-Me*-GLC	0.148	0.039
2,3-*O-Me*-GLC	1.296	1.344
2,6-*O-Me*-GLC	0.734	0.929
3,6-*O-Me*-GLC	0.687	0.418
2-*O-Me*-GLC	0.108	-
3-*O-Me*-GLC	+	-
6-*O-Me*-GLC	0.149	-
UNME-GLC	0.072	-
TOTAL GLUCOSE	**38.661**	**40.172**
2,3,4,6-*O-Me*-GAL	0.070	0.256
2,3,4-*O-Me*-GAL	+	-
2,4,6-*O-Me*-GAL	0.145	+
2,3-*O-Me*-GAL	+	-
TOTAL GALACTOSE	**0.215**	**0.256**
2,3,4,6-*O-Me*-MAN	0.055	0.051
2,3,6-*O-Me*-MAN	-	0.073
TOTAL MANNOSE	**0.055**	**0.124**
TOTAL CARBOHYDRATE	**61.004**	**54.932**

+ = less than 0.05%.

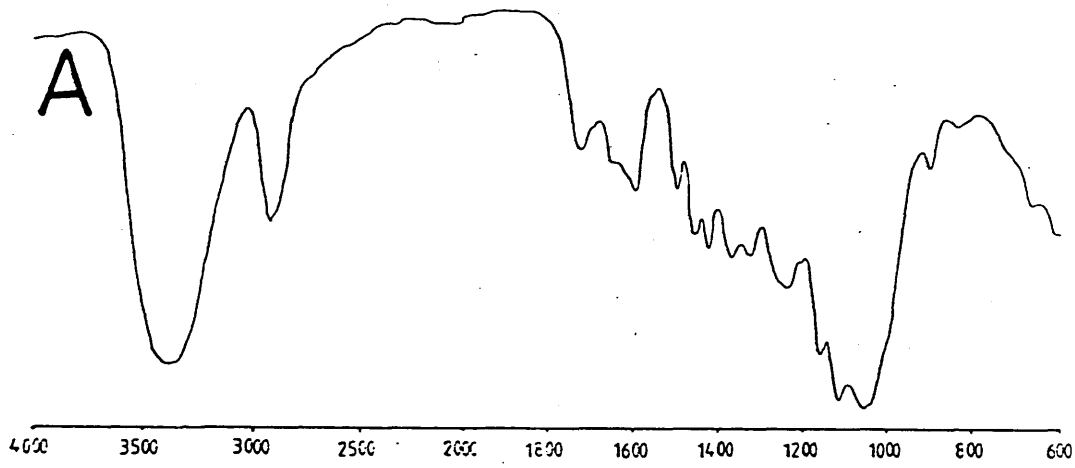
Comparison of the IR spectra of barley straw and ryegrass driselase residues (Figures 3.3.1.1 A & 3.3.1.1 B respectively), reveals little significant difference except, as expected, a higher aromatic content (1500, 1595, 1455 and 1420). Both would appear to have a similar acetyl ester content (1240) and carboxyl content (1725).

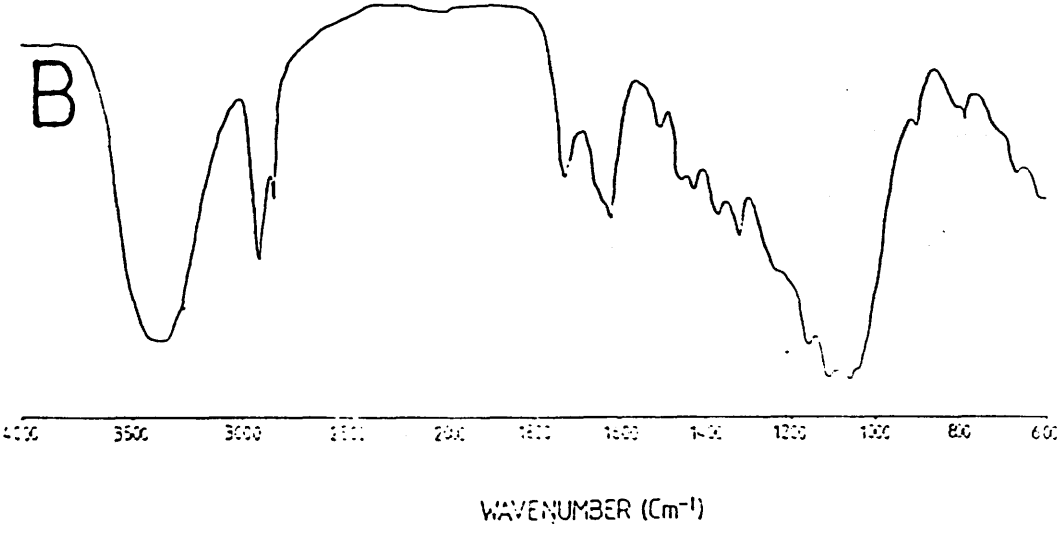
TABLE 3.3.1.3. Acetalation/Methylation analysis of driselase residues.

Results expressed as % dry weight (% of original).

ALKALI LABILE SUBSTITUTIONS	Barley driselase residue	Ryegrass driselase residue
2,5-*O-Me*-ARA	0.106 (28.36)	0.082 (7.87)
5-*O-Me*-ARA	0.166 (18.53)	0.197 (4.09)
2,3-*O-Me*-XYL	1.435 (56.47)	0.882 (12.41)
2-*O-Me*-XYL	0.447 (9.53)	0.946 (5.74)
3-*O-Me*-XYL	0.433 (11.07)	0.613 (6.58)

Figure 3.3.1.1 Infrared spectra of A) Barley driselase residue and B) Ryegrass driselase residue.


A
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
WAVENUMBER (Cm⁻¹)


B
WAVENUMBER (Cm⁻¹)

In order to determine something of their lignin structures, solid state (CP/MAS) ^{13}C NMR of the driselase residues was performed (Figures 3.3.1.2 & 3.3.1.3). Assignments of the signals (Tables 3.3.1.4 & 3.3.1.5) were based upon comparison with previously published solid state ^{13}C NMR of lignins (Maciel *et al.*, 1981; Leary *et al.*, 1988; Cyr *et al.*, 1988) and solution state ^{13}C NMR of lignins and cell walls (Ludemann & Nimz, 1973; Nimz & Ludemann, 1976; Nimz *et al.*, 1981; Himmelsbach & Barton, 1980; Scalbert *et al.*, 1986; Vanderhart & Atalla, 1984).

The major difference between ryegrass and barley straw is the very much larger signal at 168 ppm (signal 4, ferulic and coumaric COOH) in ryegrass compared to barley which could mean a considerably higher content of etherified phenolic acids. The only other significant difference is the very much higher signal, in barley, at 152.4 (signal 8 in ryegrass and 9 in barley), and 147.9 (signal 9 in ryegrass and 10 in barley) which have been assigned as C-4 coniferyl and C-3 coniferyl. It is difficult to say whether this represents any significant difference in guaiacyl content, or merely a higher total lignin content.

Figure 3.3.1.2 ^{13}C CP/MAS NMR spectrum of Barley driselase residue.

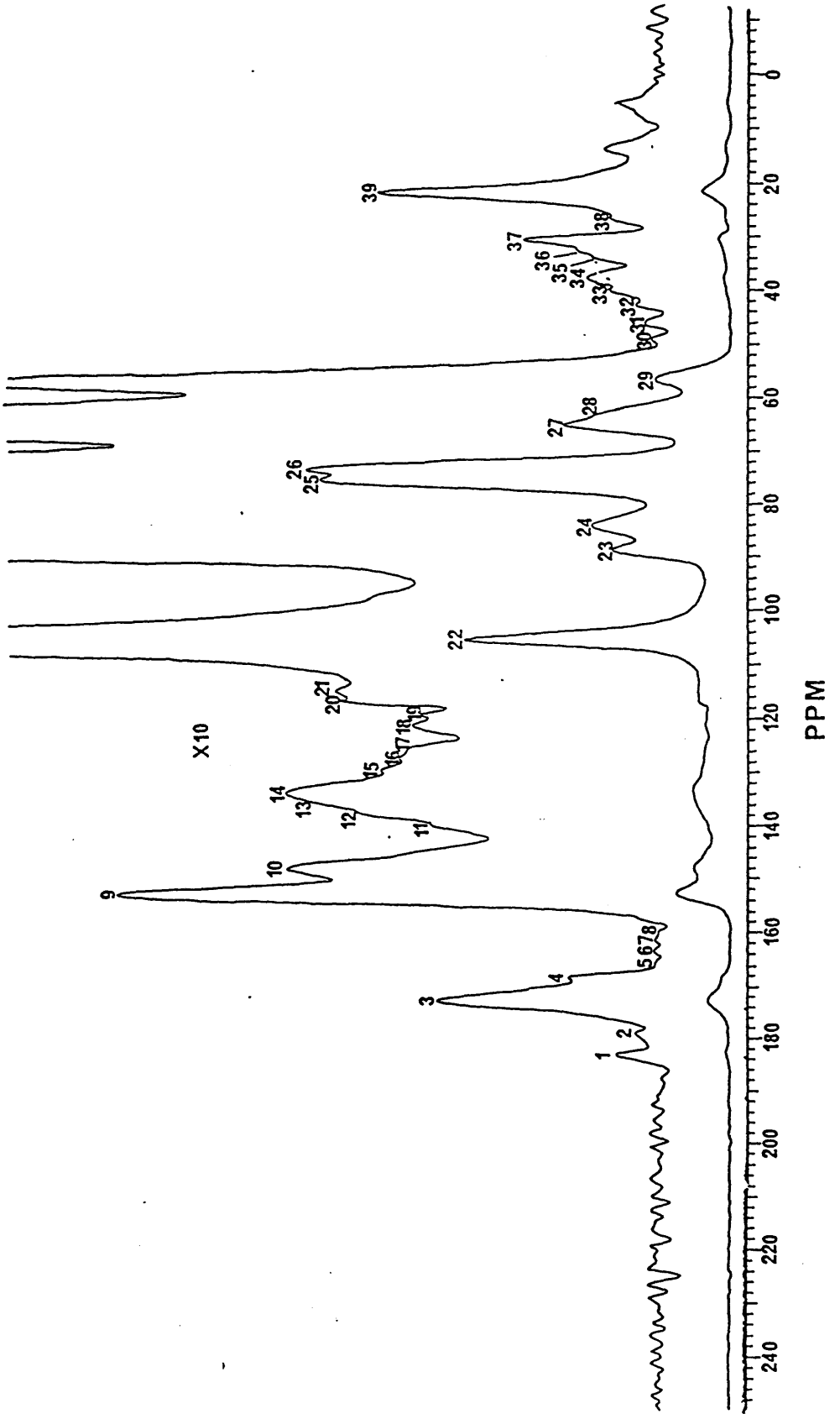

X10
PPM
0
20
40
60
80
100
120
140
160
180
200
220
240

TABLE 3.3.1.4 Assignments of signals from ^{13}C CP/MAS NMR of Barley driselase residue.

SIGNAL No	δ.	ASSIGNMENT.
1	183	
2	179	Acetyl C=O
3	173	
4	168	Ferulate/*p*-coumarate COOH
5	165.9	
6	163	C-4 in *p*-coumarate, C-4 etherified
7	162	or unsubstituted
8	160	
9	152.8	C-4 coniferyl C-3/5 syringyl
10	147.8	C-3 coniferyl (etherified C-4)
11	140	C-1 in coniferyl (Various)
12	137	C-4 in sinapyl
13	135	C-3 in phenyl coumarin
14	133.6	
15	129	C-2/6 *p*-coumaryl
16	127	
17	125	C-1 cinnamyl
18	121	C-α Ferulic ether
19	119	C-5 coniferyl, C-3/5 *p*-coumaryl
20	117	C-β Ferulic ether
21	115	C-3/5 *p*-coumaryl or C-2/5 coniferyl
22	105	C-1 cellulose, C-1 xylan, C-2 & C-6 syringyl
23	89	C-4 cellulose
24	84	C-β in β-*O*-Ar ether, C-4 & 2 araf
25	76	C-2/3/5 cellulose
26	73	C-2/3/4 xylan
27	65	C-6 cellulose C-γ in coniferyl.
28	63	C-5 xylan
29	56	Methoxyl OCH_3
30	34	
31	32	
32	30	Lipid
33	27	
39	21	Acetyl CH_3

Figure 3.3.2.3 ^{13}C CP/MAS NMR spectrum of Ryegrass driselase residue.

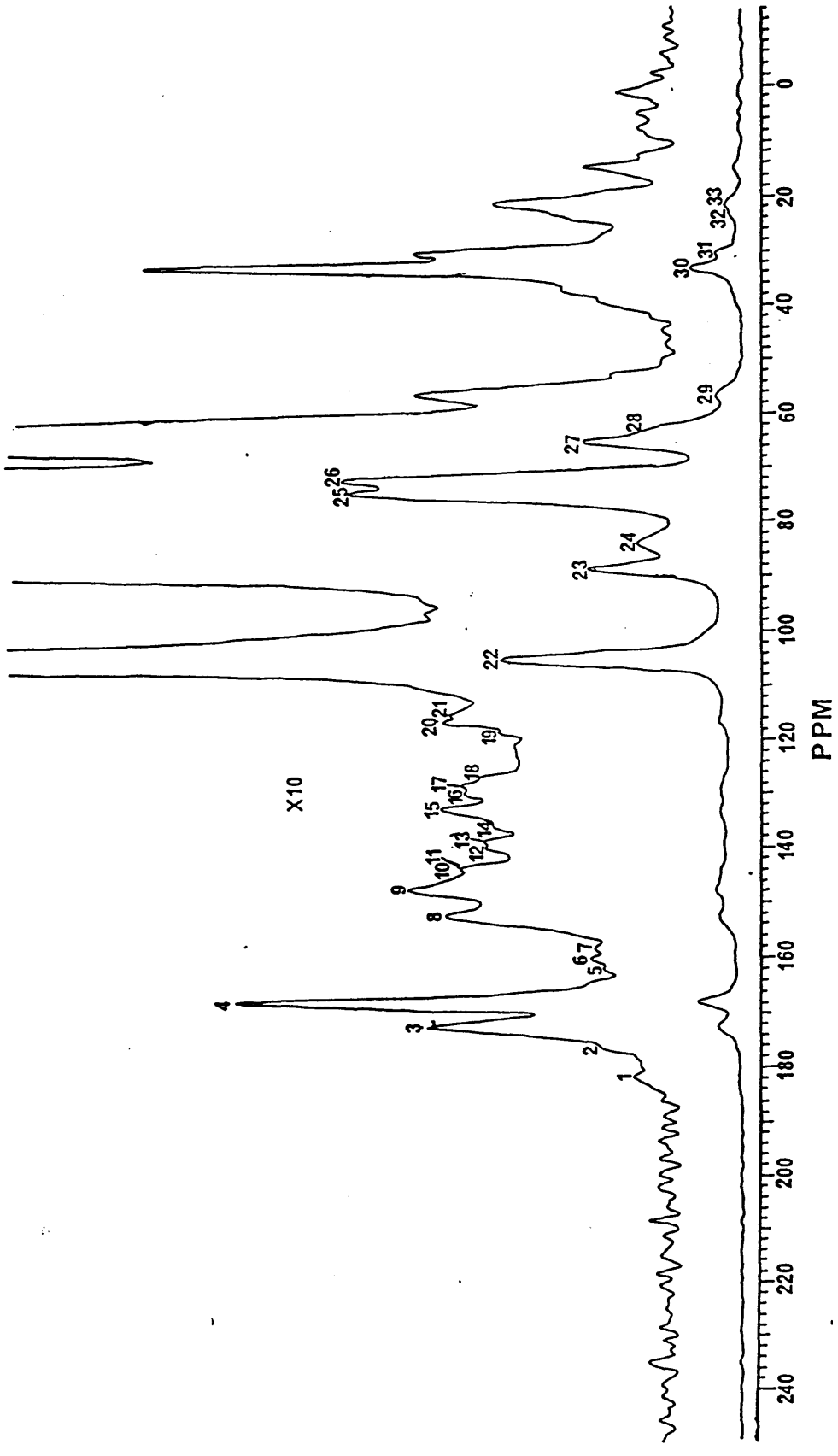

X10
PPM
0
20
40
60
80
100
120
140
160
180
200
220
240
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33

TABLE 3.3.1.5. Assignments of signals from ^{13}C CP/MAS NMR of Ryegrass driselase residue.

SIGNAL NO	PPM	ASSIGNMENT.
1	182	
2	177	Acetyl C=O
3	172.7	
4	168	Ferulate/*p*-coumarate COOH
5	162	C-4 in *p*-coumaryl, C-4 etherified
6	160	or unsubstitued
7	158.5	
8	152.4	C-4 coniferyl, C-3/5 etherified sinapyl
9	147.9	C-3 coniferyl (etherified C-4)
10	146	C-3 coniferl etherified,
11	144	C-4 phenyl coumarin
12	141	C-1 coniferyl (various), C-4 in sinapyl
13	139	C-3 phenyl coumarin
14	136.3	
15	133	
16	130	C-2/6 *p*-coumaryl
17	128	
18	126.5	C-1 coniferyl alcohol
19	119	C-5 coniferyl, C-3/5 *p*-coumaric
20	117	C-ß Ferulic ether
21	115	C-3/5 *p*-coumaryl or C-2/5 coniferyl
22	105	C-1 cellulose, C-1 xylan, C-2 syringyl
23	89	C-4 cellulose
24	84	C-ß in ß-*O*-Ar ether C-4/2 in α-L-araf
25	78	C-2/3/5 cellulose
26	75	C-2/3/4 ß(1-4) xylan
27	66	C-6 cellulose C-ɣ in coniferyl
28	63	C-5 ß (1-4) xylan
29	57	Methoxyl OCH_3
30	33	
31	30	Lipid
32	24	
33	21	Acetyl CH_3

Molecular sieve chromatography was used in an attempt to isolate and, hopefully, characterise any phenolic-carbohydrate fragments solubilised by the driselase treatment. The soluble fraction was found to be a complex mixture of carbohydrate and possibly phenolic-carbohydrate material, over a wide range of molecular weights. Unfortunately, such a low resolution technique was found to be incapable of completely resolving any of the phenolic-carbohydrate components. Attempts to purify such fractions by higher resolution techniques (PC, TLC, GC and HPLC) proved unsuccsessful.

3.4 PYROLYSIS ANALYSIS.

3.4.1 Introduction.

As described previously (section 1.3.4.4), pyrolysis is a technique whereby complex molecules are rapidly heated in order to produce volatile, easily analysed fragments. These fragments may then be passed directly into a MS or GC (with subsequent identification of peaks by MS).

It was thought that pyrolysis analysis of the barley and ryegrass fractions may provide further structural information and complement the 'wet' chemical data. In order to obtain as much information as possible, a number of different pyrolytic techniques were used. Curie point pyrolysis was used for Py-GC and Py-MS (electron impact, EI). The high frequency coils in the Curie point pyrolysis unit, unfortunately, interfere with the mass spectrometer's magnetic field and have therefore to be placed at a distance from the inlet, thus reducing transfer of higher molecular weight fragments. In order to study these, higher molecular weight, fragments, platinum filament pyrolysis, which can be performed directly in the ion source, was used.

Previous studies (Boon, 1989) have shown that electron impact (EI) ionisation severely fragments carbohydrates, whereas phenolic compounds tend to give a molecular ion under these conditions. Chemical ionisation (CI) using ammonia, however, has been found to be more suitable for carbohydrate analysis (Boon, 1989).

Tables 3.4.1, 3.4.2 and 3.4.3 show the assignments for Py-GC peaks, EI Py-MS and CI Py-MS respectively. The assignments are based on mass spectral evidence, comparison with published data (Pouwels *et al.*, 1987; Boon *et al.*, 1987; Genuit *et al.*, 1987; Boon *et al.*, 1984; Boon, 1989; Scheijen *et al.*, 1989) and comparison with traces of standard compounds.

Py-GC, Py-MS (CI and Curie point) were performed as a general analysis of the fractions. Platinum filament (EI) analysis was also performed and the time course of the pyrolysis was recorded. It was thought that the time resolved pyrolysis profiles may provide extra structural information.

Figures 3.4.25 and 3.4.26 show the time course of the pyrolysis for the barley straw and rye grass fractions respectively. The vertical axis represents the total ion current, as detected by the mass spectrometer, and the horizontal axis represents the scan number (the scan rate being 1 scan per second). The labelled areas of each time course corresponds to the appropriately lettered spectra in figures 3.4.27-3.4.42.

3.4.2 Py-GC, Py-MS (Curie point EI and DCI).

3.4.2.1 Starting materials.

Comparison of the CI spectrum (Fig 3.4.9) and the Curie point EI spectrum (Fig 3.4.5 (A)) for untreated barley straw generally agree as to carbohydrate content i.e high pentose (EI *m/z* 114; CI *m/z* 132, 150)

and significant hexose (EI *m/z* 126, 98; CI *m/z* 144, 162, 180) although CI suggests slightly higher hexose than EI. Phenolic acids *p*-coumaric and ferulic (EI *m/z* 120, 150) produced significant peaks by EI but were less evident by CI (*m/z* 182, 212) It should be noted however, that in EI *m/z* 120 and 150 (Mwt of the decarboxylated acids) may only represent esterified phenolic acids (Boon, personnal communication). The Py-GC trace (Fig 3.4.1 (A)) agrees with a relatively high phenolic acid content (peaks 8 & 9). Curie point EI Py-MS also shows large lignin marker peaks at *m/z* 180 and 210. The only peaks on Py-GC with masses corresponding to 180 and 210 (peaks 16 & 22) are rather insignificant thus giving some doubt as to the origin of *m/z* 180/210 in EI. If *m/z* 120 and 150 represent only the esterified phenolic acids, and *m/z* 164 and 194 represent etherified acids, it is interesting to note a higher content of *m/z* 164 rather than *m/z* 194, suggesting *p*-coumaric acid is more heavily etherified than ferulic acid.

The only significant differences between barley straw and ryegrass can be seen by Curie point EI (Fig 3.4.7 (A)) in that ryegrass would appear to have a slightly lower hexose, and a significantly lower phenolic content especially *m/z* 180/210. This is backed up by the Py-GC trace (Fig 3.4.3 (A)) which also suggests a lower *p*-coumaric acid content compared to ferulic acid, also seen by Curie point EI. Under CI (Fig 3.4.17) it is interesting to note that the hexose in ryegrass, produces a much higher 144 peak than barley straw, the significance of which, if any, is unknown.

3.4.2.2 Oxalate hydrolysis.

CI MS of oxalate extracted residue from barley (Fig 3.4.10) shows a massive reduction in pentose content relative to hexose. The dramatic increase in size of peak 18 in Py-GC (Fig 3.4.1 (B)) would seem to bear this out. Curie point Py-MS (Fig 3.4.5 (B)) shows an apparent decrease in *p*-coumaric acid (*m/z* 120) and ferulic acid (*m/z* 150). The phenolic markers *m/z* 180 and 210 also show a decrease although their relative proportions remain quite constant. The oxalate/methanol soluble fraction can be seen from its Curie point EI spectrum (Fig 3.4.6 (A)) to consist mainly of pentose, the CI spectrum (Fig 3.4.15) showing it to be mainly free pentose (168) the lack of 264 suggesting the lack of dimeric pentoses. Py-GC (Fig 3.4.2 (C)) demonstrates a very high phenolic acid content, also seen by Curie point EI MS. The high *m/z* 164/194 content may suggest a high content of etherified phenolic acids.

The oxalate/water soluble barley fraction is again predominantly pentose in nature but has a higher hexose content than the methanol soluble fraction. The reduction in the 168 peak on CI (Fig 3.4.16) indicates that the pentose is oligomeric in nature. Curie point EI (fig 3.4.6 (B)) shows that the phenolic acid content is much lower and Py-GC (Fig 3.4.2 (D)) suggests a radical change in ratio between ferulic and *p*-coumaric acids, *p*-coumaric being much reduced.

Ryegrass gave a similar picture with a lower phenolic content. On CI (Fig 3.4.18) it is again interesting to note that the major hexose peak for oxalate extracted residue from ryegrass is *m/z* 134 rather than m/z 144 as previously or *m/z* 180 for the correponding barley fraction.

Py-GC and Curie point MS show an increase in the *p*-CA/FA ratio suggesting a selective removal of ferulic acid.

Curie point EI (Fig 3.4.8) shows a much higher hexose content for ryegrass methanol, is supported by CI (Fig 3.4.23), which also suggests that much less of the pentose is in the free form. On Curie point EI the phenolic acid content would again seem to be much lower although the unusually high *m/z* 194 may represent a high syringyl or etherified ferulic acid content. CI of the ryegrass oxalate/water soluble fraction (Fig 3.4.24) would again suggest a higher hexose content than that for the corresponding barley fraction, the *m/z* 504 ion in CI suggesting it is more oligomeric in nature.

3.4.2.3 DMSO Extraction.

Py-GC of DMSO insoluble barley (Fig 3.4.1 (D)) shows an apparent relative increase in hexose content (increase in peak 3) whereas Curie point EI (Fig 3.4.5 (C)) and CI MS (Fig 3.4.12) of DMSO insoluble barley show an apparent decrease in pentose content (dramatic increase in *m/z* 134 in CI). Curie point EI MS would suggest little change between the DMSO residue and oxalate residue, but a slight increase in pentose and *m/z* 180 contents. DCI (Fig 3.4.11), Curie point EI (Fig 3.4.6 (C)) and Py-GC trace (Fig 3.4.1 (C)) of the DMSO soluble fraction, all clearly show a very high pentose content. The phenolic acids would appear (from Py-GC) to have a higher concentration in the DMSO soluble fraction. Py-GC also shows the presence of true lignin markers in the soluble material.

The distribution of cell wall components following DMSO extraction for ryegrass was remarkably similar to that for barley straw, the only real difference being the lower phenolic content especially in *m/z* 180/210 content. Py-GC of the residue shows a very high acetyl content.

3.4.2.4 Driselase hydrolysis.

Interpretation of the chromatograms for the driselase soluble fractions (Figures 3.4.2 (A) and 3.4.4 (A)) was not attempted due to the considerable difference between these and other fractions. It was thought that the 'gel' like nature of these fractions may have made reproducible pyrolysis difficult to achieve, possibly due to salt effects (Boon, personnal communication), and the large initial peaks, possibly from protein and buffer salts, make the peaks from solubilised cell wall components too small to allow positive identification. Similarly, Curie point EI (Fig 3.4.6 (D)) yields little information due to the high acetate peak (*m/z* 60) except, possibly, some solubilisation of hexose. CI (Fig 3.4.16), on the other hand, clearly shows the solubilisation of some hexose (*m/z* 180, 342, 402) and some pentose (*m/z* 150). Curie point EI analysis of the driselase residue (Fig 3.4.5 (D)) shows that there has been significant solubilisation of sugar, especially pentose, and Py-GC (Fig 3.4.2 (B)) shows a large increase in the lignin marker peaks (peaks 11, 12, 15, 17, 19, 20, 21, 22, 23 & 24). CI-MS (Fig 3.4.13) shows that the sugars present are mainly hexose (*m/z* 180) with some pentose (*m/z* 150). Py-GC also shows a high acetyl content in the residue. The

Curie point EI demonstrates the large change in phenolic/carbohydrate ratio.

This again holds true for the ryegrass driselase residues, although strangely enough it was the Curie point EI (Fig 3.4.8 (B)) for the ryegrass driselase soluble that showed some solubilisation of ferulic acid.

Py-GC and Curie point EI of the residue still shows some hexose and DCI still shows some pentose. The phenolic acid ratio has also changed, both Curie point and Py-GC showing a much higher *p*-CA content, compared to FA, than any other fraction.

3.4.3 Time resolved platinum filament EI.

3.4.3.1 Starting materials.

Study of the time course of the pyrolysis of untreated barley straw (Fig 3.4.25 (1)) would appear to show the presence of three components, based on their desorption from the wire. Overall chemical composition (Fig 3.4.27) agrees closely to that described earlier i.e high pentose, hexose, high *m/z* 180/210 and phenolic acids are present. The presence of dimeric lignin markers and some fatty acid/lipid like material is also evident. The ions *m/z* 298 and 328 may also suggest the presence of diferulic and truxillic acids respectively. It must be noted here that the use of the term component does not suggest any association between the materials in each component and is used to mean a component of the pyrolysis profile.

Ryegrass showed a very different pyrolysis profile with two components (Figs 3.4.26 (1) and 3.4.35), component A showed a high pentose and hexose content, some phenolic acids and lower Mwt phenolics but very little higher Mwt lignin markers. Component B showed a very similar pattern but had a slightly higher high Mwt phenolic content.

3.4.3.2 Oxalate hydrolysis.

Examination of the time course for oxalate hydrolysed barley (Fig 3.4.25 (2)) shows two major fractions. It would appear that oxalate hydrolysis has somehow disrupted the more volatile component in barley and slightly increased its volatility. Spectra for the two components are shown in Fig 3.4.28. Fraction A could be seen to consist mainly of pentose with little hexose, have a high *m/z* 180/210 content, and contain both phenolic acid and lignin markers. The less volatile fraction (B) is also mainly pentose but with an increased hexose content. The lignin markers *m/z* 180 and 210 were lower as were the phenolic acids and the dimeric phenolic acids (diferulic and truxillic) are predominantly in this fraction.

The time course for the barley oxalate/water fraction (Fig 3.4.25 (7)) clearly showed the presence of pentose and hexose (pentose predominant) (Fig 3.4.33), phenolic acids and phenolic dimers. The presence of the lignin dimers *m/z* 272, 302, 330 and 358 would strongly suggest the presence of true lignin.

The oxalate/MeOH soluble (Fig 3.4.25 (8)) can be seen to contain two distinct components. The spectra for component A (Fig 3.4.34) has an

unusual carbohydrate pattern, *m/z* 73 suggesting extensive fragmentation, very few phenolic peaks are present except very small amounts of *m/z* 180 & 272. Fraction B is remarkably similar to A with the exception of an increase in *p*-CA and FA content and the lignin dimers *m/z* 272 and 302. Neither component would appear to contain any significant amounts of diferulic or truxillic acids

The time course for oxalate hydrolysed ryegrass (Fig 3.4.26 (2)) was very similar to that for the corresponding barley fraction. The spectra for the two components (Fig 3.4.36), shows a much higher hexose content in both components, especially in component B, and a much lower phenolic content. However, contamination from an unknown source made interpretation of the higher molecular weight region of the spectrum very uncertain.

The time course for the ryegrass oxalate/water soluble fraction (Fig 3.4.26 (7)) shows three distinct components. Component A (Fig 3.4.33) is almost entirely pentose/hexose with apparently no phenolic content. Component B would seem to contain slightly more pentose in nature and contain some ferulic acid. The least volatile component C more closely resembles cell wall material with pentose and hexose and the appearance of some phenolic material. This consists predominantly of *m/z* 164 and 194 with some dimeric phenolics *m/z* 272, 198.

The ryegrass oxalate/MeOH sample (Fig 3.4.26 (8)) has dissociated into three distinct components. The more volatile component A (Fig 3.4.42) contains pentose and hexose the phenolic ions *m/z* 194 and 164 and little else. Component B has an unusual carbohydrate pattern,

suggesting complete secondary fragmentation of the pyrolysis fragments has occured. The phenolic acids (*m/z* 120/150) are present as are *m/z* 194/164. The least volatile component C, as in component B and in the corresponding barley fraction, again has a very unusual sugar pattern. The phenolic content in this component is vastly increased most noticable being the massive increase in *m/z* 164/194 content. Diferulic acid is also present as is a significant lignin content (*m/z* 272, 302, 330).

3.4.3.3 DMSO Extraction.

Examination of the pyrolysis profiles for the barley DMSO soluble and residual fractions (Figure 3.4.25 (4 & 5)) clearly show that DMSO would appear to have solubilised the compounds which generated component A in the pyrolysed oxalate residue and leave a residue which may represent component B from the oxalate residue. The DMSO solubilised material (3.4.29) can be seen to be composed predominantly of pentose with little hexose. Phenolic acids are present, as are various sterols and fatty acids. The spectrum bears a very close resemblance to oxalate hydrolysed barley component A. The DMSO residue (3.4.30), on the other hand, could be seen to more closely resemble component B from the oxalate residue. In comparison to the soluble fraction, it contains appreciably more hexose, have a reduced phenolic acid content and comparison of the lignin markers (*m/z* 272, 302, 330) for the soluble and insoluble fractions suggests slightly

different patterns which are comparable to the lignin markers for the volatile and less-volatile components in the oxalate hydrolysed residue.

Again ryegrass (Figs 3.4.26 (3 & 4) and 3.4.37 and 3.4.38) shows a similar pattern although, direct comparison of the solubilised material and residue with the oxalate hydrolysed fraction is not so easy. Component A of the soluble fraction would appear to be mainly pentose with some phenolic acids and *m/z* 180 lignin marker. Little detail can be seen in the higher Mwt region due to some lipid like material. Component B is, unfortunately, heavily contaminated and interpretation of either of the spectra is difficult.

The DMSO residue can also be seen to exist of two components. Both components are very similar except for the higher concentration of *m/z* 180 and 210 in the more volatile component

3.4.3.4 Driselase hydrolysis.

The barley driselase soluble fraction (Fig 3.4.25 (6)) consisted of three components (Fig 3.4.32), A, containing some hexose and little else, B, having a characteristic protein pattern and C, having some hexose and pentose. No significant trace of phenolics can be seen in any of the components.

The time course for the driselase residue (Figure 3.4.25 (5)) clearly shows little change from the DMSO residue. On analysis of the spectra (Figure 3.4.32) the only noticable difference is the massive increase in *m/z* 180 and 210 lignin.

The spectrum for the driselase soluble material is again difficult to interpret, although some hexose can be seen in component A. The two components for the ryegrass driselase residue (Figure 3.4.26 (5) and 3.4.39) look very similar, the only difference being a slightly higher hexose content in the second component. Again, massive contamination prevents detailed interpretation of component B. Component A agrees with the barley driselase residue in the increase in *m/z* 180/210 content on driselase treatment.

TABLE 3.4.1. Assignments of peaks from Py-G,C-M.S.

Peak No.	*M/Z.*	Assignement.	Source.
1	60	ACETIC ACID	CARBOHYDRATE.
2	96	FURALDEHYDE	PENTOSE.
3	98	ANGELICALACTONE	HEXOSE.
4	114	3-HYDROXY-2-PENTENO-1,5-LACTONE	PENTOSE.
5	108	CRESOL	
6	124	G-H	LIGNIN.
7	138	G-C	LIGNIN.
8	120	P-C=C	*p*-COUMARATE.
9	150	G-C=C	FERULIC ACID.
10	126	2-METHYL-3-HYDROXY-4-PYRAN-4-ONE	HEXOSE.
11	154	S-H	LIGNIN.
12	164	G-C=C-C	LIGNIN.
13	168	S-C	LIGNIN.
14	166	G-CO-C	LIGNIN.
15	152	G-CHO	LIGNIN.
16	180	S-C=C	LIGNIN.
17	194	S-C-C=C	LIGNIN.
18	162	1,6 ANHYDROGLUCOSE	HEXOSE.
19	182	S-CHO	LIGNIN.
20	196	S-C-CHO	LIGNIN.
21	180	G-C=C-CHO	LIGNIN.
22	210	S-C-CO-C	LIGNIN.
23	208	S-C-C-CHO	LIGNIN.
24	210	S-C=C-CH_2OH	LIGNIN.

Figure 3.4.1 Py-GC traces of A) Barley straw, B) Oxalate hydrolysed barley, C) Barley DMSO residue and D) Barley DMSO soluble.

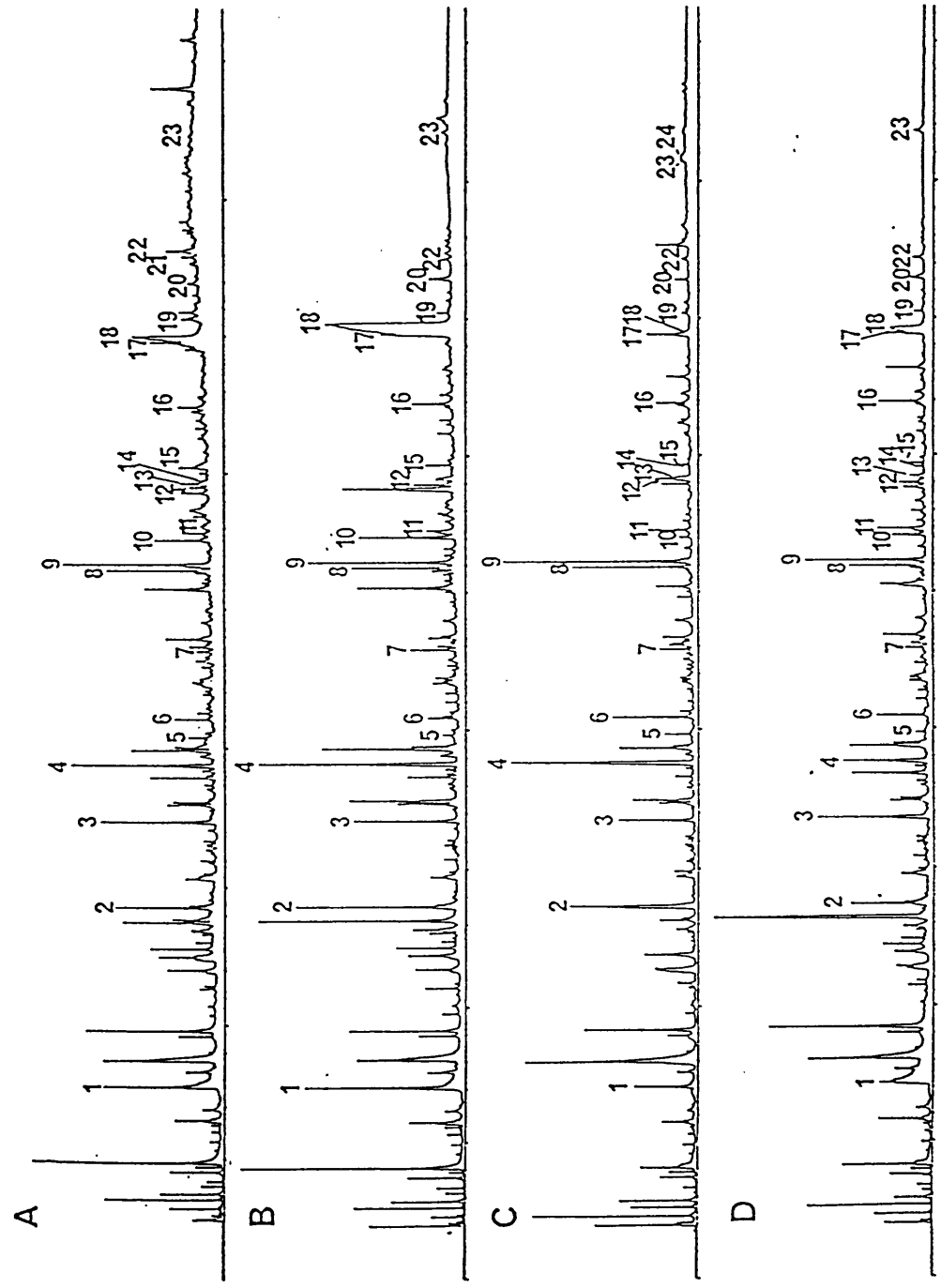

A
B
C
D

Figure 3.4.2 Py-GC traces of A) Barley driselase soluble,

B) Barley driselase residue,

C) Barley MeOH and

D) Barley H_2O.

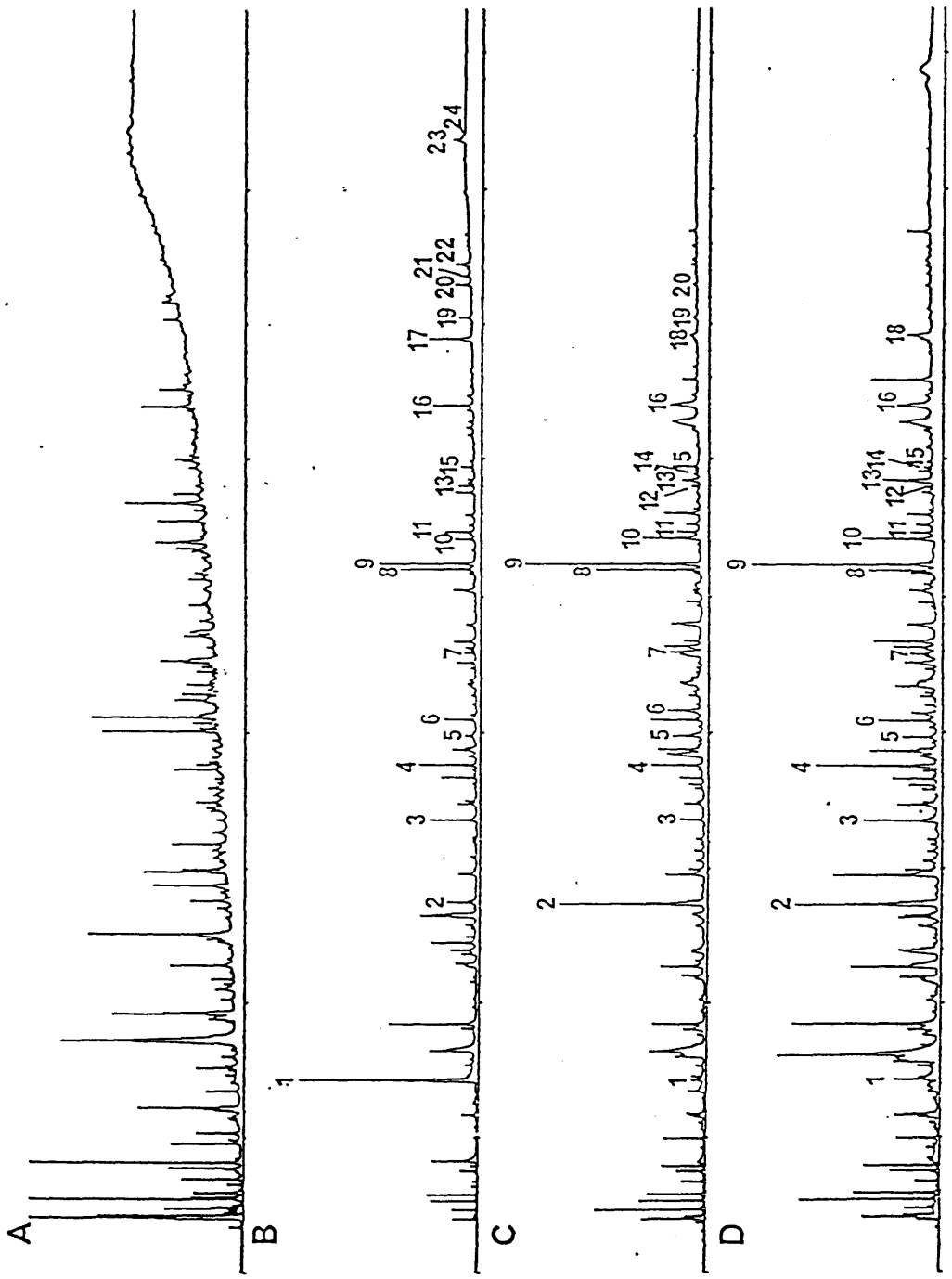

A
B
C
D

Figure 3.4.3 Py-GC traces of A) Ryegrass,

B) Oxalate hydrolysed ryegrass,

C) Ryegrass DMSO residue and

D) DMSO soluble ryegrass.

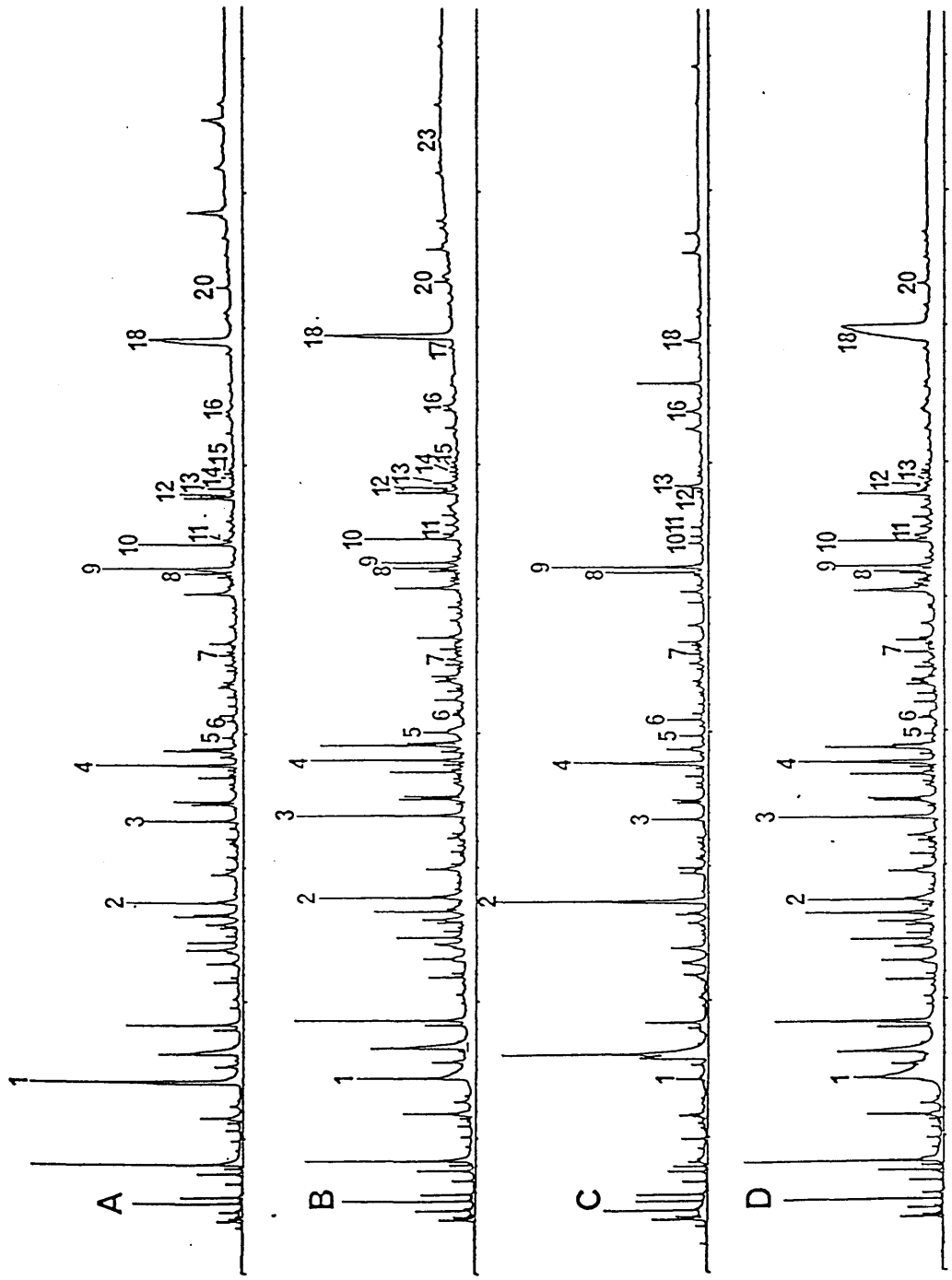

A
B
C
D

Figure 3.4.4 Py-GC traces of A) Ryegrass driselase soluble,

B) Ryegrass driselase residue,

C) Ryegrass MeOH and

D) Ryegrass H_2O.

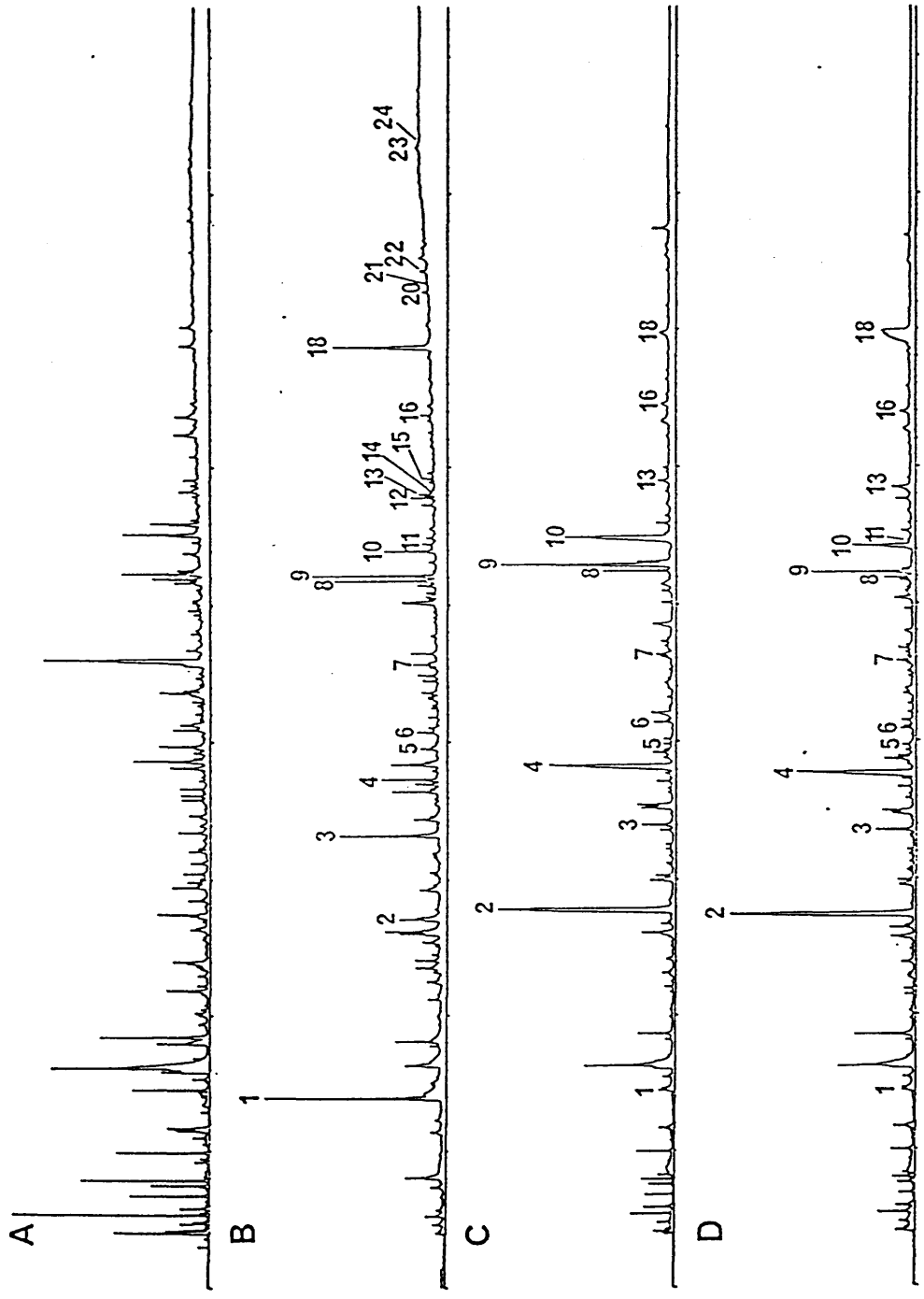

A
B
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
18
20
21
22
23
24
C
1
2
3
4
5
6
7
8
9
10
13
16
18
D
1
2
3
4
5
6
7
8
9
10
11
13
16
18

TABLE 3.4.2. Assignments of mass peaks from electron impact (EI) Py-M.S.

M/Z.	IDENTITY.
43	ACETATE
57	CELLULOSE
60	ACETIC ACID
73	CELLULOSE
85	114 FRAGMENT
86	PENTOSAN
92	PROTEIN
96	FURFURAL
98	ANGELICALACTONE
114	PENTOSAN
116	CELLULOSE
117	PROTEIN
120	*p*-COUMARIC ACID (*p*-CA)
124	G-H
126	HEXOSE
131	PROTEIN
144	GLUCAN
150	FERULIC ACID (FA)
152	G-CHO
154	S-H
164	(PCA),G-C=C-C,G-C-C-OH
166	G-CO-CH_3, G-C-C=O
180	G-C-CO-CH_3,S-C=C,G-C=C-CH_2OH
182	S-CHO, G-COO-CH_3.
194	S-C=C-C (FA).
196	S-C-C=O.
208	S-C=C-CHO.
210	S-C-CO-CH_3.
236	FATTY ACID.
256	FATTY ACID.
272	LIGNIN DIMER.
298	DIFERULIC ACID.
302	LIGNIN DIMER.
324	DIFERULIC ACID.
328	TRUXILLIC ACID.
330	LIGNIN DIMER.
342	DIFERULIC ACID.
358	LIGNIN DIMER, TRUXILLIC ACID.
388	TRUXILLIC ACID.
396	STEROL.
414	STEROL.
418	LIGNIN DIMER.
464	LIPID.
564	LIPID.

Figure 3.4.5 Curie point Py-MS of A) Barley straw, B) Oxalate hydrolysed Barley, C) Barley DMSO residue and D) Barley driselase residue.

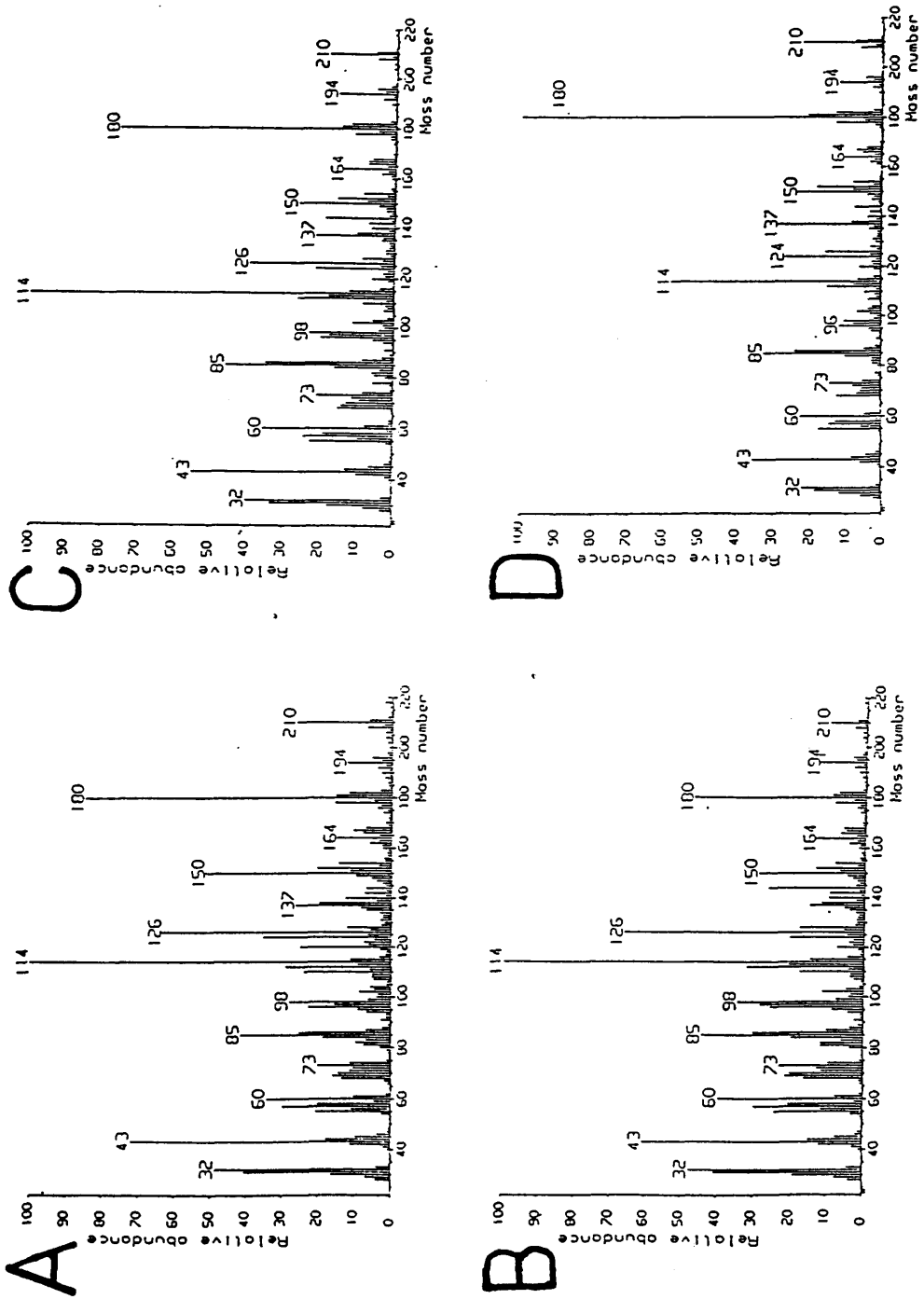

A
B
C
D
Relative abundance
Mass number

Figure 3.4.6 Curie point Py-MS of A) Barley DMSO soluble,

B) Barley driselase soluble,

C) Barley MeOH and

D) Barley H_2O.

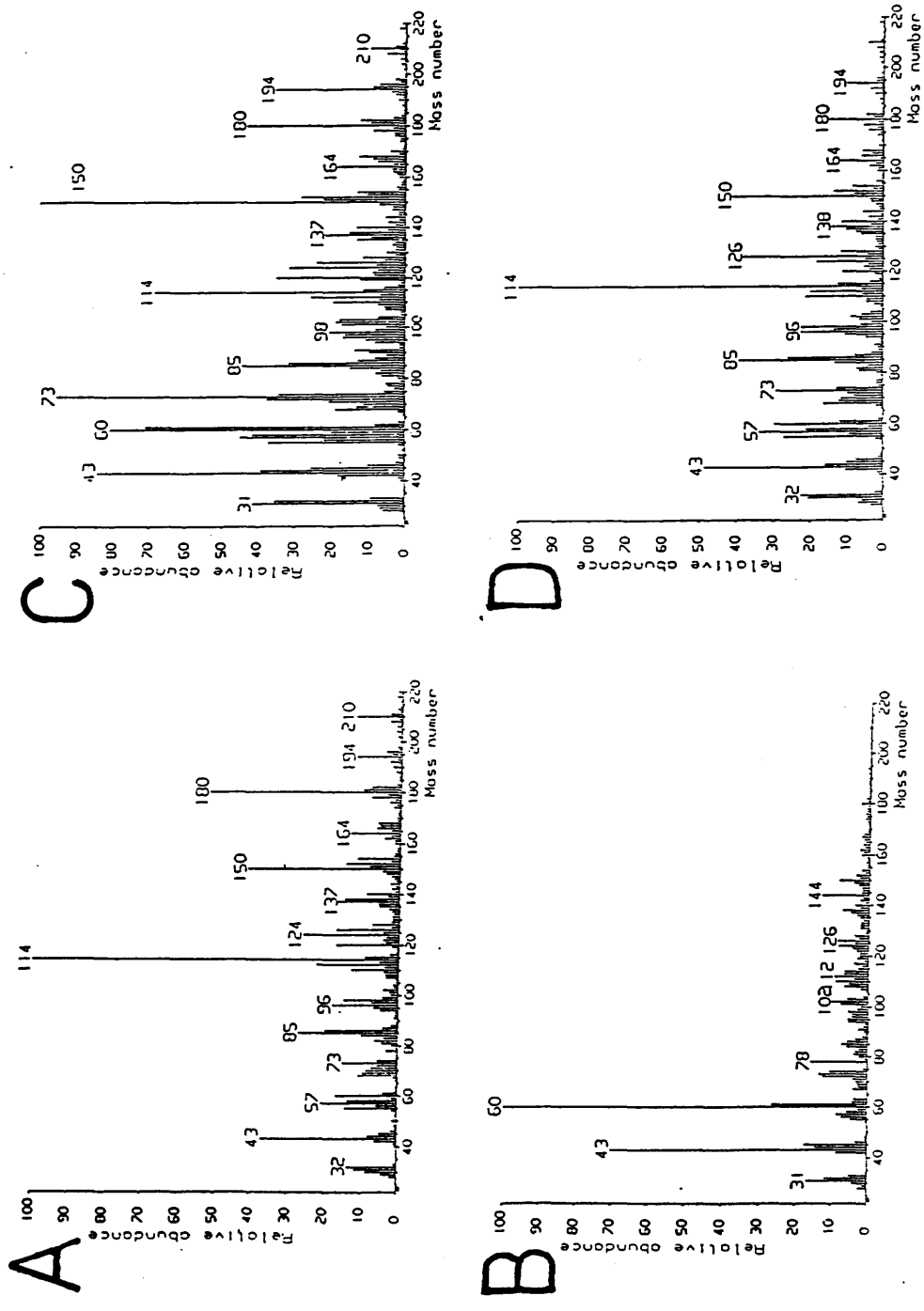

A
B
C
D
Relative abundance
Mass number

Figure 3.4.7 Curie point Py-MS of A) Ryegrass,

B) Oxalate hydrolysed ryegrass,

C) Ryegrass DMSO residue and

D) Ryegrass driselase residue.

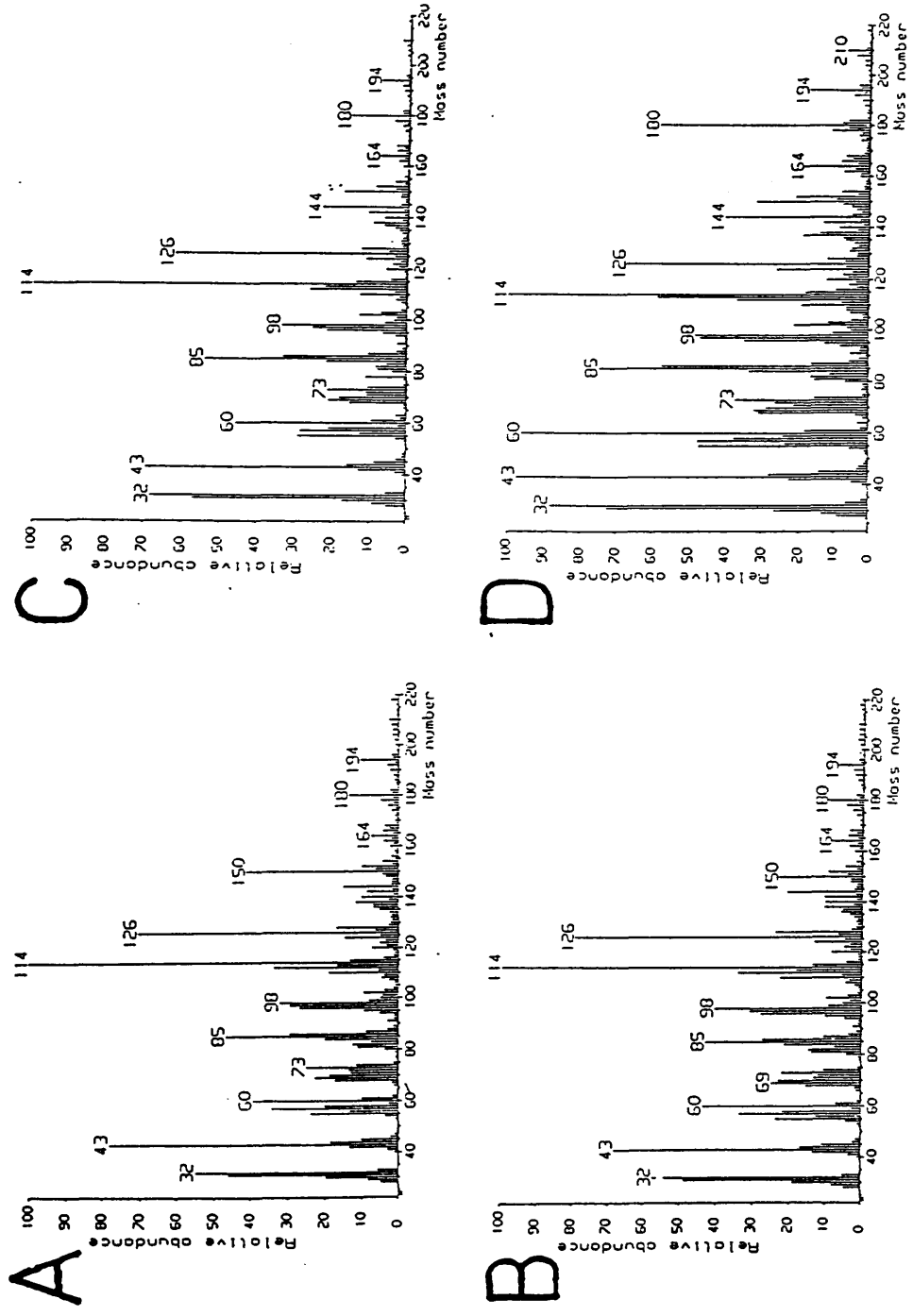

A
B
C
D
Relative abundance
Mass number

Figure 3.4.8 Curie point Py-MS of A) Ryegrass DMSO soluble,

B) Ryegrass driselase soluble,

C) Ryegrass MeOH and

D) Ryegrass H_2O.

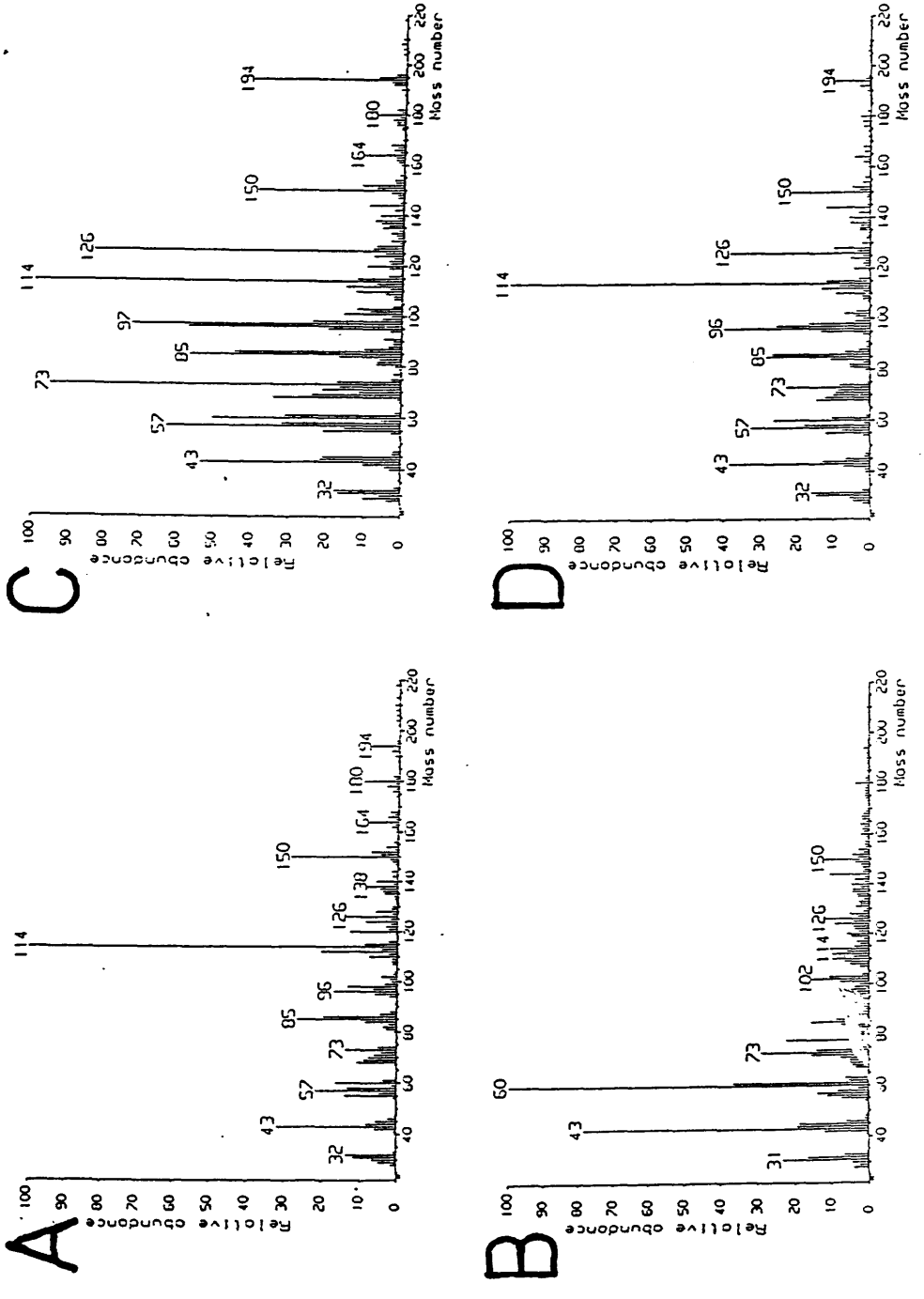

A
B
C
D
Relative abundance
Mass number

TABLE 3.4.3. Assignments of mass peaks from Direct Chemical Ionisation (CI) Py-M.S.

M/Z.	IDENTITY.	*M/Z.*	IDENTITY.
54	$(NH_4)_3$	168	FREE PENTOSE.
78	ACETIC ACID	170	S-CHO.
120	P-C=C	176	GALCOOH.
121	VINYLPHENOL	180	HEXOSE,HEXOSAN.
124	G-H	181	VINYLPHENYL.
126	HEXOSE	182	*p*-COUMARIC ACID.
132	ANHYDROPENTOSE	193	$S-C=C-CH_3$.
134	CELLULOSE	200	G-CHO.
135	G-C	210	$S-CO-CH_3$.
144	GLUCOSE/GLYCAN	212	FERULIC ACID.
146	RHAMNOSE,DEOXYHEXOSE	222	CELLULOSE.
150	PENTOSAN	264	PENTOSE-PENTOSE.
151	VINYLPHENOL	282	PENTOSE-PENTOSE.
154	S-H	312	HEXOSE-PENTOSE.
162	GLUCOSE/GLYCAN	342	HEXOSE-HEXOSE.
163	$G-C=C-CH_2$	402	CELLULOSE.
164	DEOXYHEXOSE	414	PENTOSE-PENTOSE-PENTOSE.
165	S-C	504	HEXOSE-HEXOSE-HEXOSE.

Figure 3.4.9 Platinum filament DCI Py-MS spectrum of Barley straw, scans 23-43.

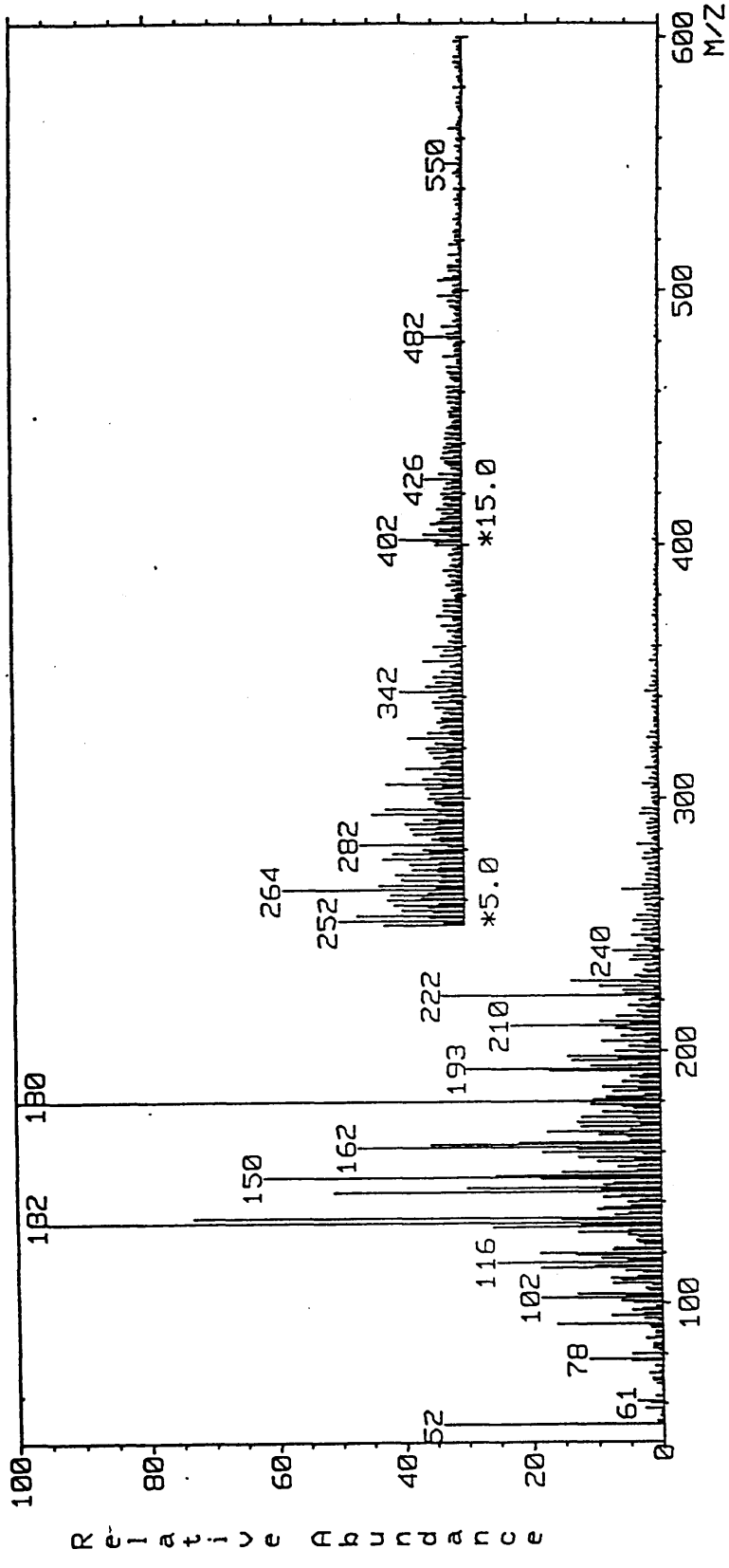

Relative Abundance
M/Z
100
80
60
40
20
0
100
200
300
400
500
600
52
61
78
102
116
150
162
180
182
193
210
222
240
252
264
282
342
402
426
482
550
*15.0
*5.0

Figure 3.4.10 Platinum filament DCI Py-MS spectrum of Oxalate hydrolysed barley straw, scans 25-39.

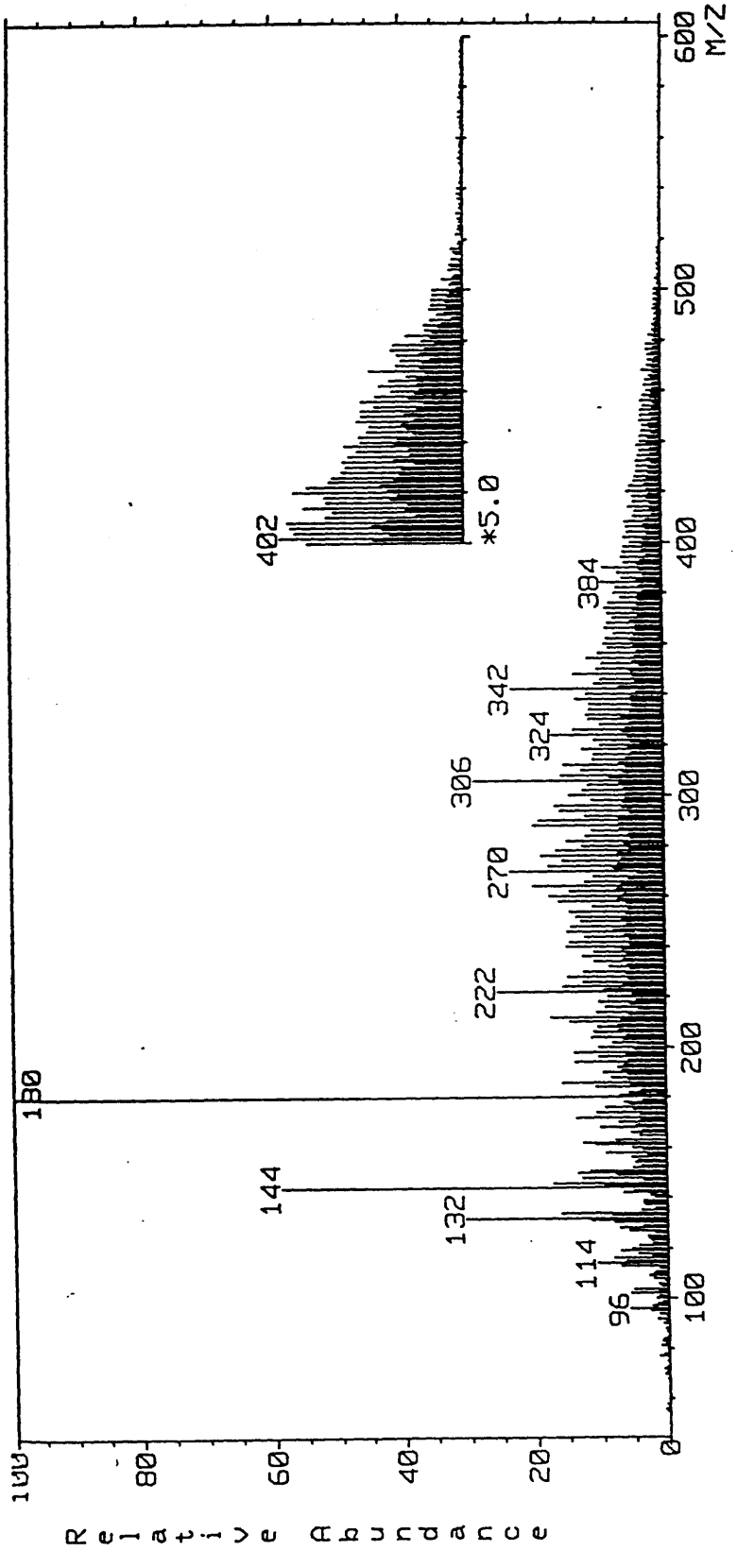

Relative Abundance
100
80
60
40
20
0
100
200
300
400
500
600
M/Z
180
144
132
114
96
222
270
306
324
342
384
402
*5.0

Figure 3.4.11 Platinum filament DCI Py-MS spectrum of DMSO soluble barley, scans 25-59.

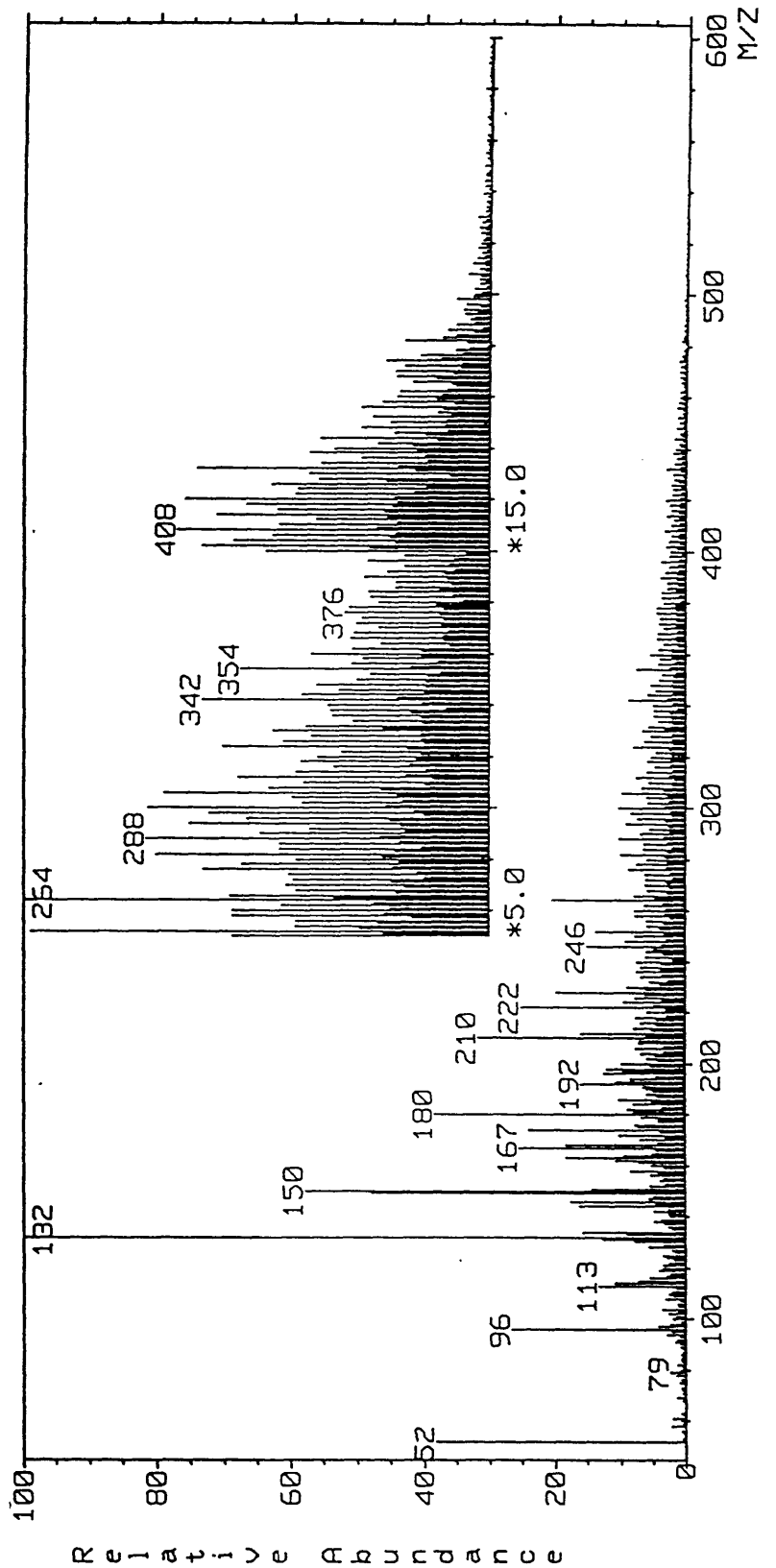

Relative Abundance
M/Z
100
80
60
40
20
0
100
200
300
400
500
600
52
79
96
113
132
150
167
180
192
210
222
246
254
288
342
354
376
408
*5.0
*15.0

Figure 3.4.12 Platinum filament DCI Py-MS spectrum of Barley DMSO residue, scans 23-46.

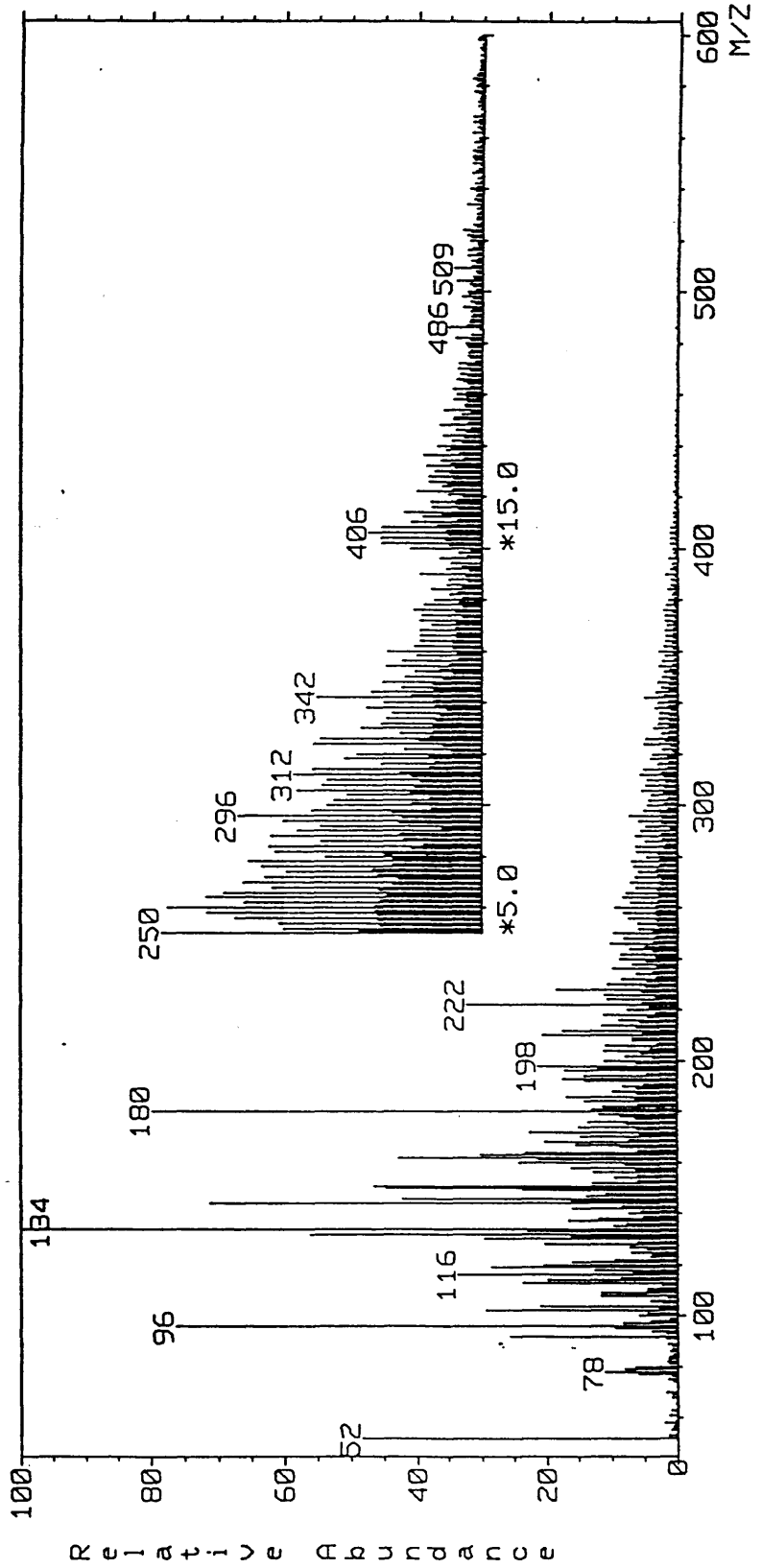

Relative Abundance
100
80
60
40
20
0
100
200
300
400
500
600
M/Z
52
78
96
116
134
180
198
222
250
296
312
342
406
486
509
*5.0
*15.0

Figure 3.4.13 Platinum filament DCI Py-MS spectrum of Barley driselase residue, scans 19-44.

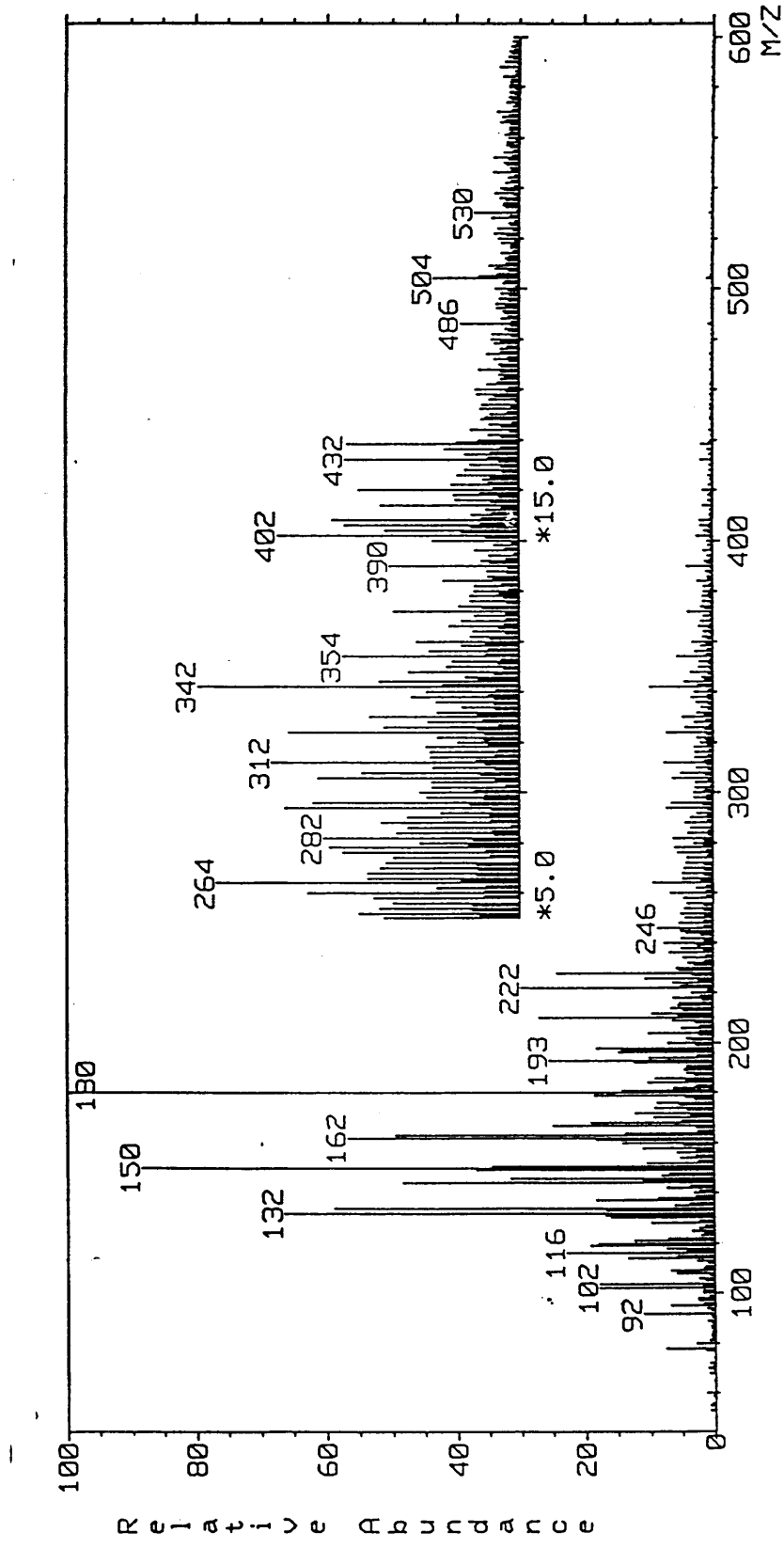

Relative Abundance
M/Z
100
80
60
40
20
0
100
200
300
400
500
600
130
150
162
132
116
102
92
193
222
246
264
282
312
342
354
390
402
432
486
504
530
*5.0
*15.0

Figure 3.4.14 Platinum filament DCI Py-MS spectrum of driselase soluble barley, scans 30–50.

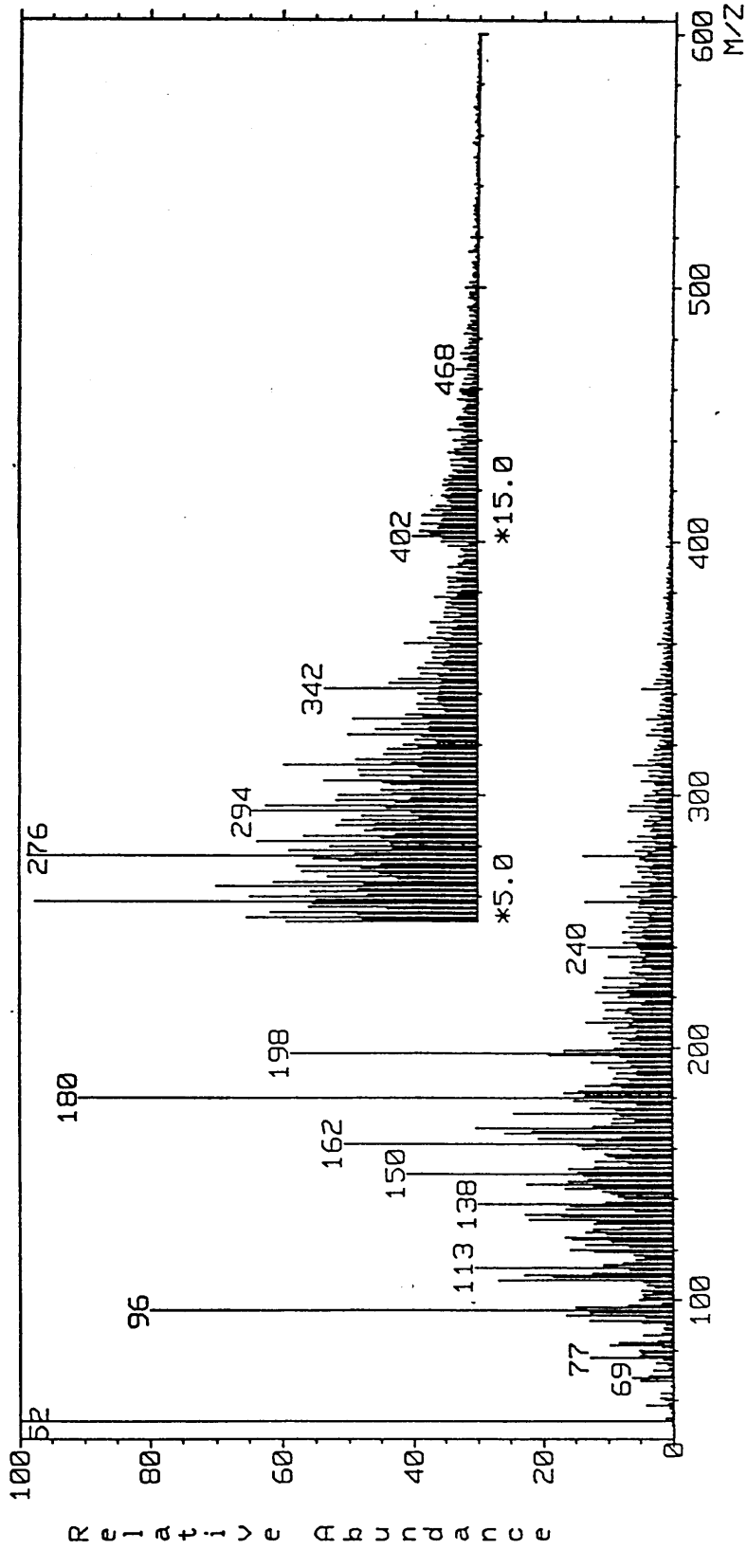

Relative Abundance
M/Z
100
80
60
40
20
0
100
200
300
400
500
600
52
69
77
96
113
138
150
162
180
198
240
276
294
342
402
468
*5.0
*15.0

Figure 3.4.15 Platinum filament DCI Py-MS spectrum of Barley MeOH, scans 17-50.

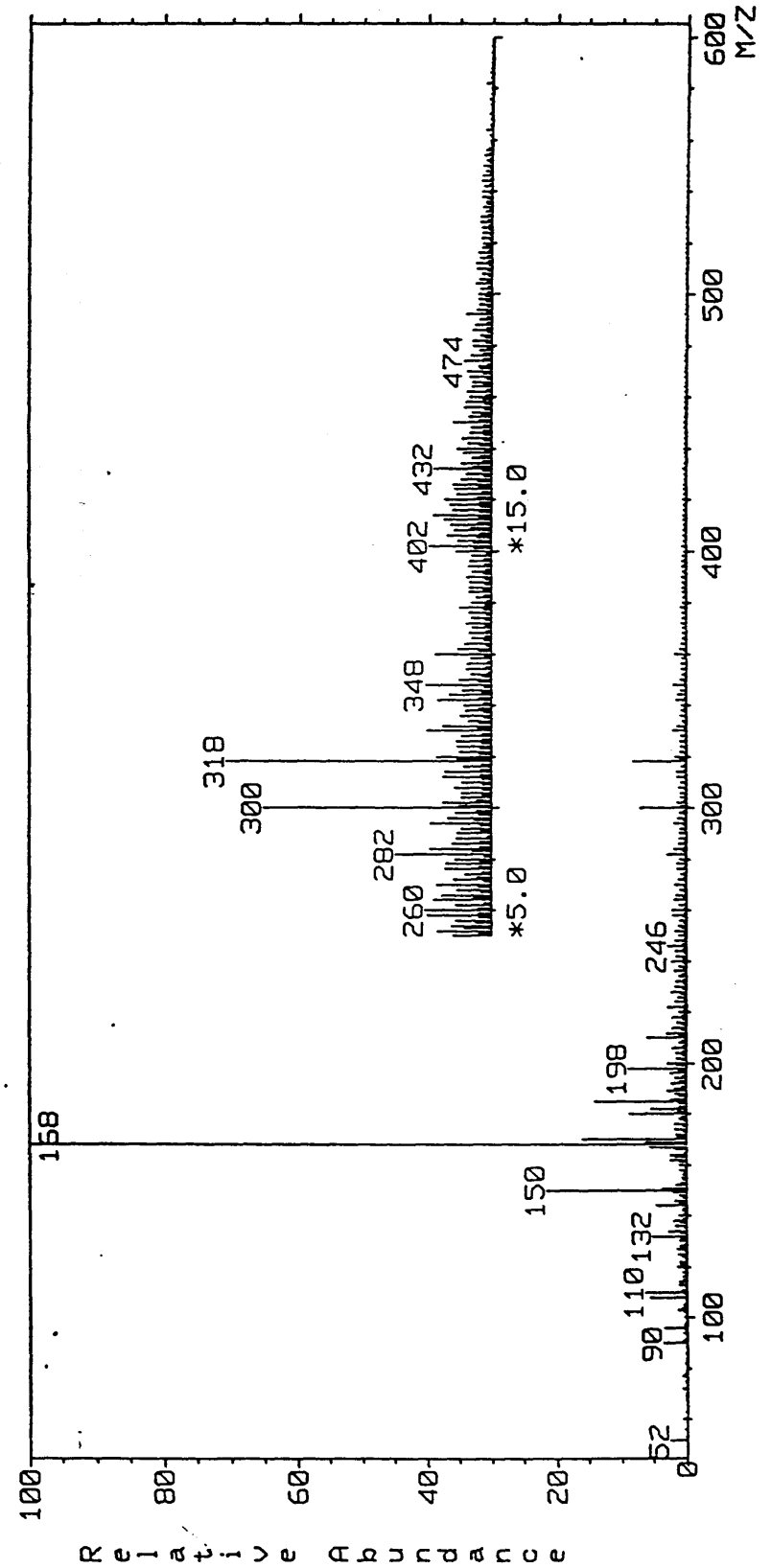

Relative Abundance
100
80
60
40
20
0
100
200
300
400
500
600
M/Z
52
90
110
132
150
168
198
246
260
282
300
318
348
402
432
474
*5.0
*15.0

Figure 3.4.16 Platinum filament DCI Py-MS spectrum of Barley H_2O, scans 32-61.

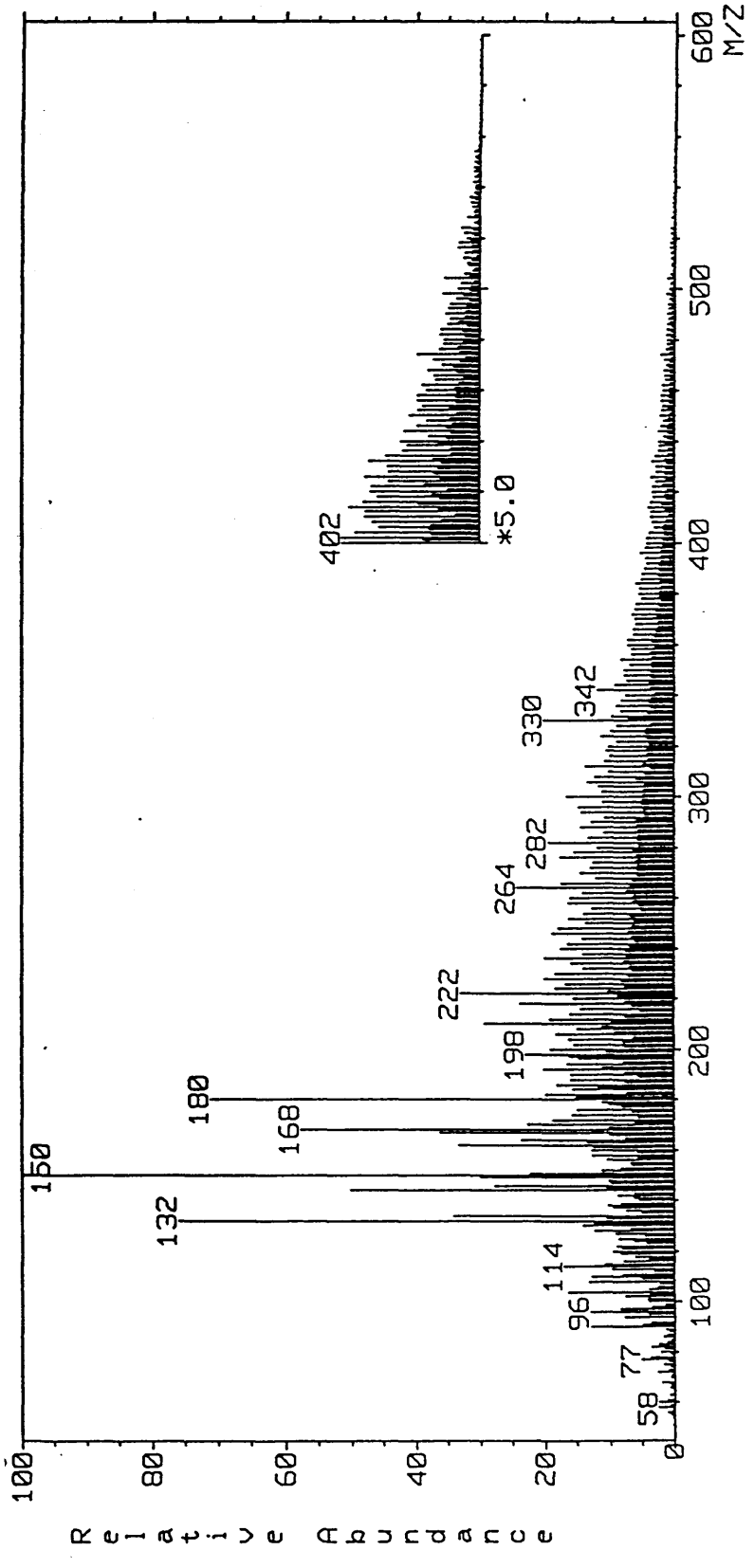

Relative Abundance
100
80
60
40
20
0
100
200
300
400
500
600
M/Z
58
77
96
114
132
150
168
180
198
222
264
282
330
342
402
*5.0

Figure 3.4.17 Platinum filament DCI Py-MS spectrum of Ryegrass, scans 28-31.

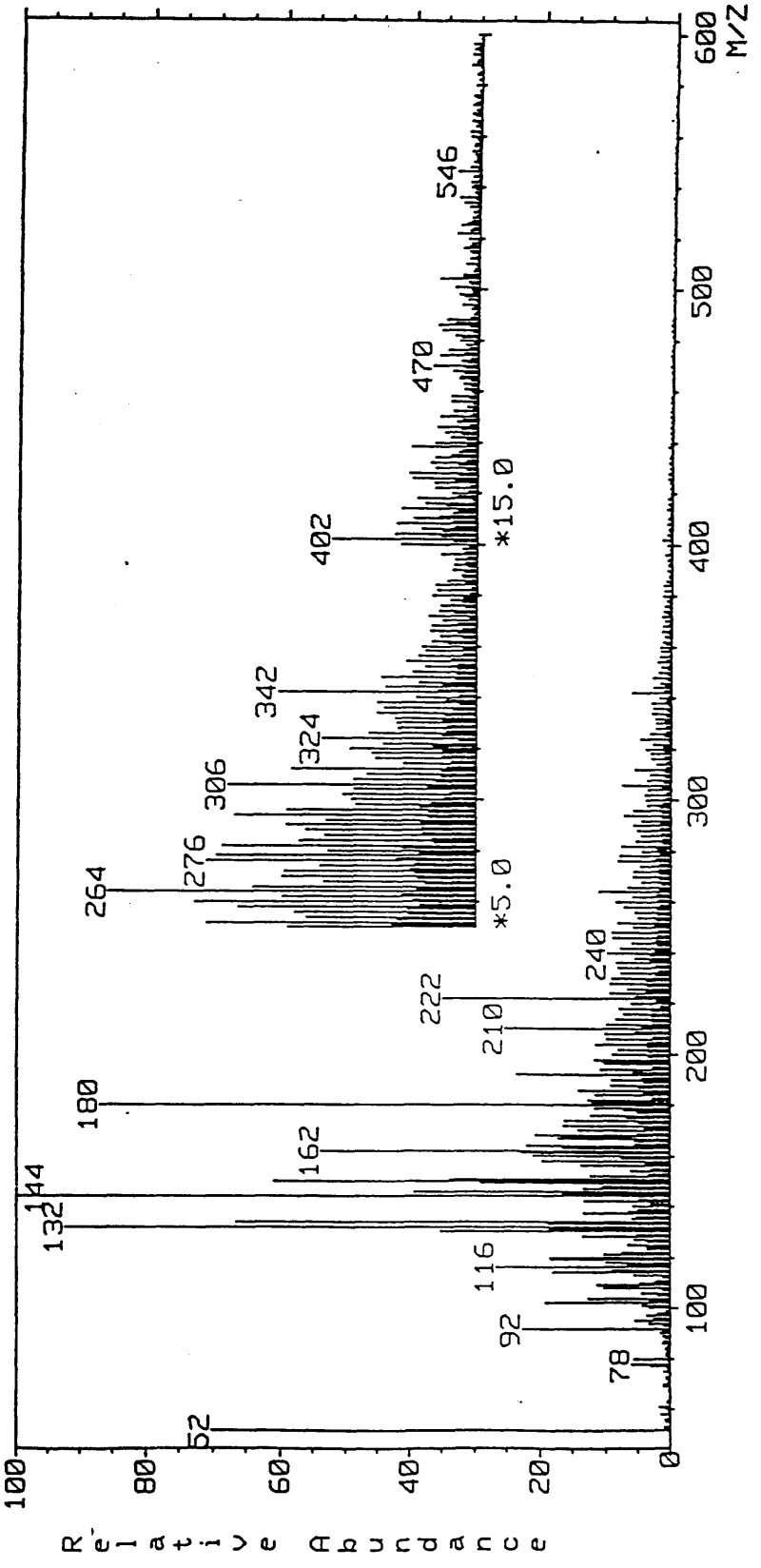

Relative Abundance
M/Z
100
80
60
40
20
0
100
200
300
400
500
600
52
78
92
116
132
144
162
180
210
222
240
264
276
306
324
342
402
470
546
*15.0
*5.0

Figure 3.4.18 Platinum filament DCI Py-MS spectrum of Oxalate hydrolysed ryegrass, scans 24-37.

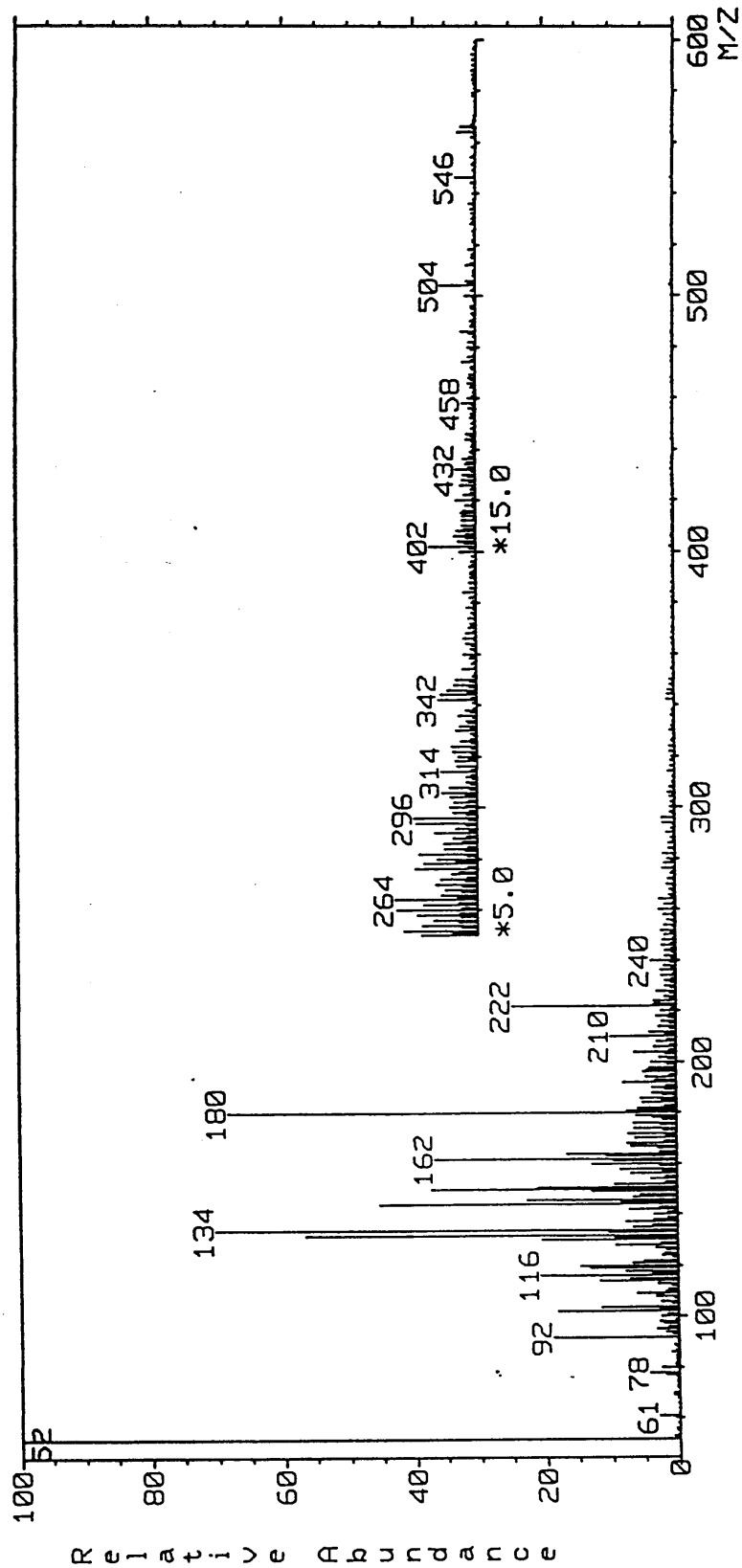

Relative Abundance
M/Z
100
80
60
40
20
0
100
200
300
400
500
600
BP
61
78
92
116
134
162
180
210
222
240
264
296
314
342
402
432
458
504
546
*5.0
*15.0

Figure 3.4.19 Platinum filament DCI Py-MS spectrum of DMSO soluble ryegrass, scans 25-46.

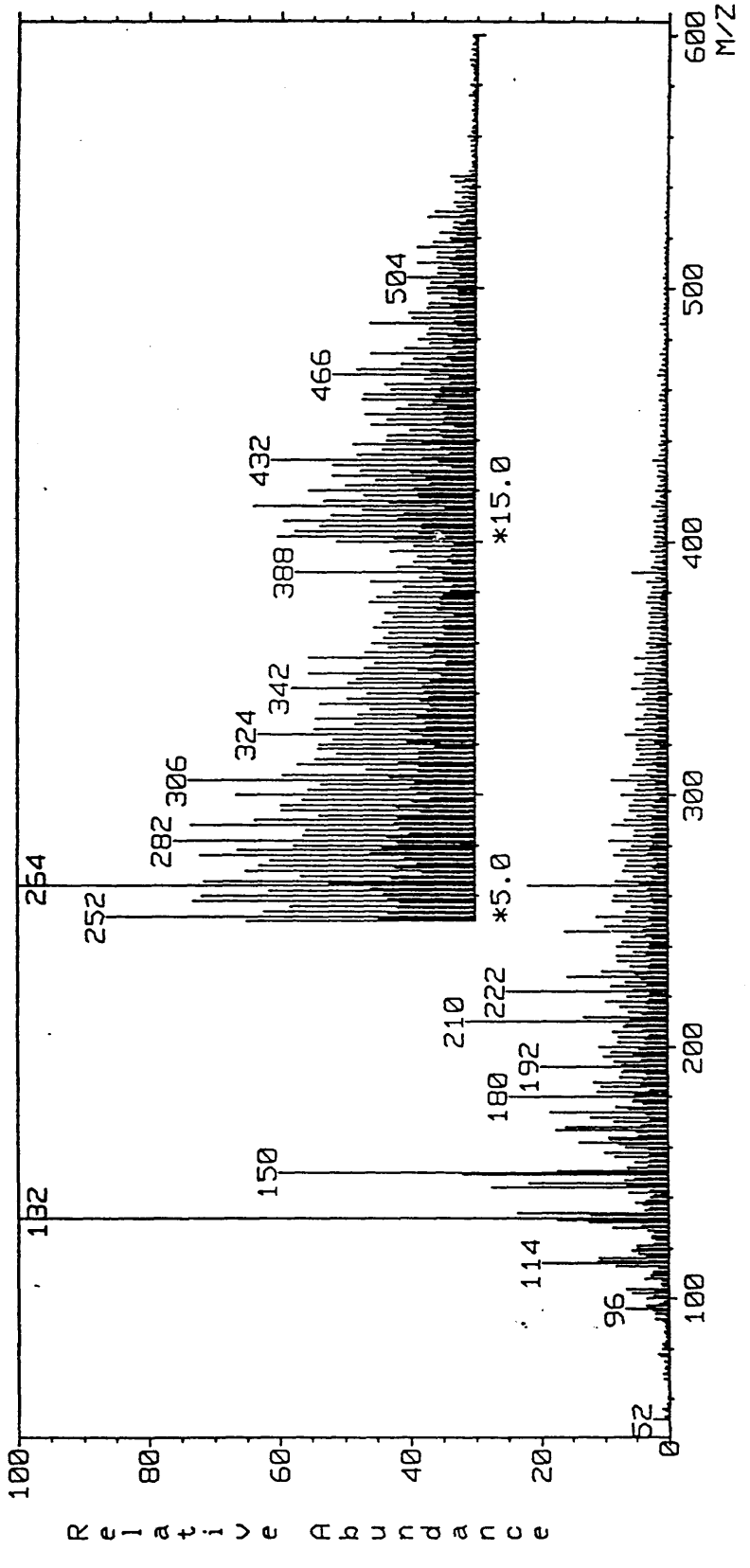

Relative Abundance
M/Z
100
80
60
40
20
0
100
200
300
400
500
600
52
96
114
132
150
180
192
210
222
252
254
282
306
324
342
388
432
466
504
*5.0
*15.0

Figure 3.4.20 Platinum filament DCI Py-MS spectrum of Ryegrass DMSO residue, scans 26-52.

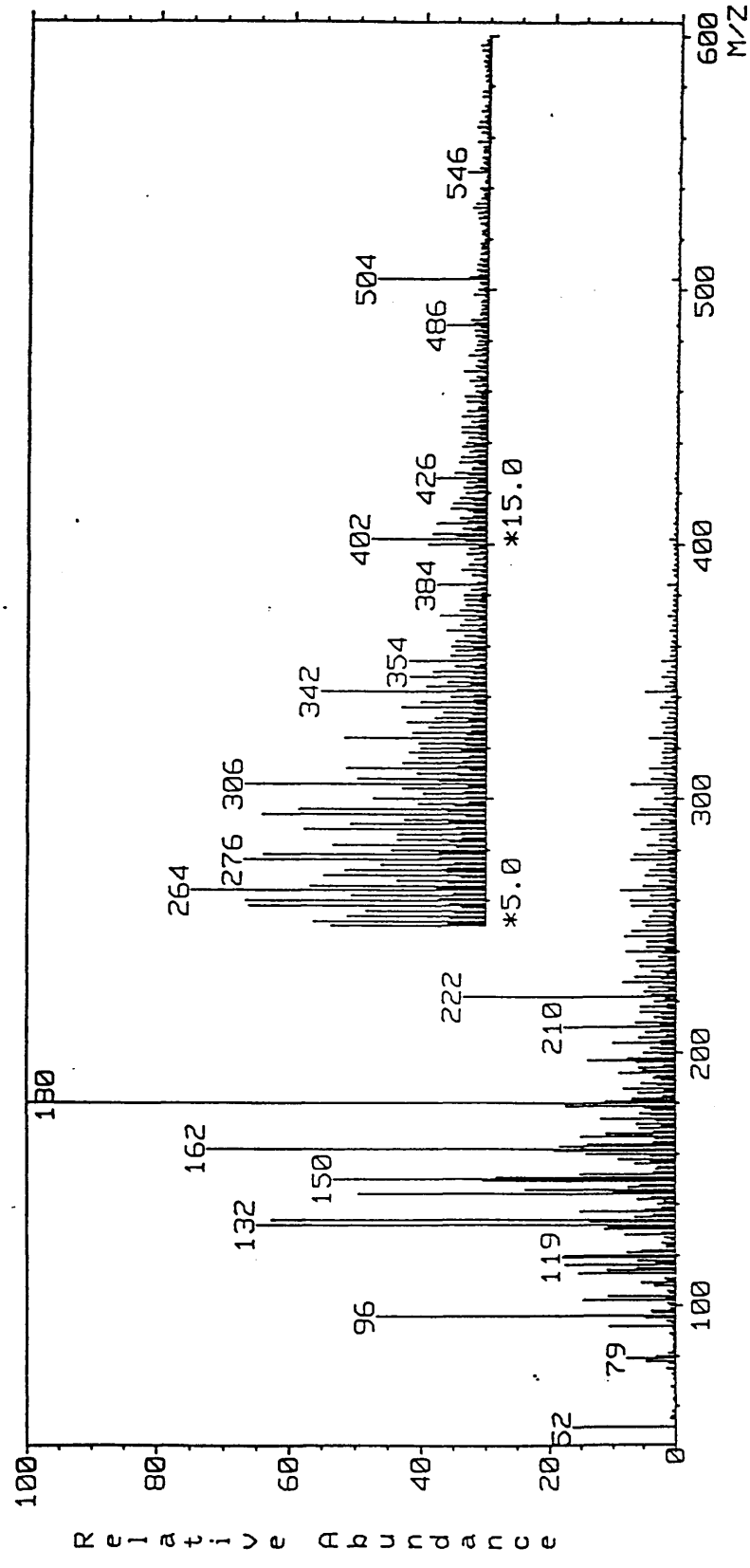

Relative Abundance
M/Z
100
80
60
40
20
0
100
200
300
400
500
600
52
79
96
119
132
150
162
180
210
222
264
276
306
342
354
384
402
426
486
504
546
*5.0
*15.0

Figure 3.4.21 Platinum filament DCI Py-MS spectrum of Ryegrass driselase residue, scans 22-41.

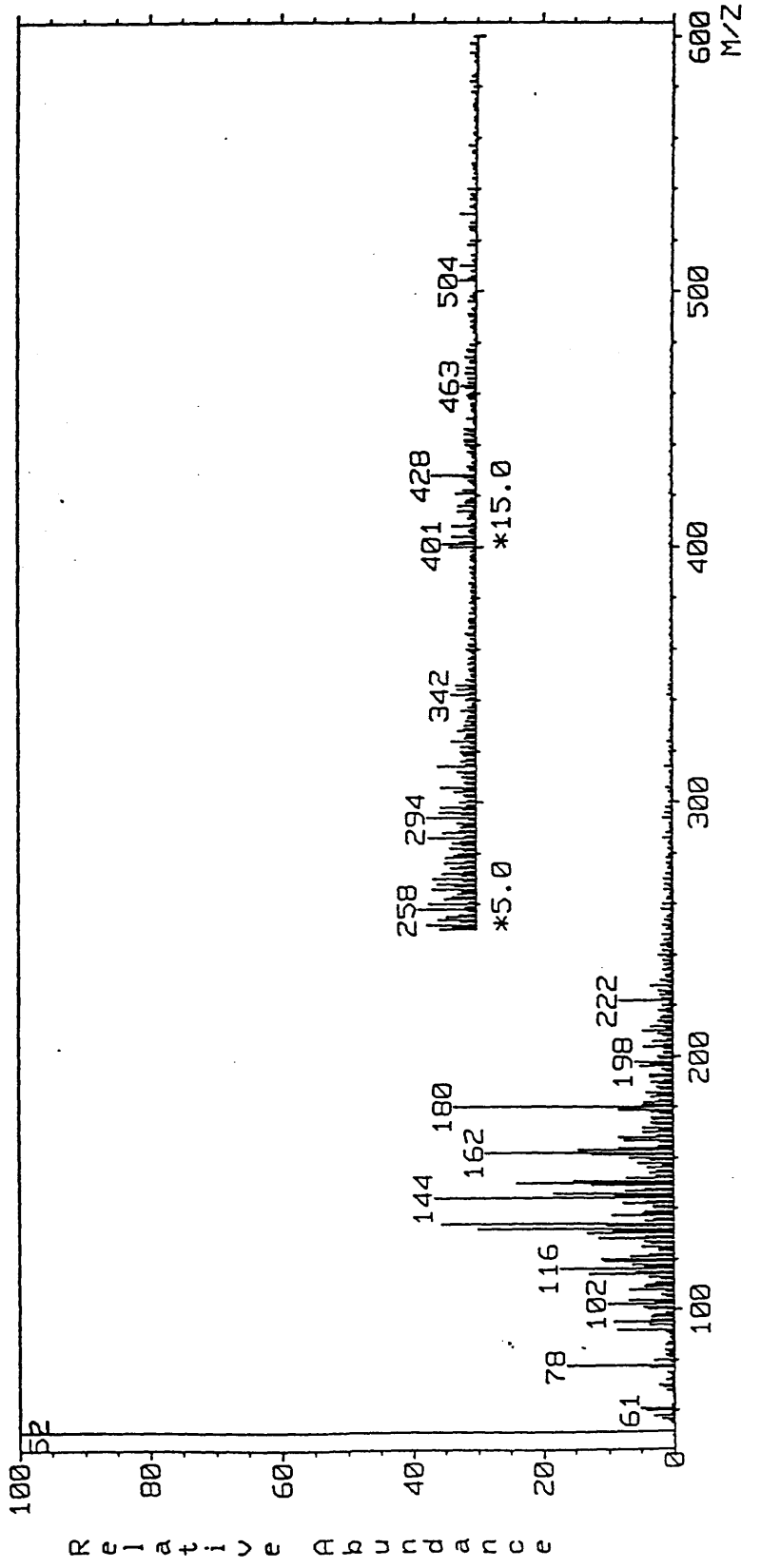

Relative Abundance
M/Z
100
80
60
40
20
0
100
200
300
400
500
600
52
61
78
102
116
144
162
180
198
222
258
294
342
401
428
463
504
*5.0
*15.0

Figure 3.4.22 Platinum filament DCI Py-MS spectrum of driselase soluble ryegrass, scans 19-43.

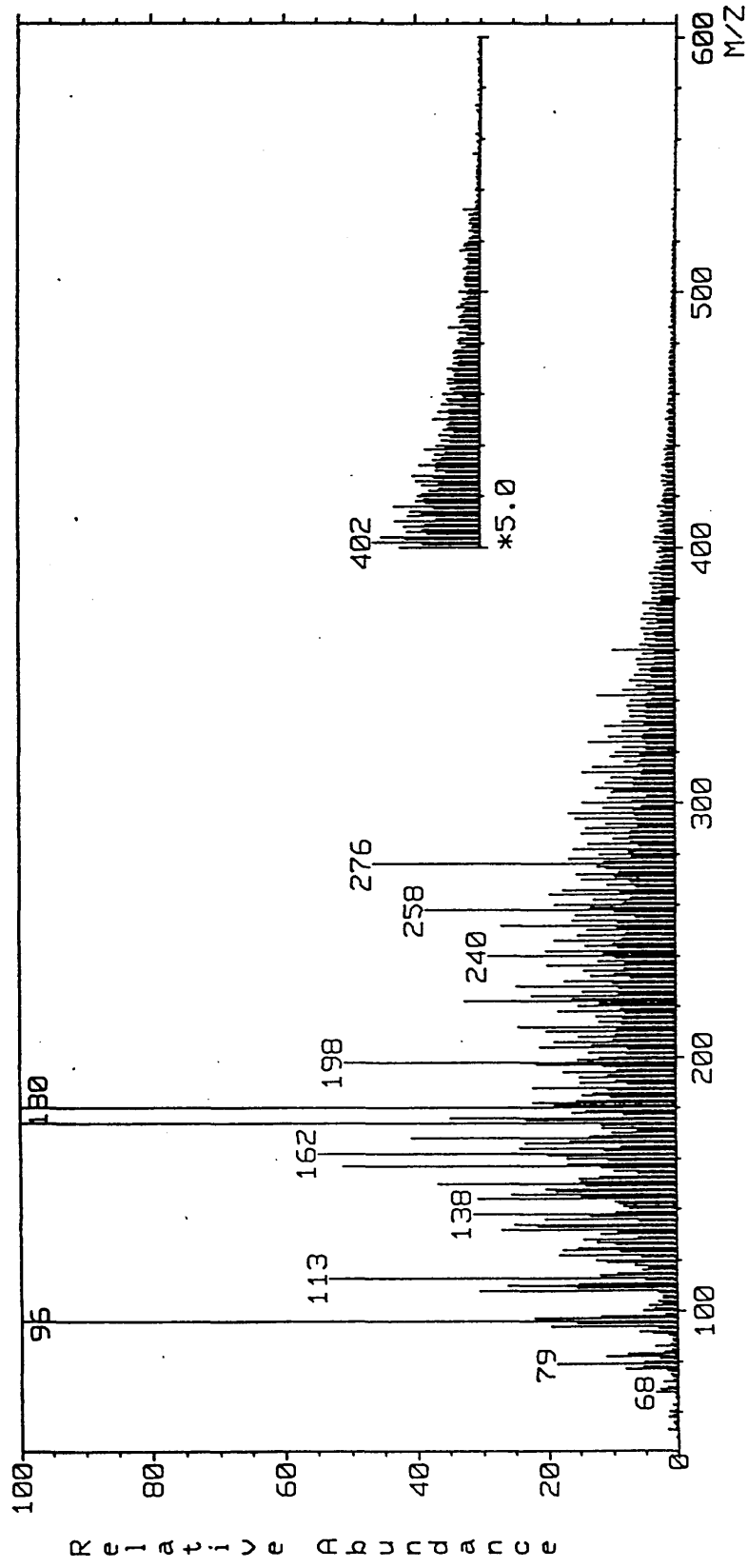

Relative Abundance
100
80
60
40
20
0
100
200
300
400
500
600
M/Z
68
79
96
113
138
162
180
198
240
258
276
402
*5.0

Figure 3.4.23 Platinum filament DCI Py-MS spectrum of Ryegrass MeOH, scans 22-60.

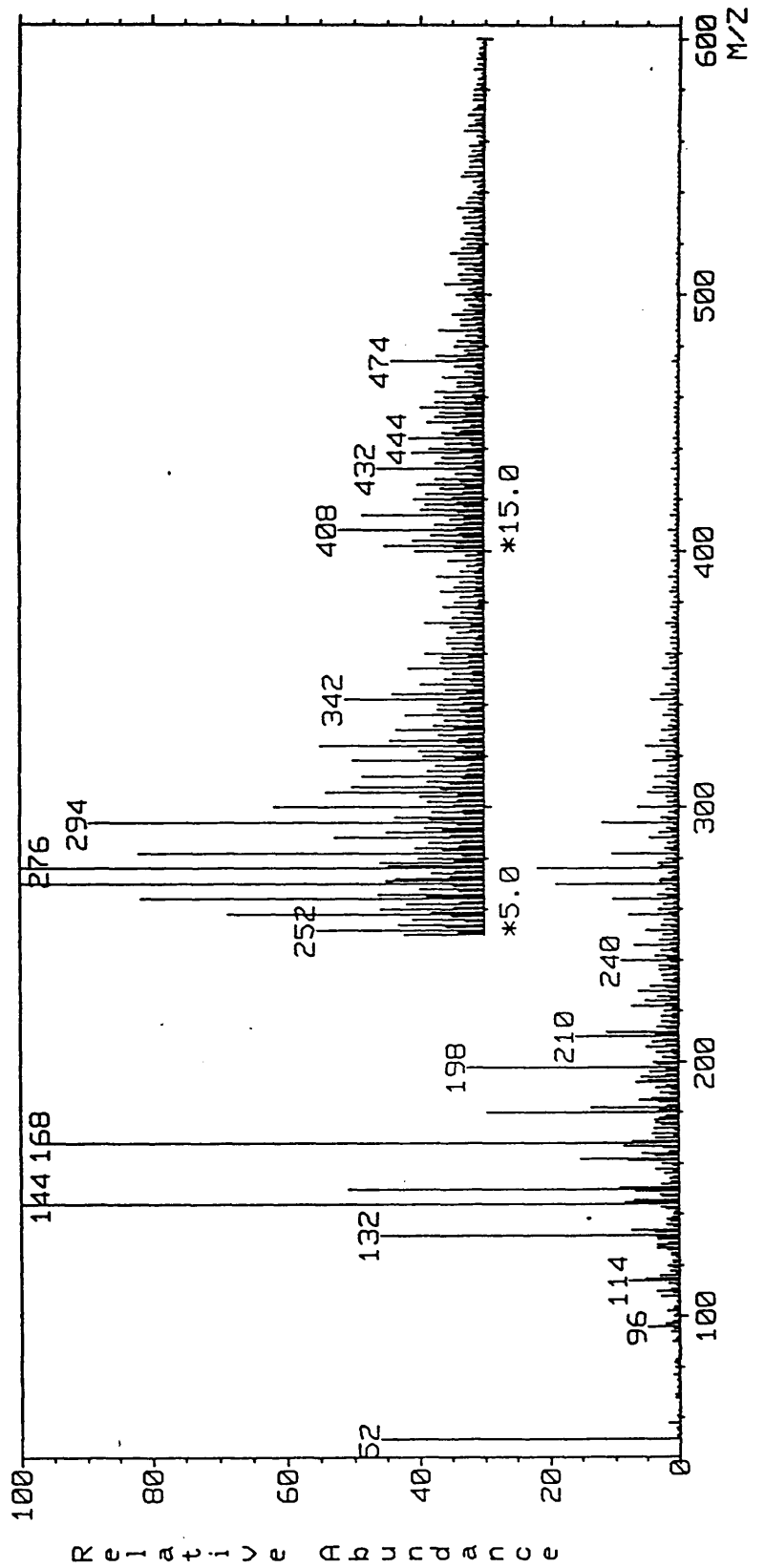

Relative Abundance
M/Z
100
80
60
40
20
0
100
200
300
400
500
600
52
96
114
132
144
168
198
210
240
252
276
294
342
408
432
444
474
*5.0
*15.0

Figure 3.4.24 Platinum filament DCI Py-MS spectrum of Ryegrass H_2O, scans 32-54.

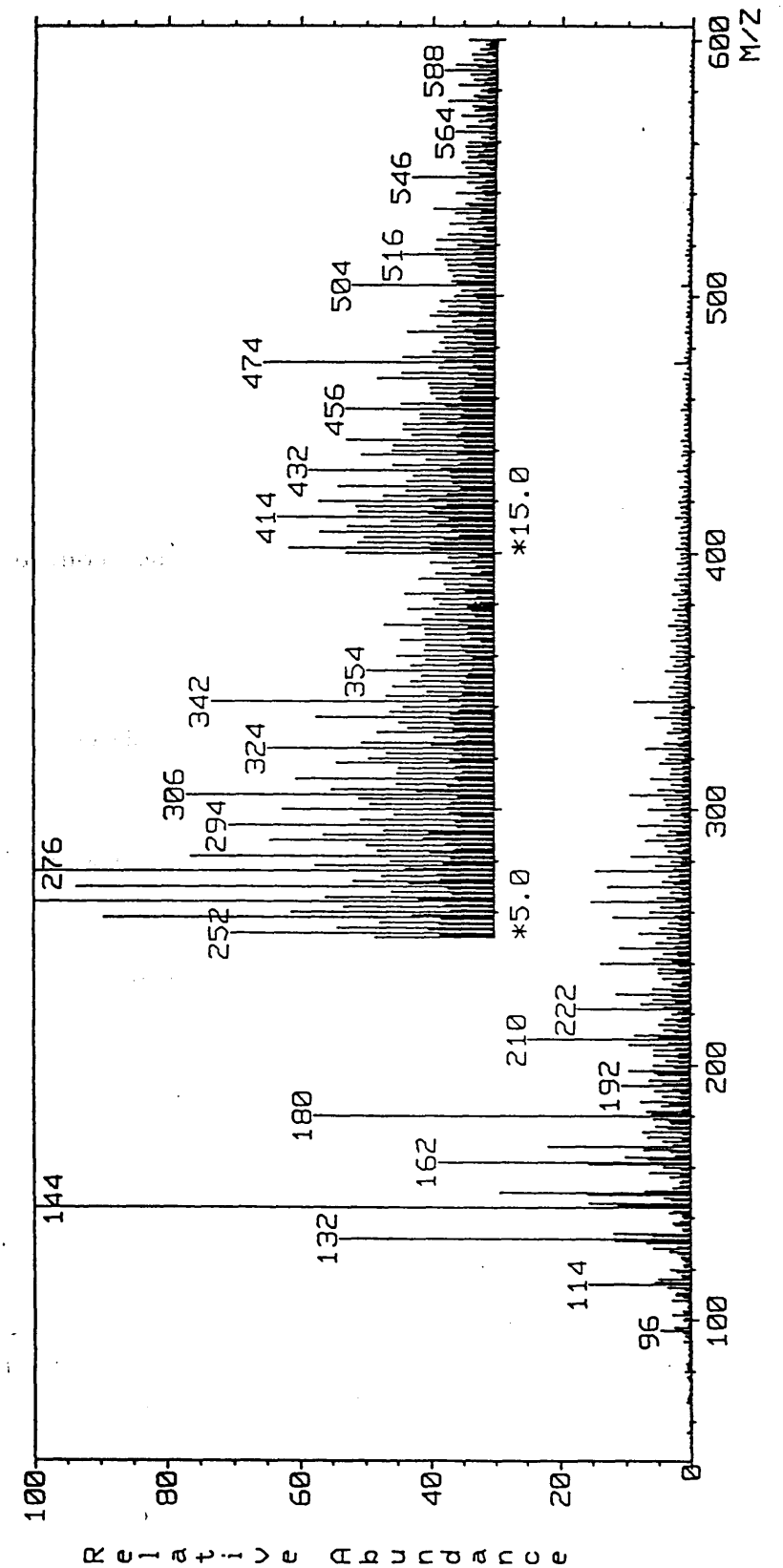

Relative Abundance
M/Z
100
80
60
40
20
0
100
200
300
400
500
600
96
114
132
144
162
180
192
210
222
252
276
294
306
324
342
354
414
432
456
474
504
516
546
564
588
*5.0
*15.0

Figure 3.4.25 Platinum filament EI Py-MS time course of barley fractions.

1) Barley straw.

2) Oxalate hydrolysed barley straw.

3) DMSO soluble barley straw.

4) Oxalate hydrolysed DMSO extracted barley straw.

5) Barley straw driselase residue.

6) Barley straw driselase soluble.

7) Barley straw H_2O.

8) Barley MeOH.

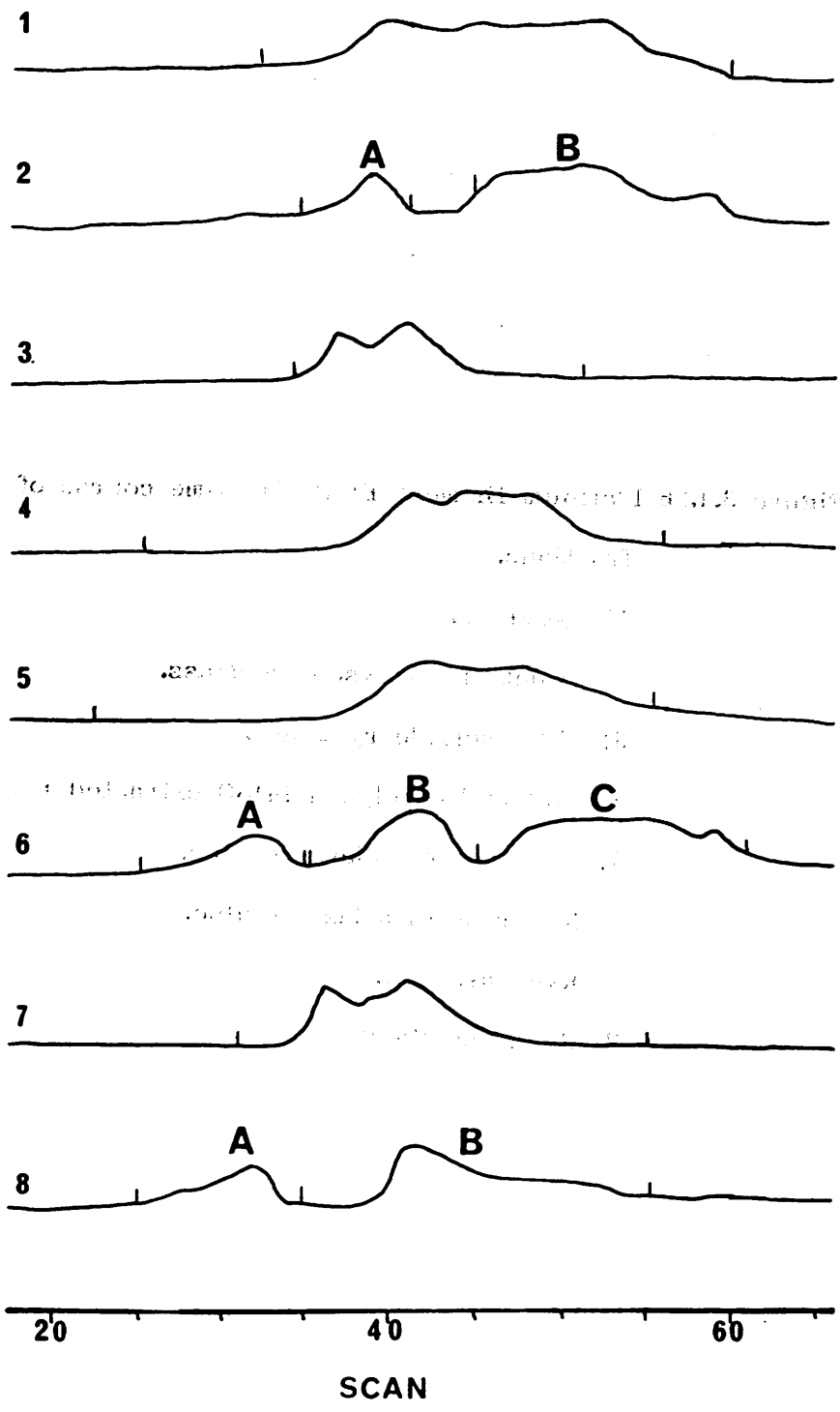

1
2
A
B
3
4
5
6
A
B
C
7
8
A
B
20
40
60
SCAN

Figure 3.4.26 Platinum filament EI Py-MS time course of ryegrass fractions.

1) Ryegrass.

2) Oxalate hydrolysed ryegrass.

3) DMSO soluble ryegrass.

4) Oxalate hydrolysed DMSO extracted ryegrass.

5) Ryegrass driselase residue.

6) Ryegrass driselase soluble.

7) Ryegrass H_2O.

8) Ryegrass MeOH.

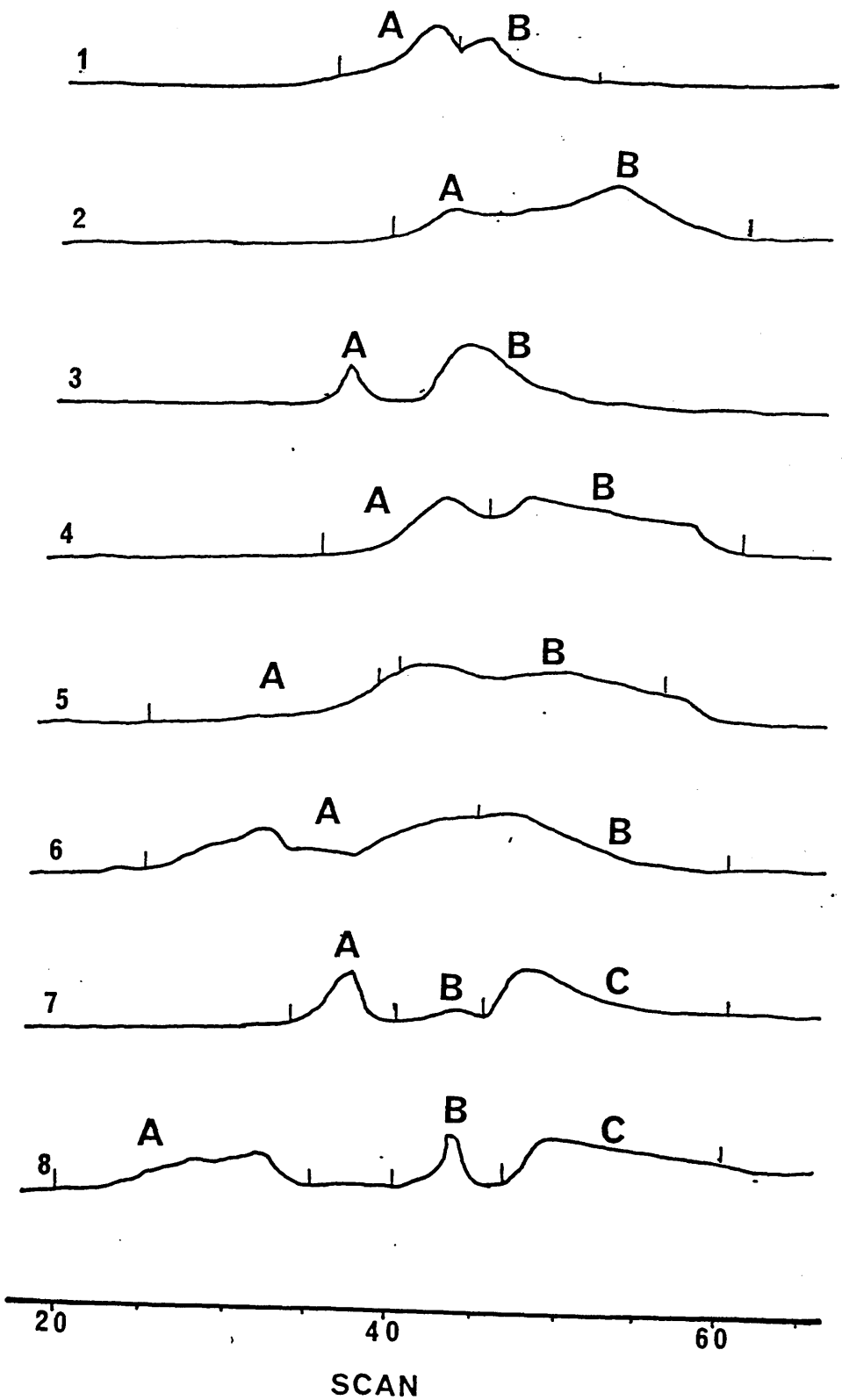

1
A
B
2
A
B
3
A
B
4
A
B
5
A
B
6
A
B
7
A
B
C
8
A
B
C
20
40
60
SCAN

Figure 3.4.27 Platinum filament EI Py-MS spectrum of Barley straw, scans 32-59.

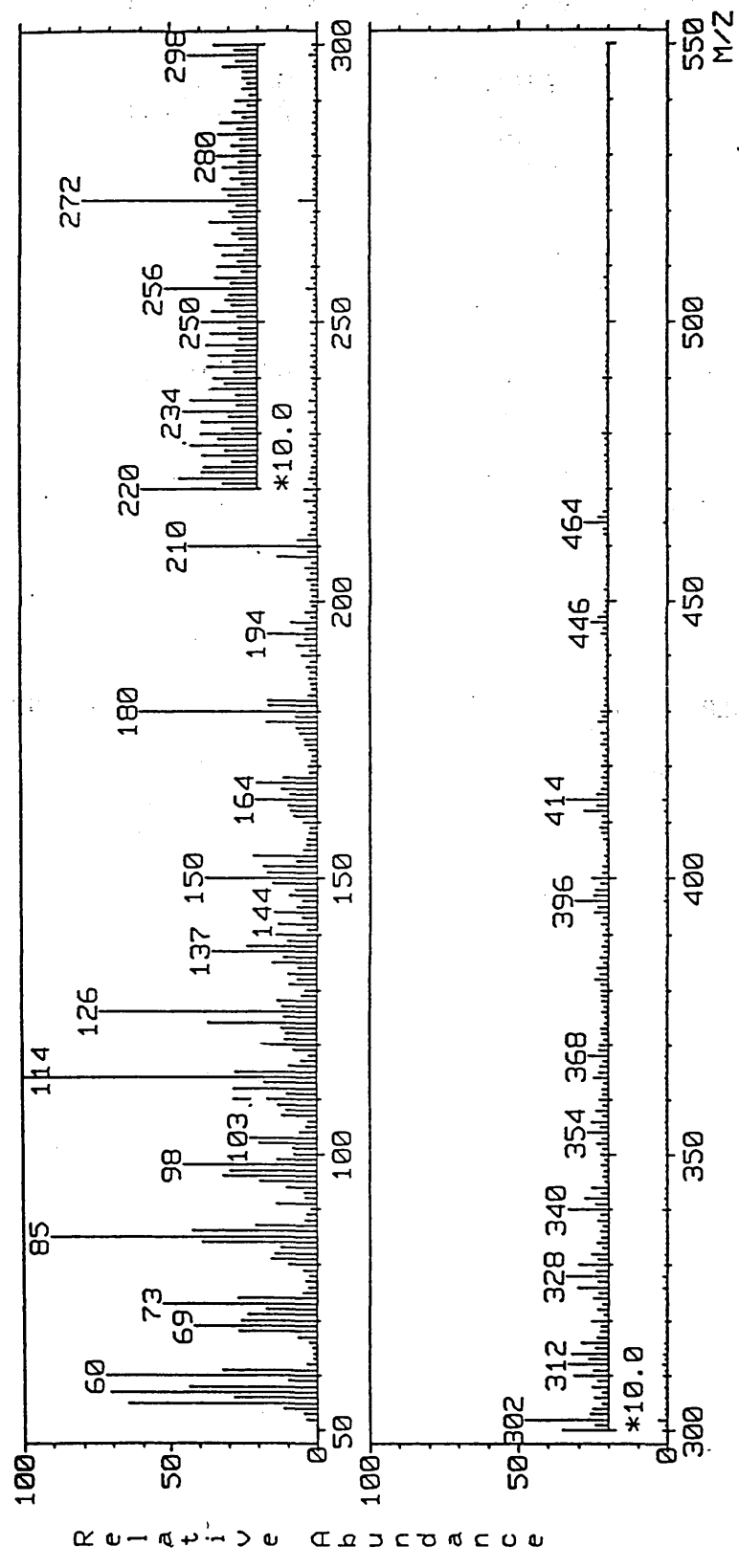
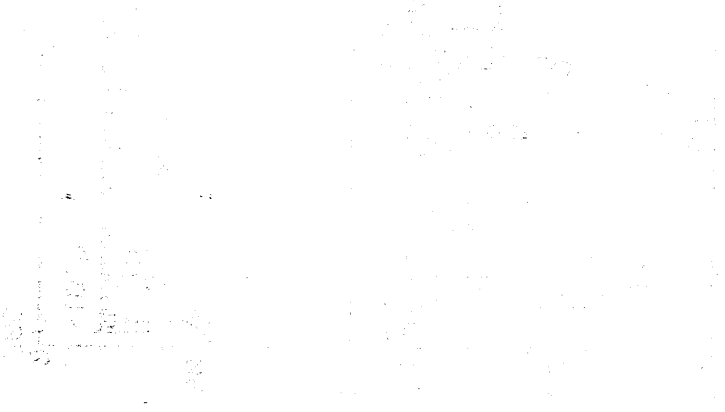
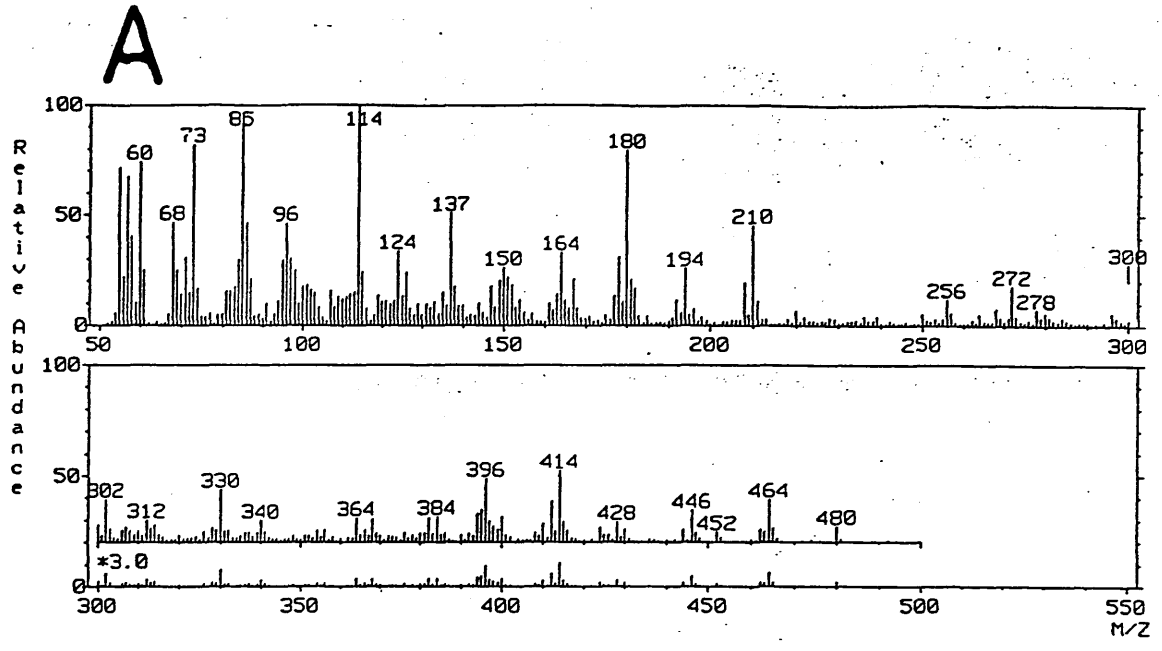

Relative Abundance
M/Z
*10.0
*10.0

Figure 3.4.28 Platinum filament EI Py-MS of oxalate hydrolysed barley straw A) scans 33-41 and B) 44-75.




A
Relative Abundance
100
50
0
60
73
85
114
68
96
124
137
150
164
180
194
210
256
272
278
300
50
100
150
200
250
300
100
50
0
302
312
330
340
364
384
396
414
428
446
452
464
480
*3.0
300
350
400
450
500
550
M/Z

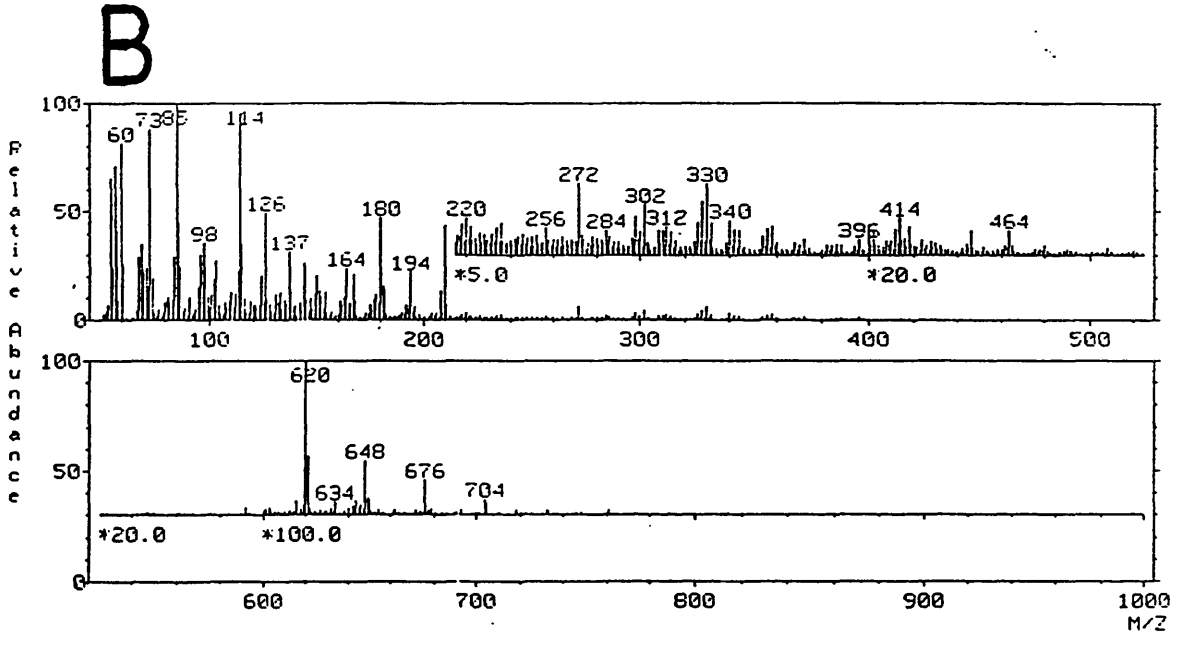

B
Relative Abundance
100
50
0
60
73
85
114
98
126
137
164
180
194
220
256
272
284
302
312
330
340
396
414
464
*5.0
*20.0
100
200
300
400
500
100
50
0
620
634
648
676
704
*20.0
*100.0
600
700
800
900
1000
M/Z

Figure 3.4.29 Platinum filament EI Py-MS spectrum of DMSO soluble barley, scans 33-50.

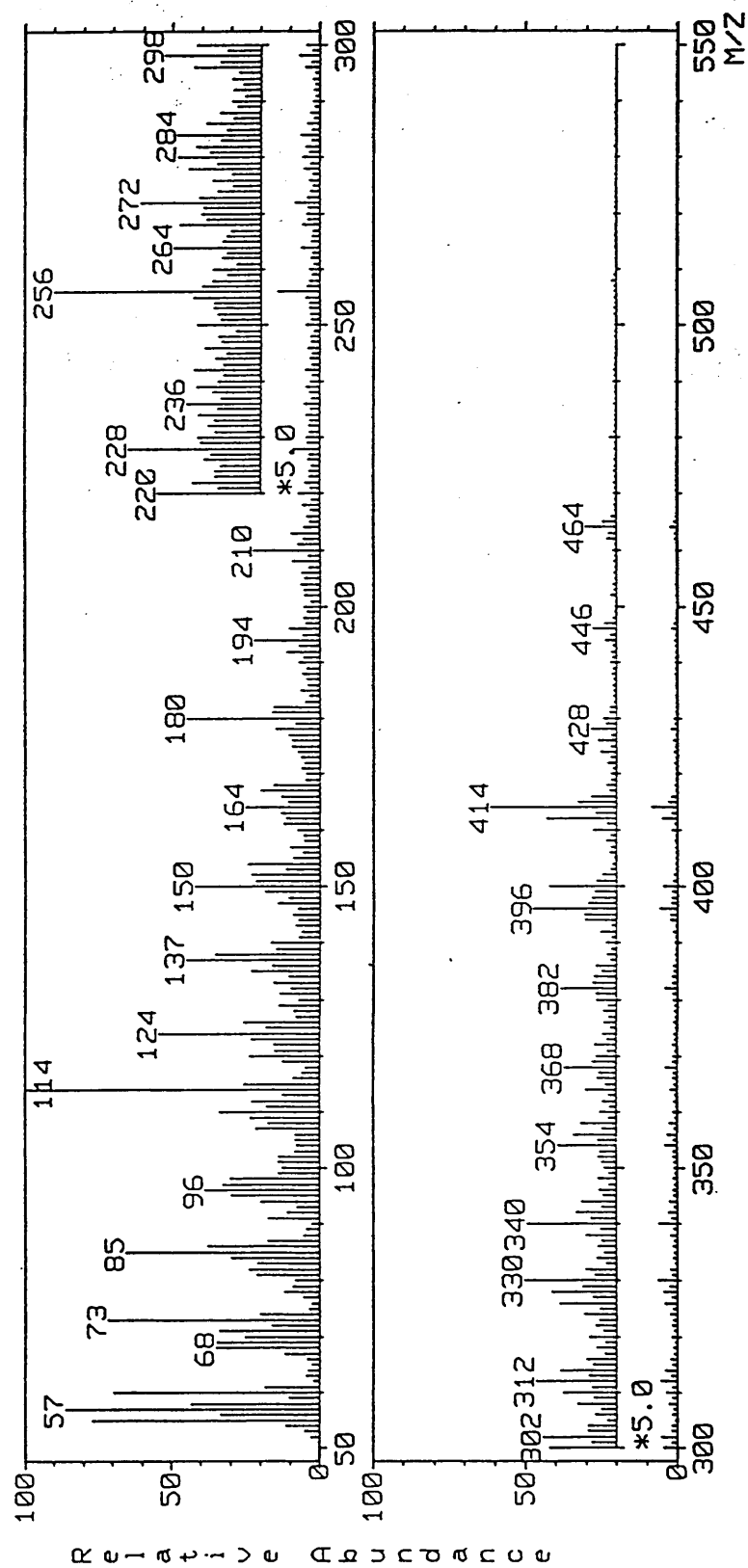

Relative Abundance
M/Z
*5.0
*5.0

Figure 3.4.30 Platinum filament EI Py-MS spectrum of Barley DMSO residue, scans 25-55.

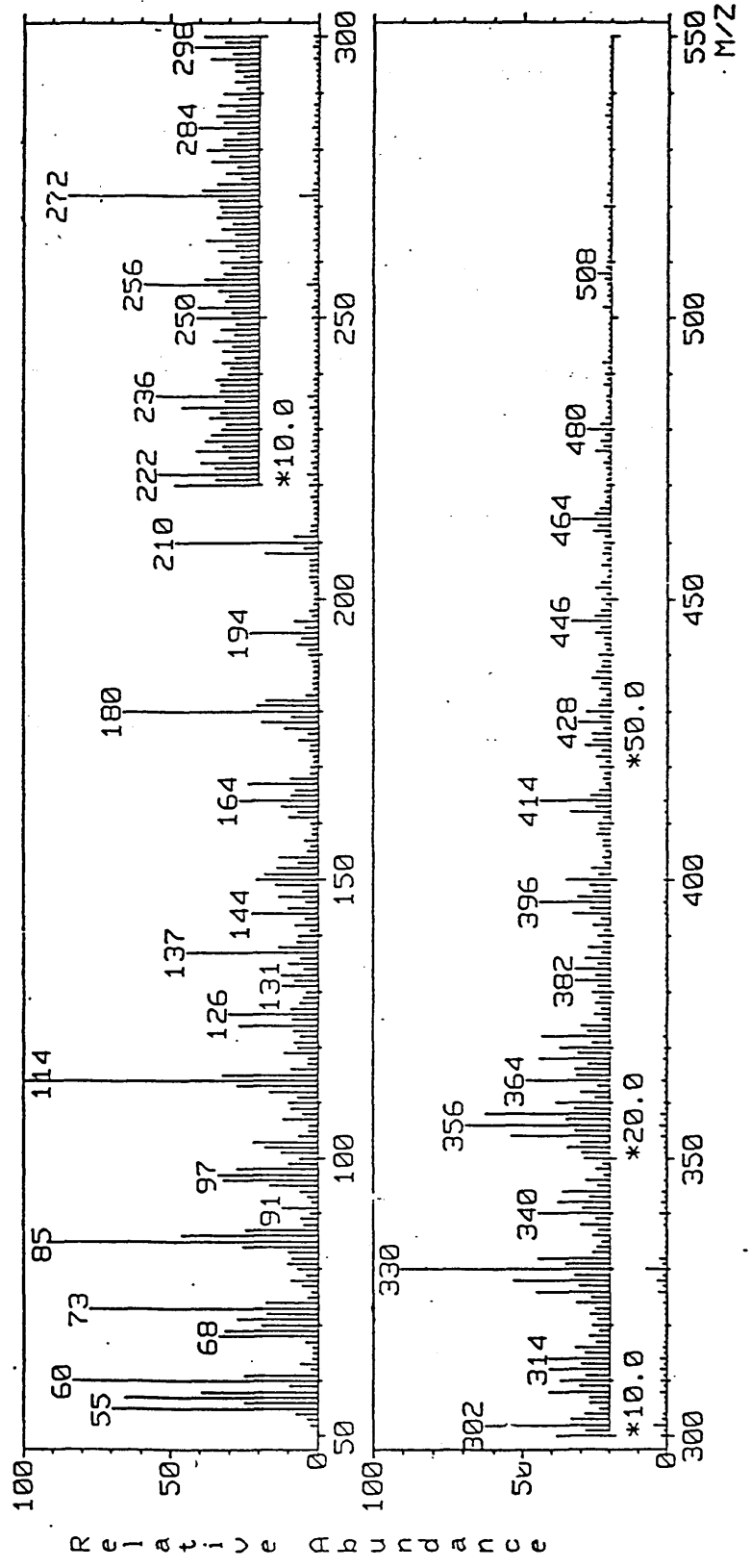

Relative Abundance
M/Z
*10.0
*20.0
*50.0

Figure 3.4.31 Platinum filament EI Py-MS of driselase soluble barley straw A) scans 25-34, B) 35-45 and C) 46-60.

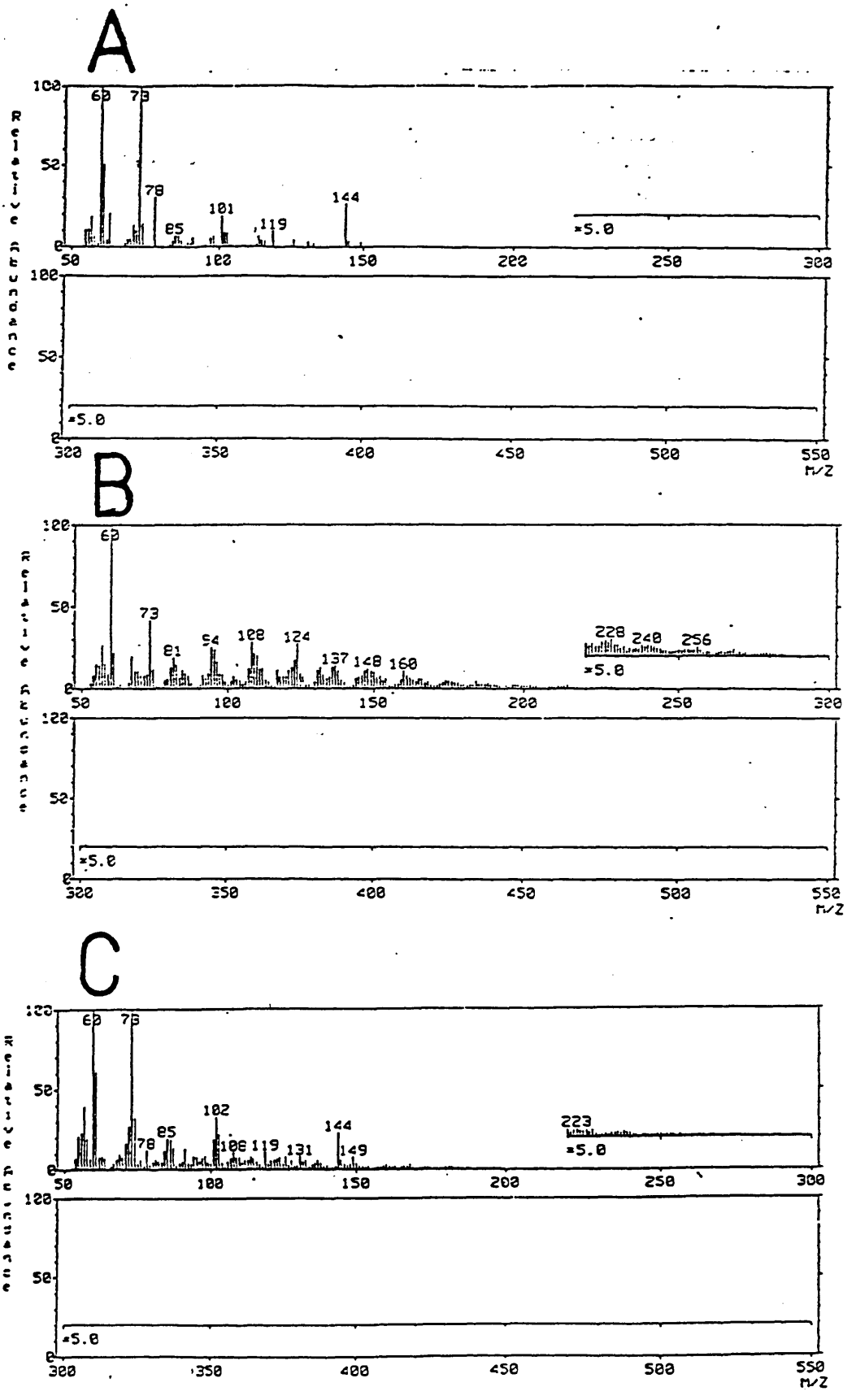

A
100
50
0
69
73
78
85
101
119
144
×5.0
50
100
150
200
250
300
100
50
0
×5.0
300
350
400
450
500
550
M/Z
B
100
50
0
69
73
81
94
108
124
137
148
160
228
240
256
×5.0
50
100
150
200
250
300
100
50
0
×5.0
300
350
400
450
500
550
M/Z
C
100
50
0
69
73
78
85
102
108
119
131
144
149
223
×5.0
50
100
150
200
250
300
100
50
0
×5.0
300
350
400
450
500
550
M/Z

Figure 3.4.32 Platinum filament EI Py-MS spectrum of Barley driselase residue, scans 22-56.

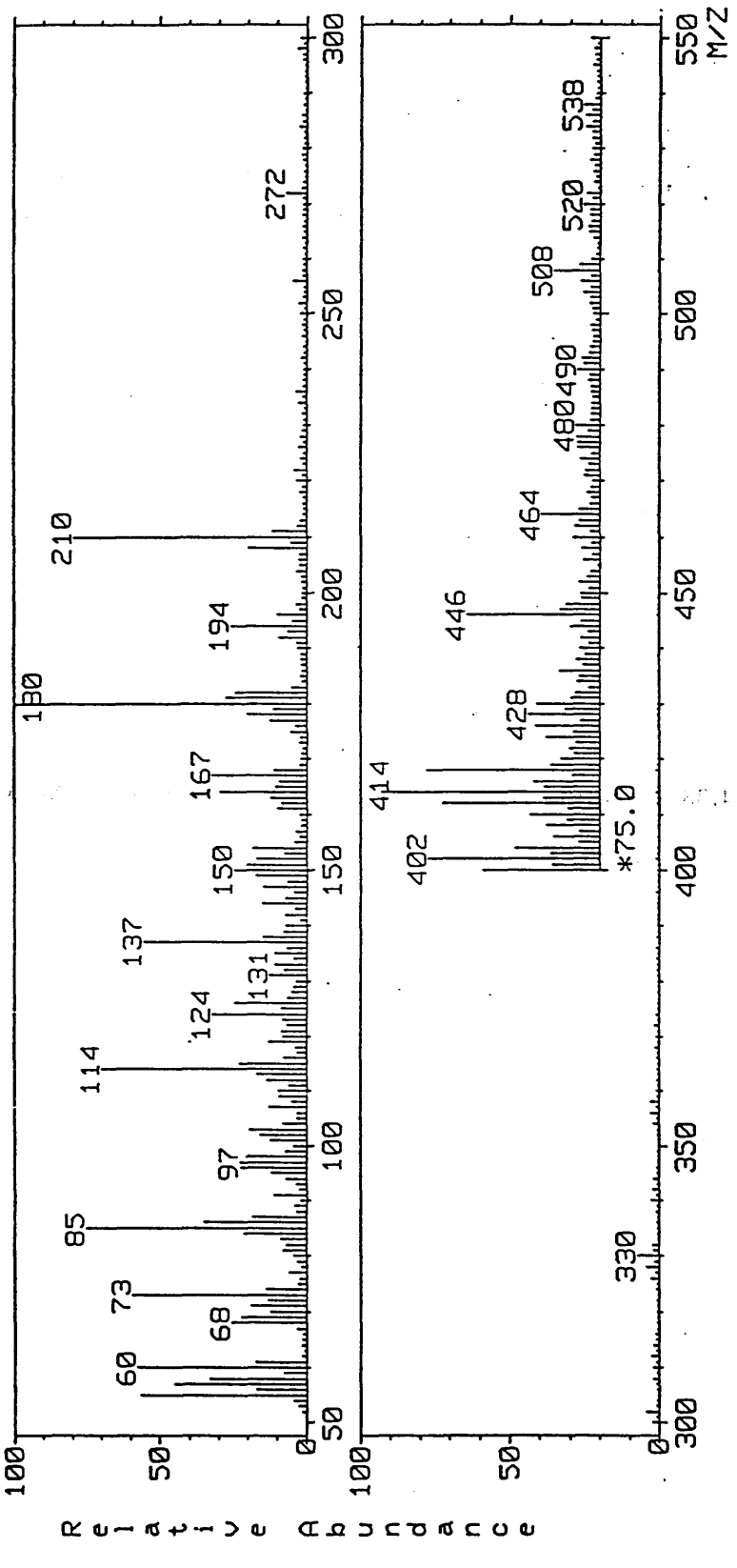
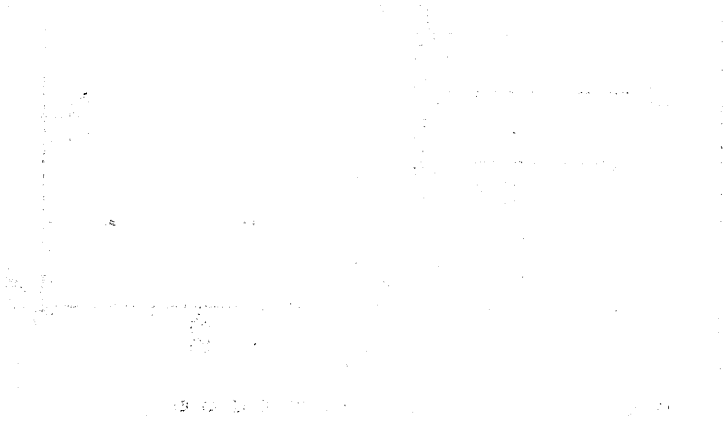
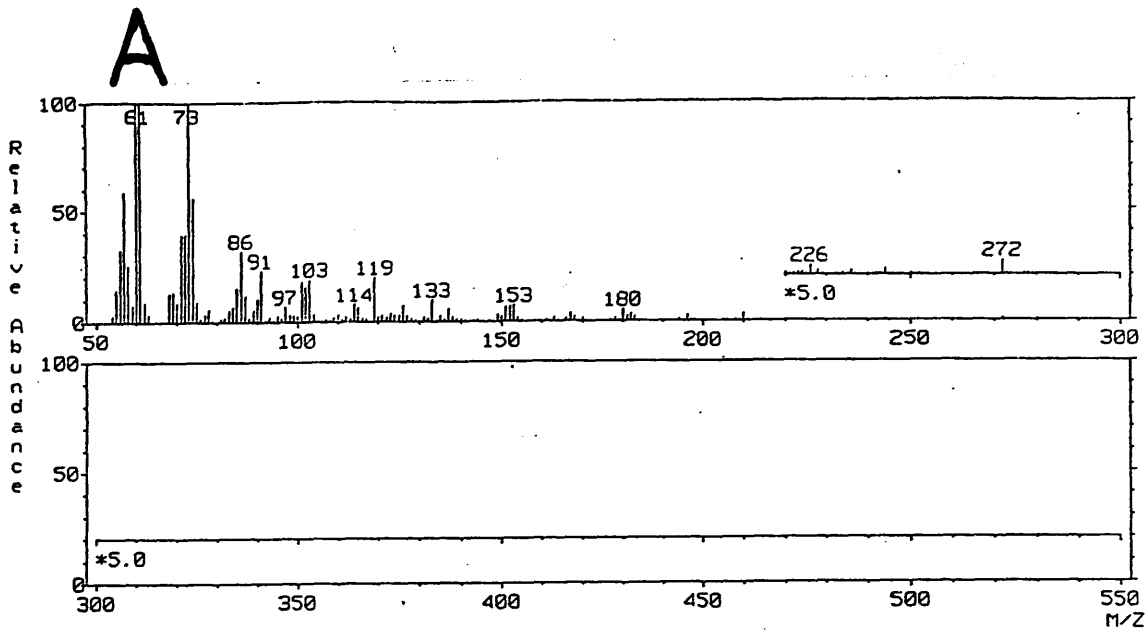

Relative Abundance
100
50
0
50
100
150
200
250
300
60
68
73
85
97
114
124
131
137
150
167
180
194
210
272
100
50
0
300
330
350
400
414
402
428
446
464
480
490
508
520
538
550
M/Z
*75.0

Figure 3.4.33 Platinum filament EI Py-MS of barley MeOH A) scans 25-35, B) 35-55.




A
Relative Abundance
100
50
0
61
73
86
91
97
103
114
119
133
153
180
226
272
*5.0
50
100
150
200
250
300
100
50
0
*5.0
300
350
400
450
500
550
M/Z

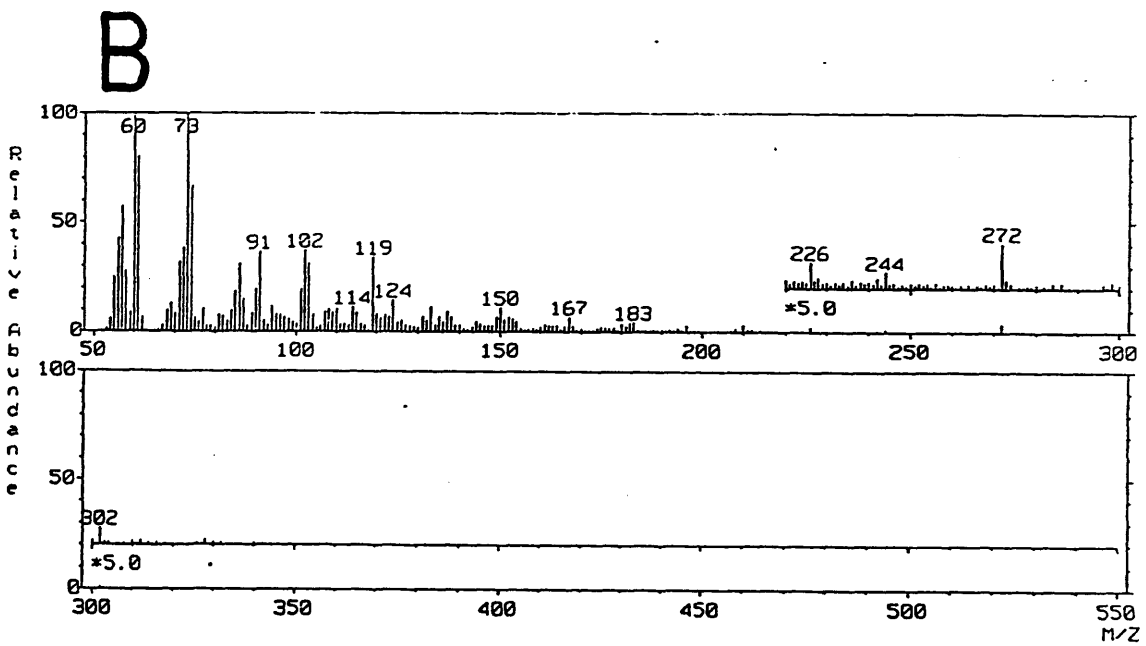

B
Relative Abundance
100
50
0
60
73
91
102
119
114
124
150
167
183
226
244
272
*5.0
50
100
150
200
250
300
100
50
0
302
*5.0
300
350
400
450
500
550
M/Z

Figure 3.4.34 Platinum filament DCI Py-MS spectrum of Barley H_2O, scans 31-55.

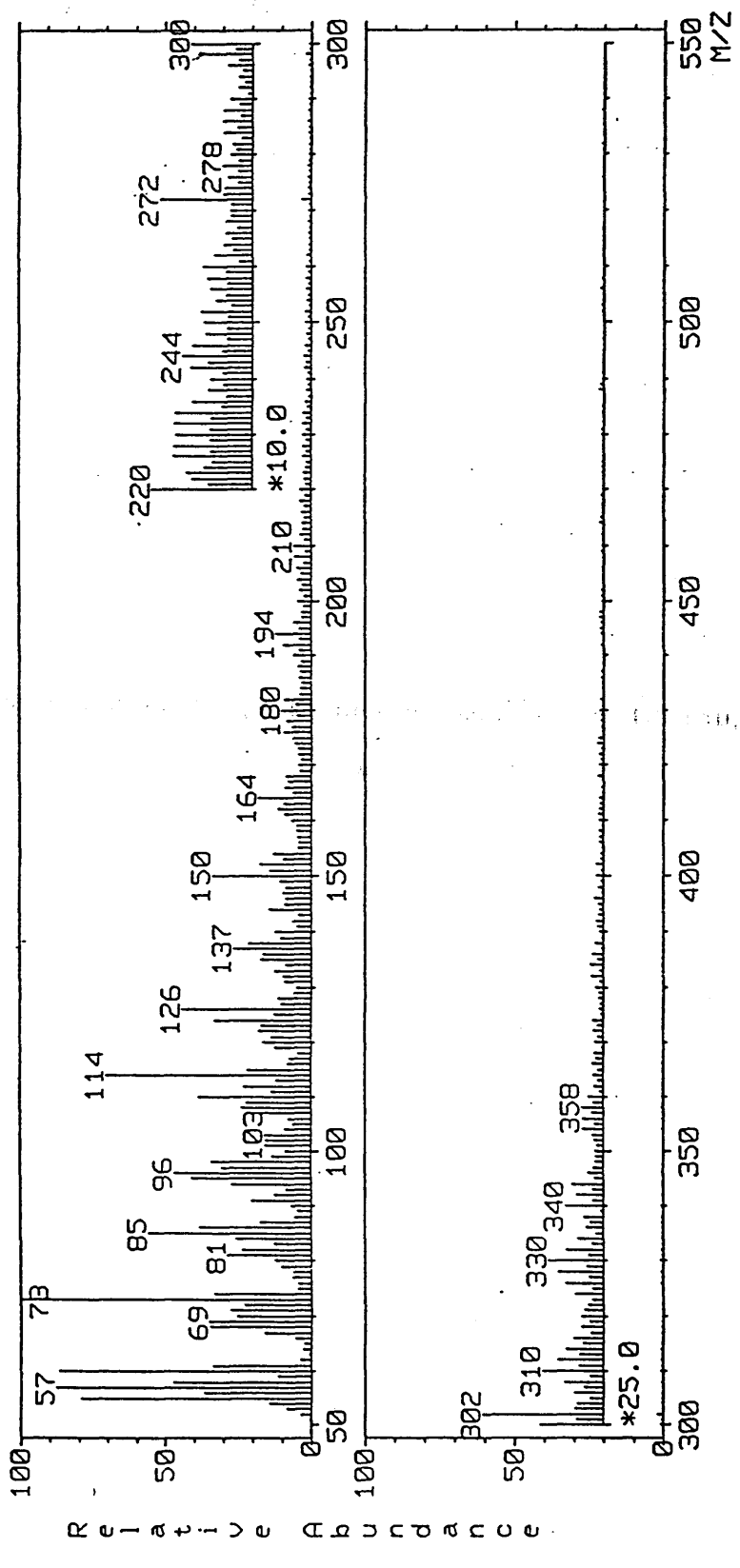
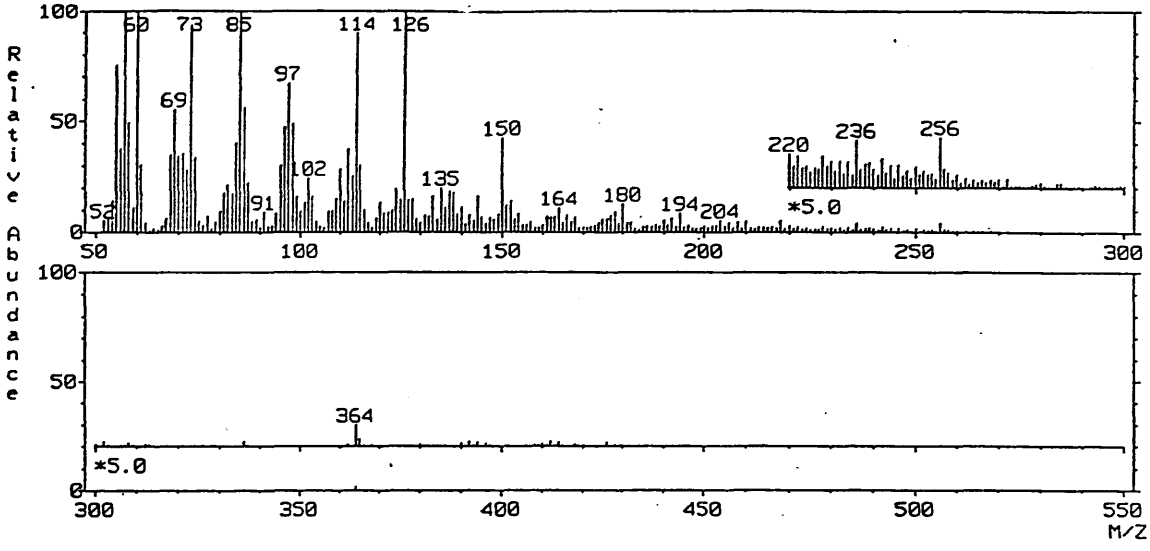

Relative Abundance
M/Z
*10.0
*25.0
57
73
69
81
85
96
103
114
126
137
150
164
180
194
210
220
244
272
278
302
310
330
340
358

Figure 3.4.35 Platinum filament EI Py-MS of Ryegrass A) scans 35-42, B) 43-50.

A


Relative Abundance
100
50
0
60
73
85
114
126
97
69
150
220
236
256
52
91
102
135
164
180
194
204
*5.0
50
100
150
200
250
300
364
*5.0
300
350
400
450
500
550
M/Z

B

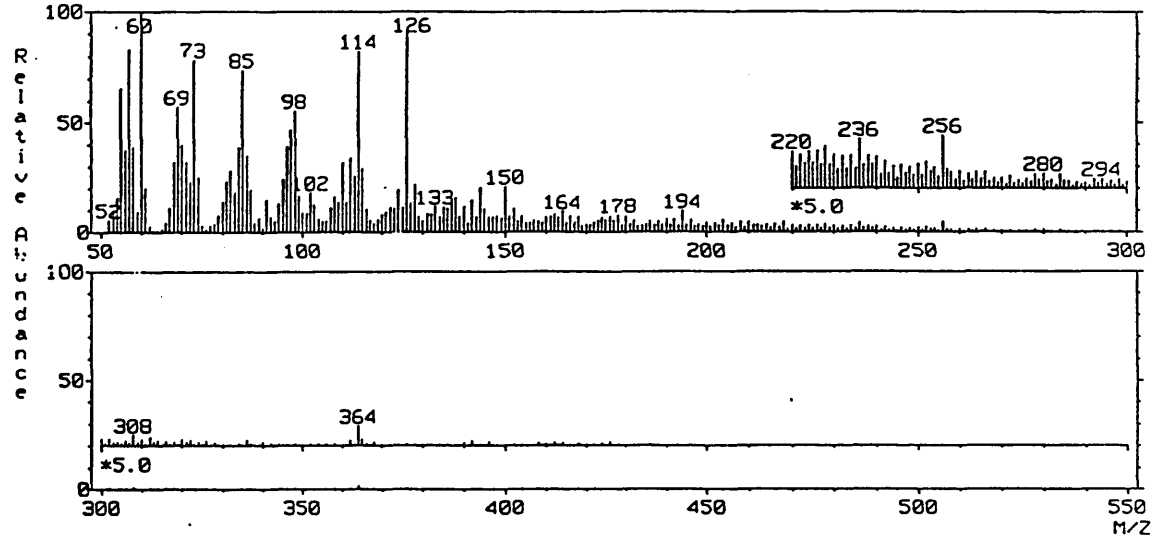
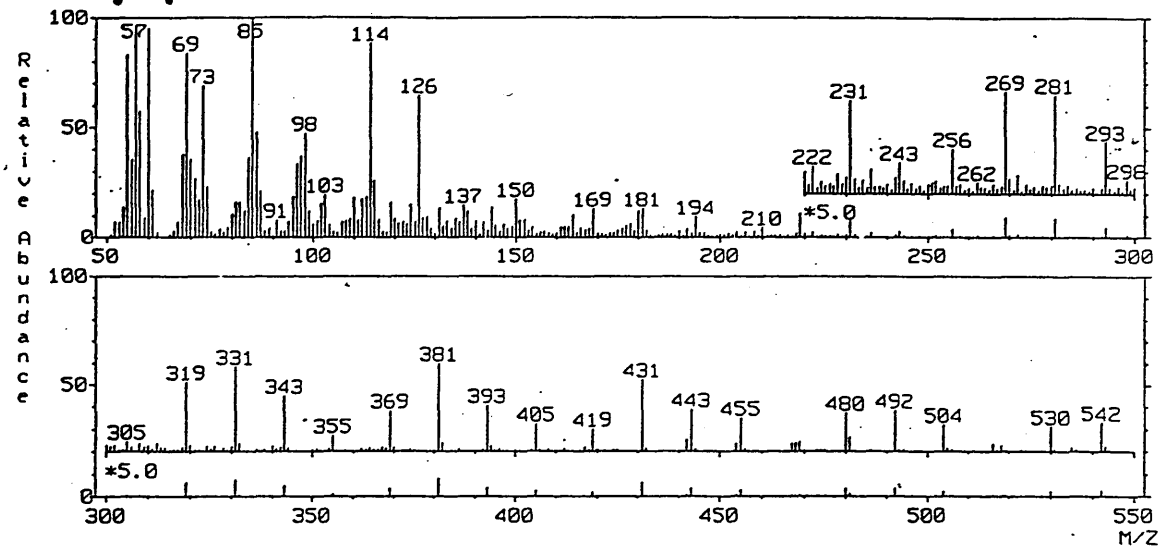
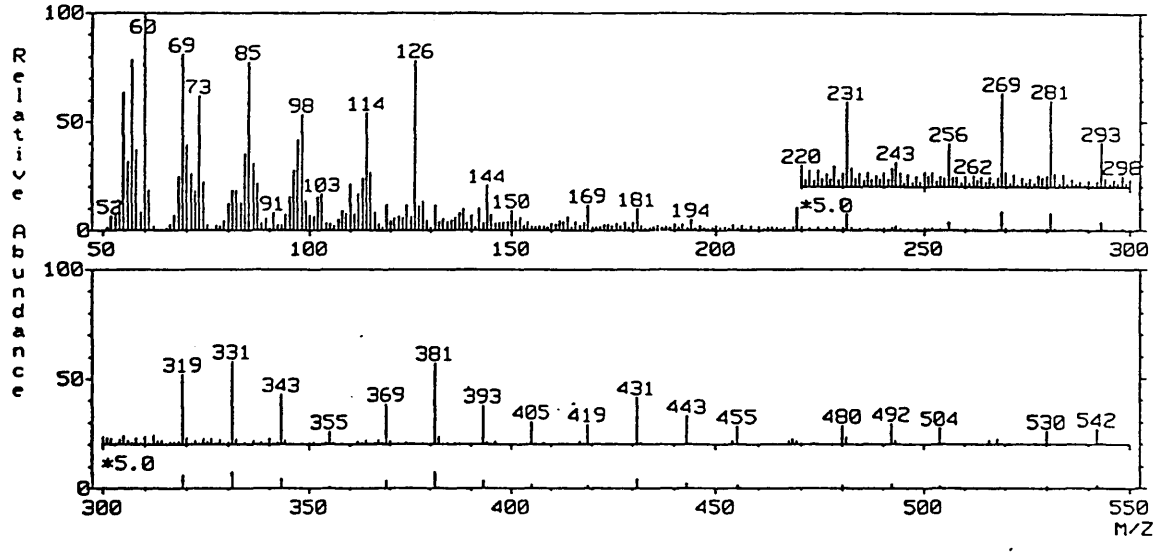

Relative Abundance
100
50
0
60
73
85
114
126
69
98
150
220
236
256
280
294
52
102
133
164
178
194
*5.0
50
100
150
200
250
300
308
364
*5.0
300
350
400
450
500
550
M/Z

Figure 3.4.36 Platinum filament EI Py-MS of oxalate hydrolysed ryegrass A) scans 38-45, B) 45-59.

A


Relative Abundance
100
50
0
50
69
73
85
91
98
103
114
126
137
150
169
181
194
210
222
231
243
256
262
269
281
293
298
*5.0
50
100
150
200
250
300
100
50
0
305
319
331
343
355
369
381
393
405
419
431
443
455
480
492
504
530
542
*5.0
300
350
400
450
500
550
M/Z

B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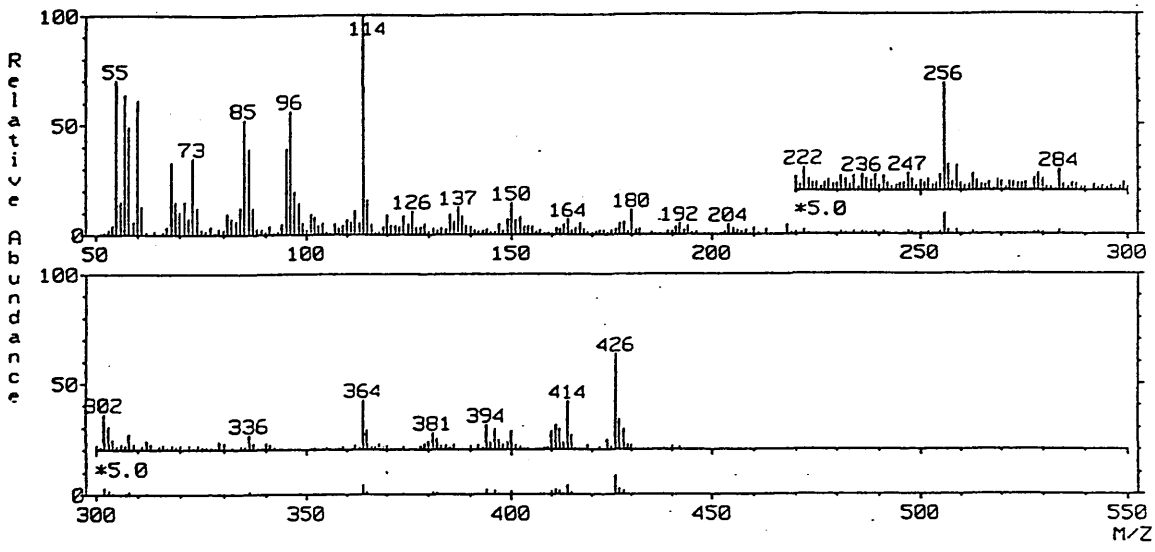
53
60
69
73
85
91
98
103
114
126
144
150
169
181
194
220
231
243
256
262
269
281
293
298
*5.0
50
100
150
200
250
300
100
50
0
319
331
343
355
369
381
393
405
419
431
443
455
480
492
504
530
542
*5.0
300
350
400
450
500
550
M/Z

Figure 3.4.37 Platinum filament EI Py-MS of DMSO soluble ryegrass A) scans 35-40, B) 41-55.

A


Relative Abundance
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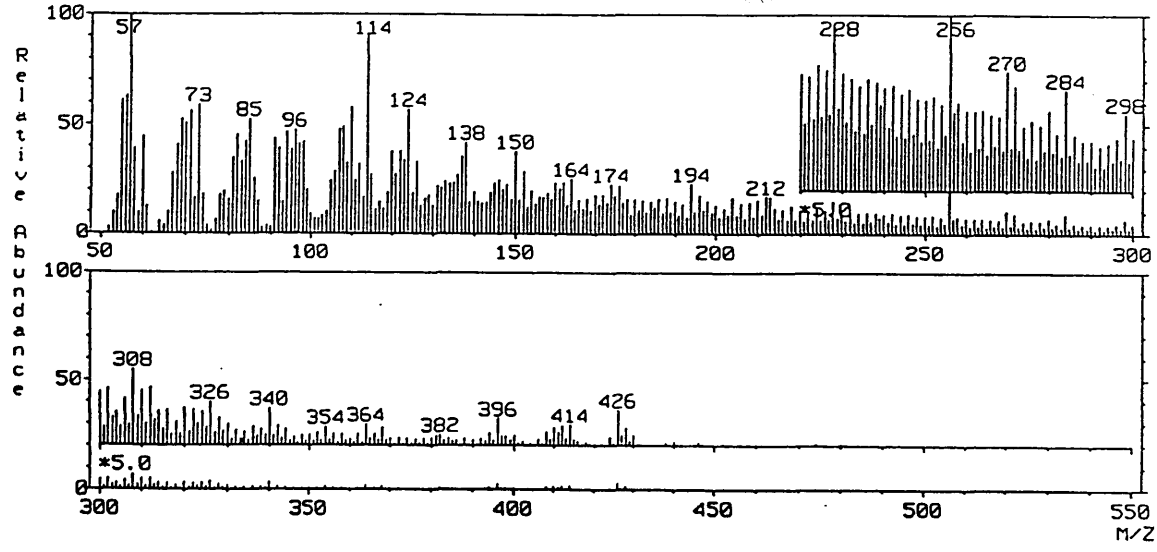
50
0
55
73
85
96
114
126
137
150
164
180
192
204
222
236
247
256
284
*5.0
50
100
150
200
250
300
302
336
364
381
394
414
426
*5.0
300
350
400
450
500
550
M/Z

B


Relative Abundance
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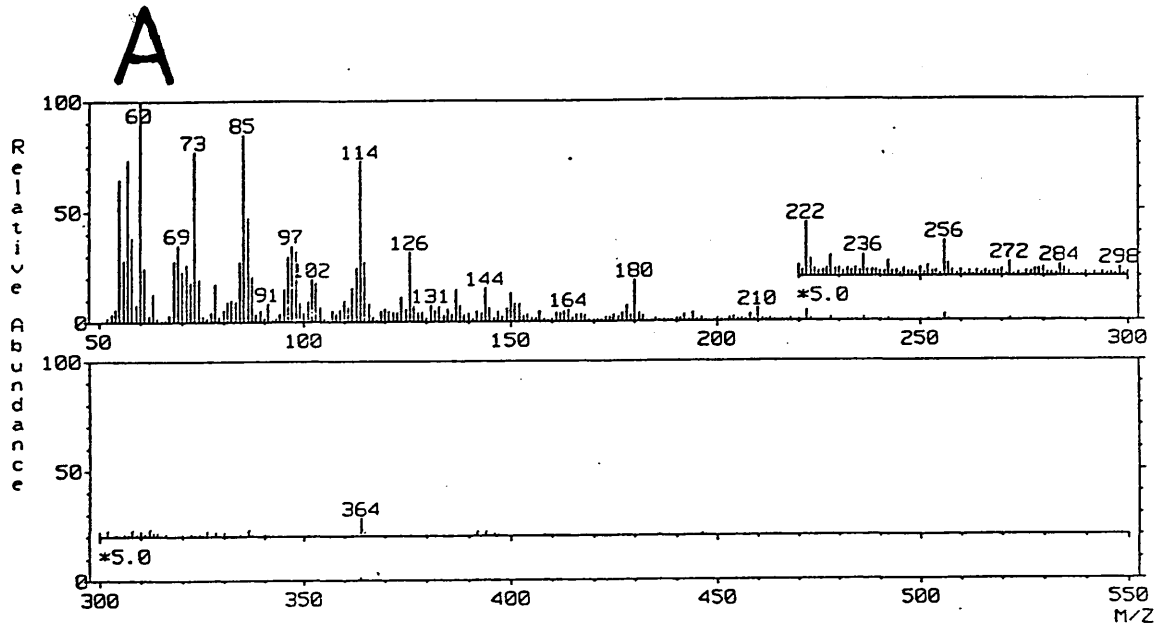
0
57
73
85
96
114
124
138
150
164
174
194
212
228
256
270
284
298
*5.0
50
100
150
200
250
300
308
326
340
354
364
382
396
414
426
*5.0
300
350
400
450
500
550
M/Z

Figure 3.4.38 Platinum filament EI Py-MS of ryegrass DMSO residue A) scans 35-44, B) 40-47


A
Relative Abundance
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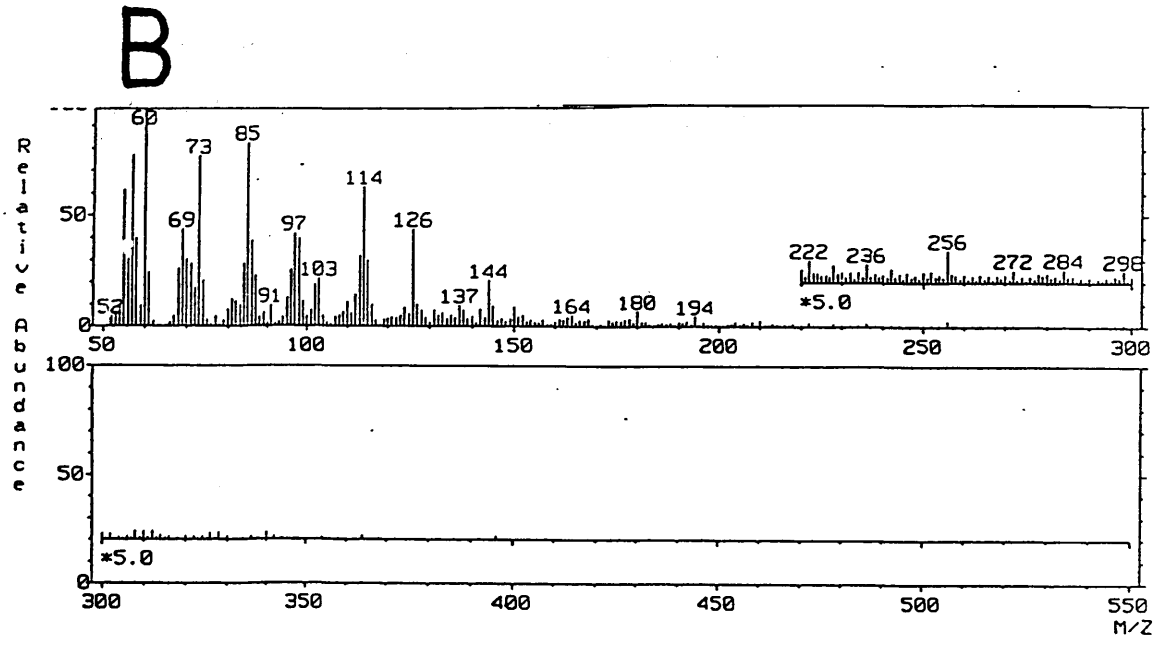
50
0
60
73
85
69
97
114
126
102
91
131
144
164
180
210
222
236
256
272
284
298
*5.0
50
100
150
200
250
300
364
*5.0
300
350
400
450
500
550
M/Z


B
Relative Abundance
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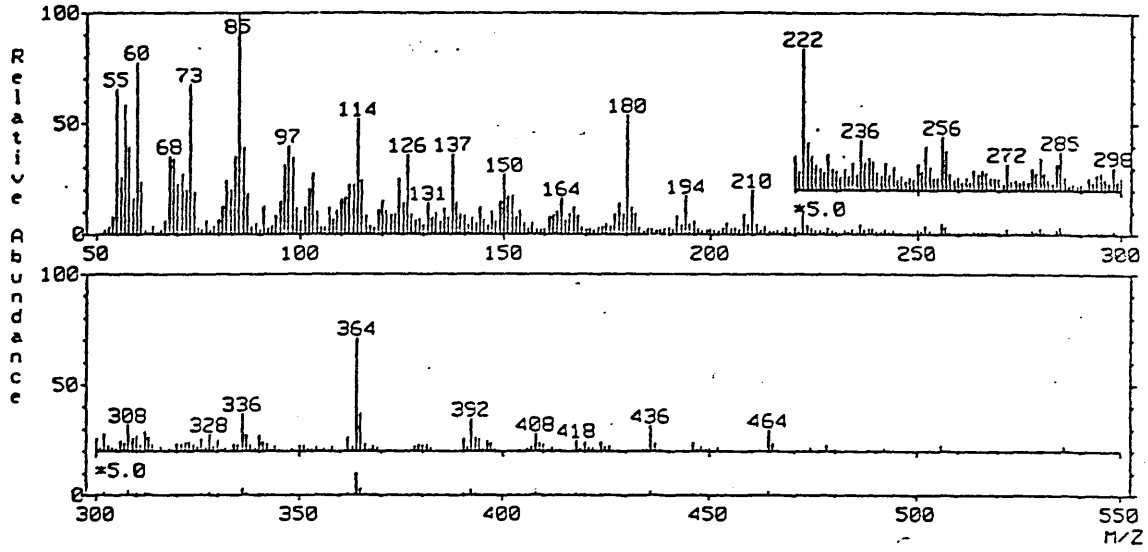
0
60
73
85
69
97
114
126
103
91
144
137
164
180
194
52
222
236
256
272
284
298
*5.0
50
100
150
200
250
300
*5.0
300
350
400
450
500
550
M/Z

Figure 3.4.39 Platinum filament EI Py-MS of ryegrass driselase residue A) scans 25-44, B) 40-47

A


Relative Abundance
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50
0
85
60
55
73
68
97
114
126
137
131
150
164
180
194
210
222
236
256
272
285
298
*5.0
50
100
150
200
250
300
364
308
328
336
392
408
418
436
464
*5.0
300
350
400
450
500
550
M/Z

B

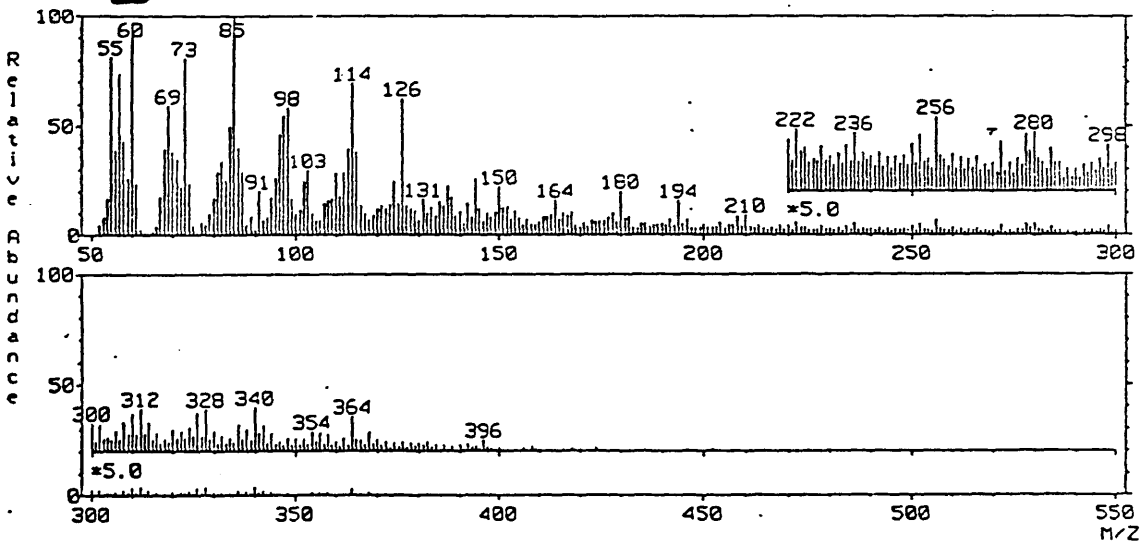
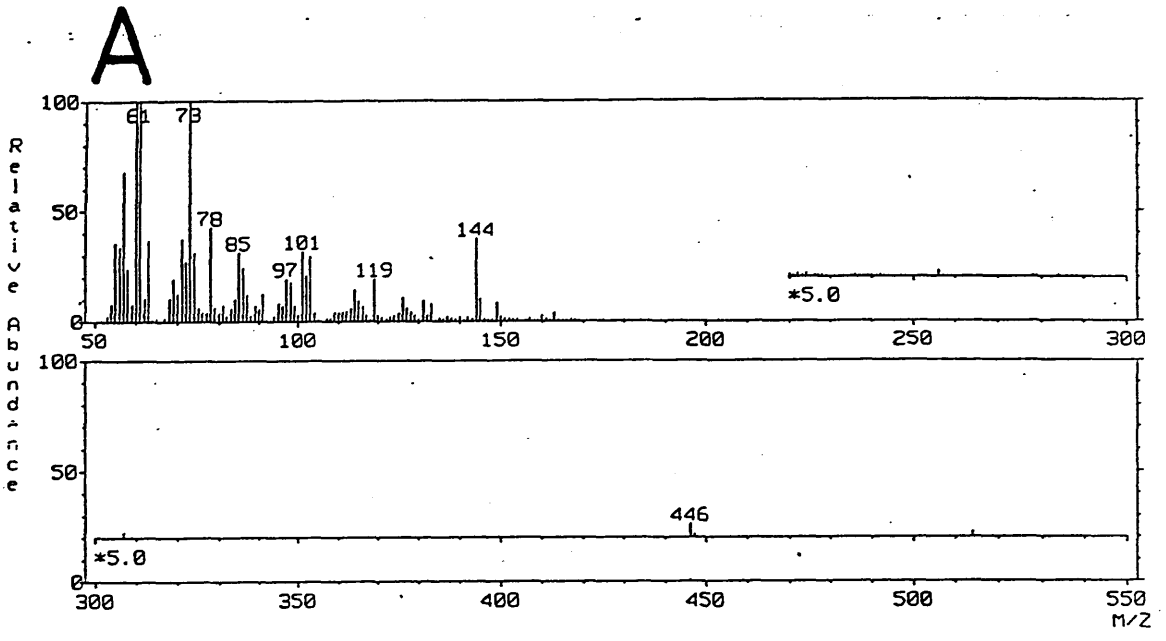

Relative Abundance
100
50
0
55
60
73
85
69
98
114
126
103
91
131
150
164
180
194
210
222
236
256
280
298
*5.0
50
100
150
200
250
300
312
328
340
364
354
396
300
*5.0
350
400
450
500
550
M/Z

Figure 3.4.40 Platinum filament EI Py-MS of driselase soluble ryegrass A) scans 25–38, B) 40–47.


A
Relative Abundance
100
50
0
61
73
78
85
97
101
119
144
446
*5.0
50
100
150
200
250
300
350
400
450
500
550
M/Z

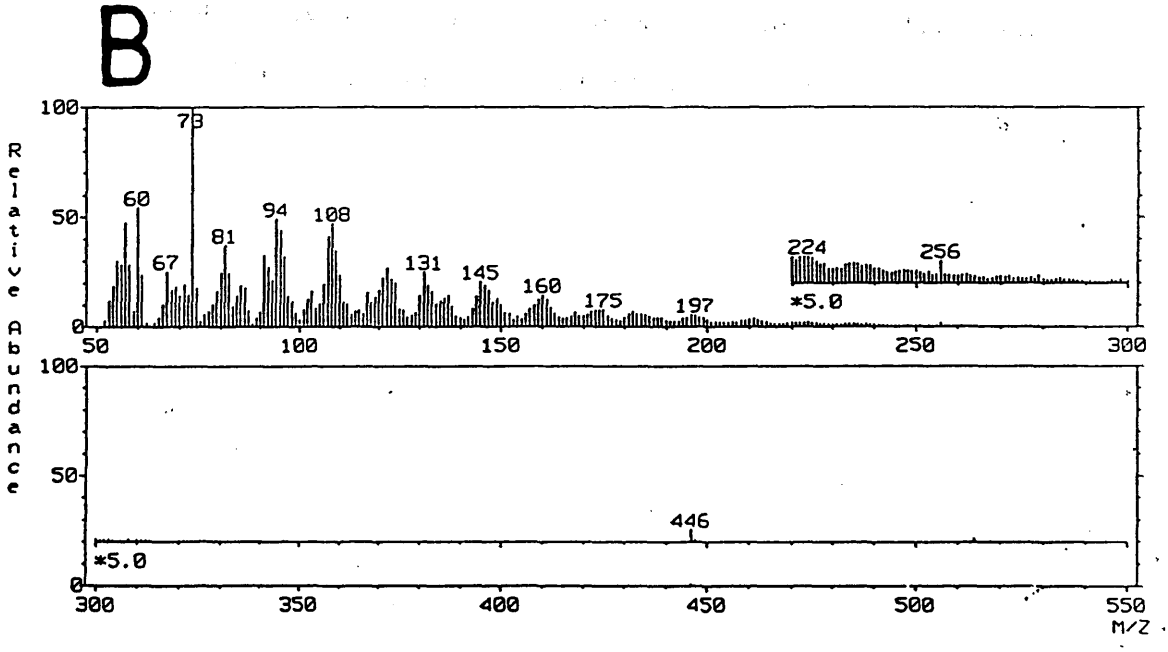

B
Relative Abundance
100
50
0
73
60
67
81
94
108
131
145
160
175
197
224
256
446
*5.0
50
100
150
200
250
300
350
400
450
500
550
M/Z

Figure 3.4.41 Platinum filament EI Py-MS of ryegrass MeOH

A) scans 33-40, B) 40-45 and C) 45-60.

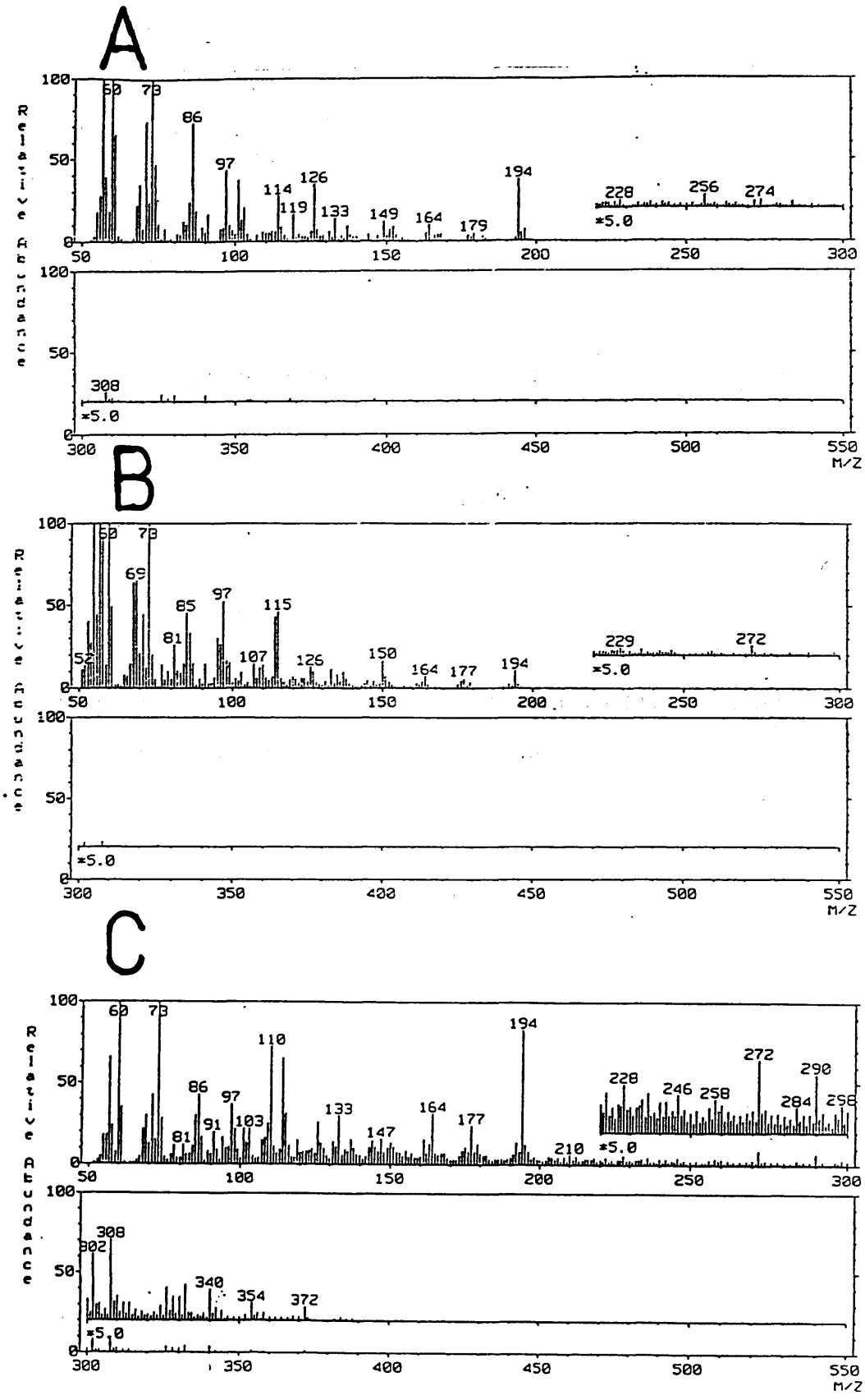
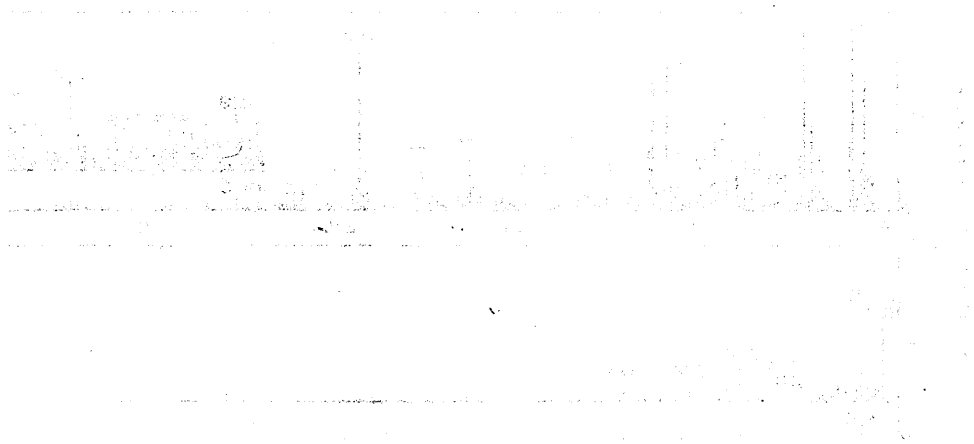
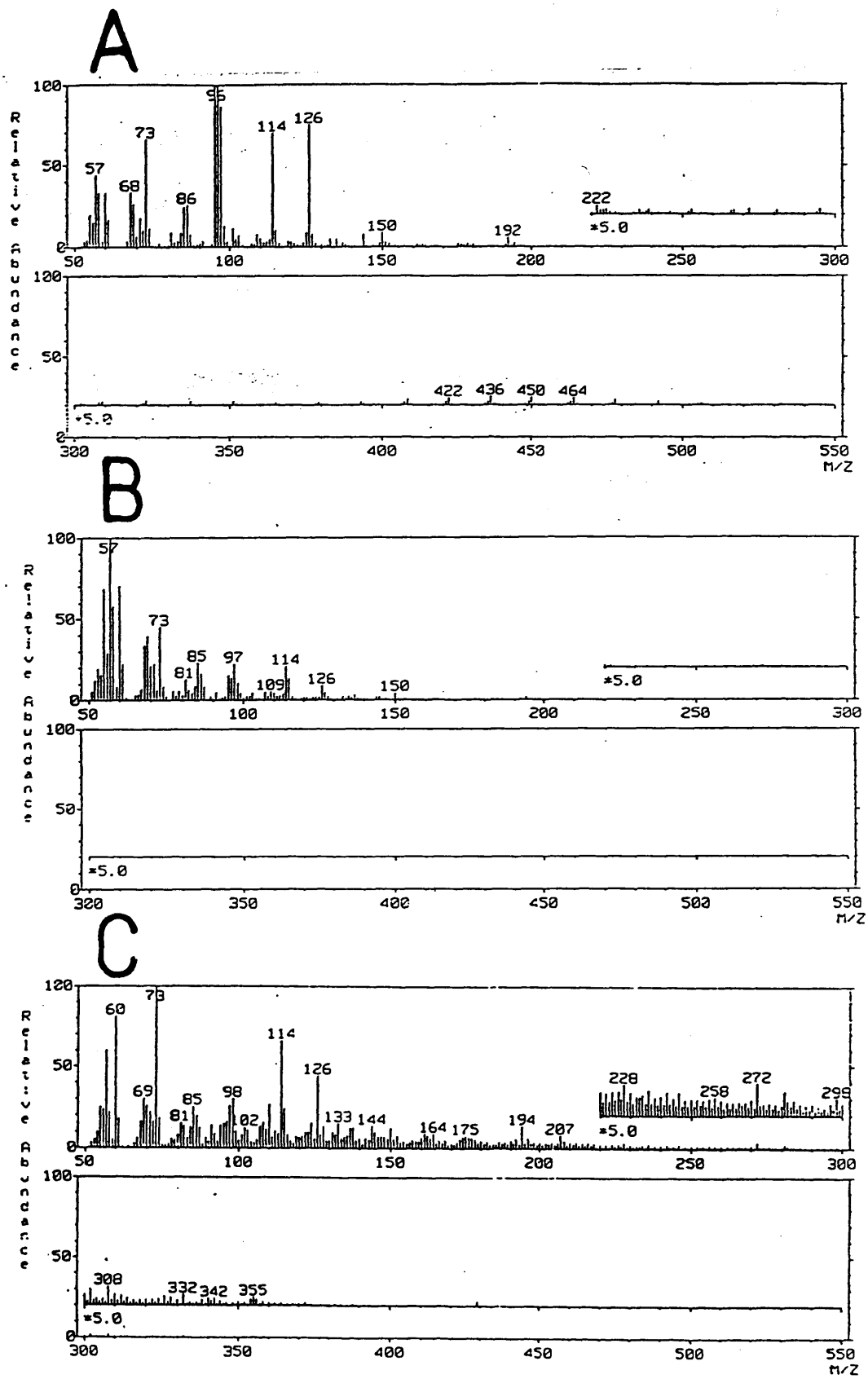

A
B
C
Relative abundance
M/Z
*5.0

Figure 3.4.42 Platinum filament EI Py-MS of ryegrass H_2O A) scans 20-35, B) 40-47 and C) 48-60.




A
Relative Abundance
100
50
0
95
73
114
126
57
68
86
150
192
222
*5.0
422 436 450 464
*5.0
50 100 150 200 250 300
300 350 400 450 500 550
M/Z
B
Relative Abundance
57
73
85
97
114
81
109
126
150
*5.0
*5.0
C
Relative Abundance
73
60
114
126
69
85
98
81
102
133
144
164
175
194
207
228
258
272
299
*5.0
300
332
342
355
*5.0

3.4.4 Discussion.

Interpretation of the pyrolysis results, suggested that the starting materials were, structurally, very similar with ryegrass having a lower lignin content. Oxalic acid hydrolysis would appear to bring about a loss of pentose and phenolic acids from the starting materials. There would also seem to be a loss of some lignin. Of the material solubilised, the bulk of the phenolic acids and pentose (mainly monomeric) is solubilised in the methanol fraction with more of the higher Mwt phenolics and oligomeric carbohydrate remaining in the water soluble material.

DMSO extraction apparently solubilised a fraction rich in pentose with some lignin (especially in barley) and phenolic acids. The residue is mainly hexose in nature, heavily acetylated and heavily lignified.

Hydrolysis of the DMSO residues with driselase solubilised both pentose and hexose and, in ryegrass at least, some ferulic acid. The residues are both heavily acetylated and lignified, both still retained some pentose and hexose. Throughout the fractionation scheme it is apparent that ferulic acid was preferentially removed, increasing the *p*-coumaric/ferulic acid ratio until it is almost 1:1 in the driselase residues compared to approximately 1:2 in the starting materials.

The precise physical processes involved in pyrolysis make structural interpretation of the pyrolysis profiles for platinum filament EI MS difficult to interpret. It is difficult to say whether the separated components truly represent discrete cell wall fractions or represent fragments which are volatilised at the same time.

At first glance, subtle differences in the phenolic fingerprints of the barley DMSO soluble and insoluble fractions were noticed. More detailed examination of the relative intensities of the assigned phenolic ions (Table 3.4.4) clearly demonstrates that significant differences in the lignin structure between the DMSO soluble and the residue may exist.

The precise mechanism of the pyrolytic breakdown of lignin, however, is unknown, although tentative proposals have been made (Genuit et al., 1987). It would be exceptionally difficult to draw any significant structural conclusions from these results.

Very subtle differences in the phenolic component of the ryegrass fractions were also noticed. Contamination of many of the ryegrass fractions, however, prevented direct comparison of the phenolic ions. Therefore it is not possible to say whether these differences truly exist and, if so, are they the same as those found in barley straw. It was felt that these differences warrented further investigation, therefore it was decided to analysis the lignin struture in greater detail (section 3.5).

It is interesting to note that the DMSO and driselase residue lignin fingerprints are essentially very similar thus suggesting that driselase does not solubilise any significant lignin.

In general, pyrolysis analysis of the cell wall fractions by each different pyrolytic technique have given consistant and comparable results. The degree of structural information provided and the

comparitively short analysis time must strongly commend pyrolysis analysis as a valuable tool in cell wall studies.

Table 3.4.4 Relative intensities of phenolic markers from Py-MS (EI) for barley fractions.

		% relative ion intensities.					% lignin total				
Fragment	Mwt	BS	DMSO SOL	DMSO RES	DRIS RES	H2O	BS	DMSO SOL	DMSO RES	DRIS RES	H2O	
p-Coumaric acid	120	19	23	7	8	16	5.7	6.3	2.1	1.9	8	A
G-H	124	36	56	26	32	32	10.7	15.3A	8	7.5	16	A
Ferulic acid	150	39	43	21	25	33	11.6	11.8	6.4	5.9	16.5	A
G-CHO	152	18	22	14	17	17	5.4	6	4.3	4	8.5	A
S-H	154	21	23	13	17	12	6.3	6.3	4	4	6	A
p-CA G-CCC G-COH	164	20	26	27	30	19	6	7.1	8.3	7.1	9.5	
G-COMe G-CCO	166	11	12	7	9	9	3.3	3.3	2.1	2.1	4.5	A
G-CCOMe S-CC G-CCH2OH	180	60	46	68	100	10	17.9	12.6	20.8	23.5	5	B
S-CHO G-COOMe	182	16	16	18	23	8	4.8	4.4	5.5	5.4	4	B
S-CCC	194	16	21	23	27	12	4.8	5.7	7	6.4	6	
S-CCO	196	8	10	7	9	6	2.4	2.7	2.1	2.1	3	A
S-CCCHO	208	13	9	17	19	5	3.9	2.5	5.2	4.5	2.5	B
S-CCOMe	210	43	23	49	80	6	12.8	6.3	15	18.8	3	B
Lignin dimer	272	6	8	6.5	7	3	1.8	2.2	2	1.6	1.5	A
Lignin dimer	302	2.8	4.6	4	4	4	0.8	1.3	1.2	0.9	2	A
Diferulic acid	324	0.5	2	1	1	0.9	0.1	0.5	0.3	0.2	0.5	
Lignin dimer, TA	328	1.2	4	3	3	1.3	0.4	1.1	0.9	0.7	0.7	
Lignin dimer	330	1	6	7	7	1.9	0.3	1.6	2.1	1.6	1	
Lignin dimer,DFA	342	0.7	2.4	1.7	2	0.8	0.2	0.7	0.5	0.5	0.4	
Lignin dimer,TA	358	0.3	2.2	4	2	0.6	0.1	0.6	1.2	0.5	0.3	
Lignin dimer,TA	388	0.1	0.2	0.6	0	0.2	0	0.1	0.2	0	0.1	
Lignin dimer	418	0.1	0.1	0	0	0.1	0	0	0	0	0.1	
Total		334.9	365.9	326.9	425	199.6						
% phenolic acids		18.81	22.74	12.36	10.35	27.35						A

A = high in DMSO soluble, low in DMSO residue and driselase residue,intermediate in untreated barley straw.
B = low in DMSO soluble, high in DMSO residue and driselase residue,intermediate in untreated barley straw.

3.5.THIOACIDOLYSIS.

3.5.1 Introduction.

As mentioned previously (section 1.3.3.2), thioacidolysis is a solvolytic technique which specifically cleaves alkyl-aryl ether linkages as demonstrated in Figure 3.5.1. This technique only allows analysis of *p*-hydroxyphenyl (H), syringyl (S) and guaiacyl (G) content in alkyl aryl ether linked (uncondensed) lignin. A modification of this method, by premethylation of the sample with diazomethane allows analysis of the 'internal' and 'terminal' lignin monomers to be differentiated (Lapierre & Rolando, 1988).

Pyrolysis analysis indicated possible differences between the structures of the solubilised and residual lignins. It was thought that thioacidolysis analysis of the DMSO solubilised fractions and the driselase residues would provide further information on these structural differences.

3.5.2. RESULTS.

Thioacidolysis was performed on permethylated barley DMSO soluble, ryegrass DMSO soluble and driselase insoluble ryegrass fractions. The results of the thioacidolysis analysis are shown in Table 3.5.1.

Determination of the G-OMe contents were not, initially, attempted

due to an overlapping of the G-OMe peak with that of a xylan degradation product. S-OMe values were, similarly, not determined for the ryegrass samples because of the very low amounts present.

In order to establish whether it would be possible to attempt a rough estimate of G-OMe content by selective ion monitoring (SIM) by GC-MS, the mass spectrum of each component was studied and the ions *m/z* 211, 269, 241 and 299 were then chosen to represent G-OMe, G-OH, S-OMe and S-OH respectively. The intensity of each ion was then taken as a relative measure of content as shown in Table 3.5.2.. Figure 3.5.2 shows the GC-MS trace (total ion current) of the thioacidolysis sample for ryegrass driselase residue.

Figure 3.5.1 Principles of thioacidolysis analysis.

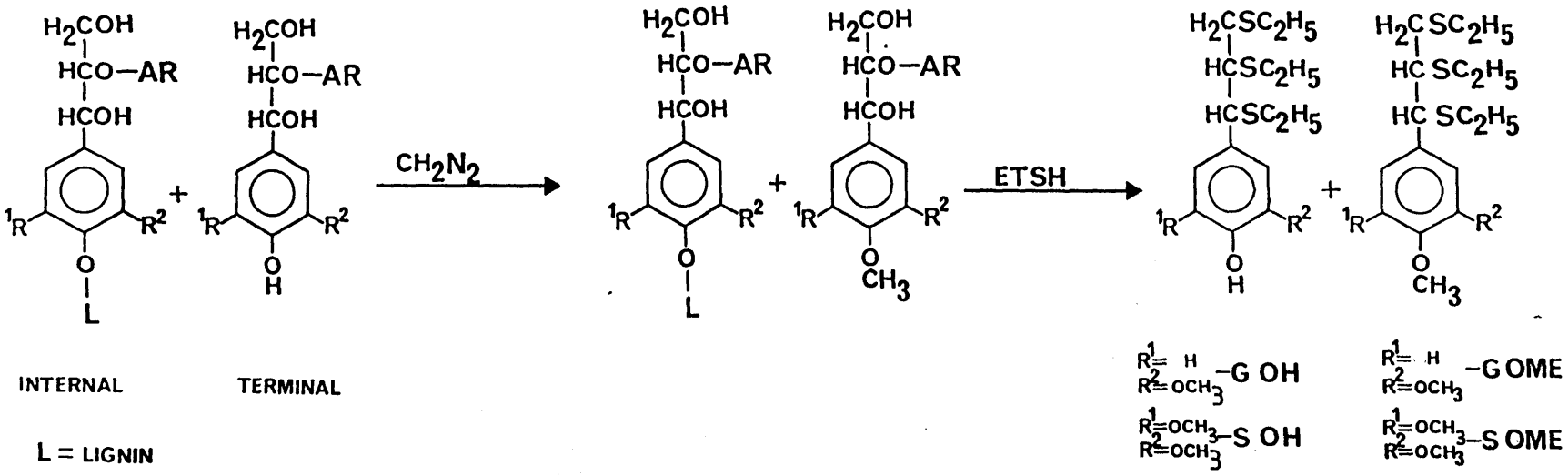

H_2COH
HCO—AR
HCOH
^1R
R^2
O
L
H
CH_2N_2
ETSH
$H_2CSC_2H_5$
$HCSC_2H_5$
CH_3
INTERNAL
TERMINAL
L = LIGNIN
R^1= H, R^2=OCH_3 –G OH
R^1=OCH_3, R^2=OCH_3 –S OH
R^1= H, R^2=OCH_3 –G OME
R^1=OCH_3, R^2=OCH_3 –S OME

Figure 3.5.2 GC trace of thioacidolysis analysis.

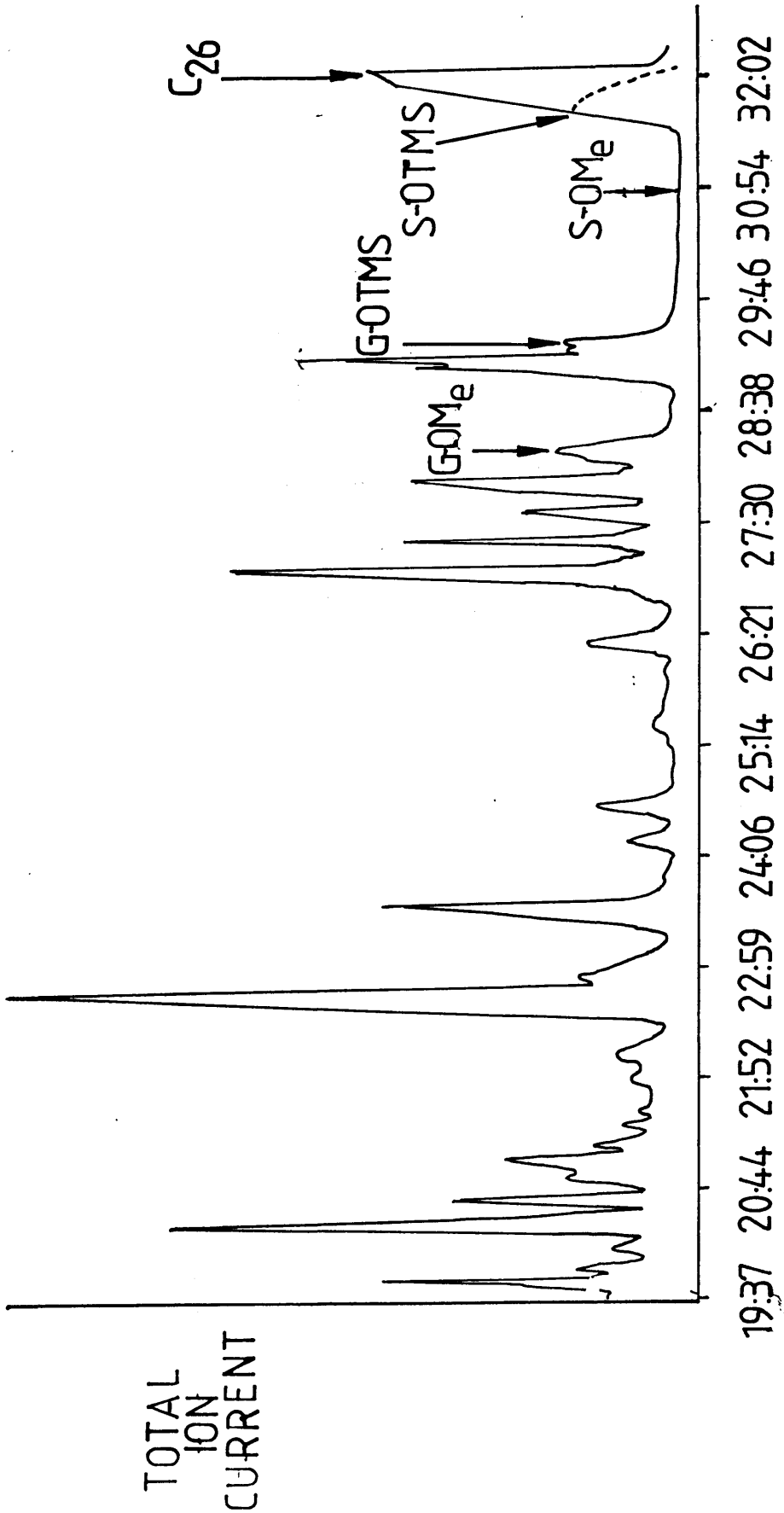

TOTAL ION CURRENT
19:37
20:44
21:52
22:59
24:06
25:14
26:21
27:30
28:38
29:46
30:54
32:02
TIME (mins)
C26
G-OTMS
S-OTMS
G-OMe
S-OMe

TABLE 3.5.1 Thioacidolysis results.

	Barley straw DMSO soluble	Ryegrass DMSO soluble	Ryegrass driselase residue.
G-OH	1.26	0.27	1.33
	(36.50)	(7.80)	(38.55)
S-OH	1.77	0.41	1.11
	(47.20)	(10.90)	(29.38)
G-OMe	N.D	N.D	N.D
S-OMe	0.25	N.D	N.D
	(6.50)		
S-OH/G-OH	1.40	1.52	0.83
	(1.29)	(1.39)	(0.76)

Results expressed as % weight of sample (μmole/g^{-1} of sample).

N.D - not determined.

TABLE 3.5.2 Selected ion monitoring analysis of thioacidolysis samples.

	Barley straw DMSO soluble	Ryegrass DMSO soluble	Ryegrass driselase residue.
G-OH	113940	42151	81149
G-OMe	98674	71158	64167
ΣG	212614	113309	145316
G-OMe/G-OH	0.87	1.63	0.79
S-OH	126974	65847	54455
S-OMe	13289	6038	4276
ΣS	140263	71885	58731
S-OMe/S-OH	0.10	0.10	0.08
ΣS + ΣG	352877	185194	204047
S-OH/G-OH	1.10	1.56	0.67
ΣS/ΣG	0.66	0.63	0.40

Results expressed as ion intensities.

3.5.3. Discussion.

It is evident from the thioacidolysis results (Table 3.5.1) is that a significant difference exists in the S/G ratios of the internal (S/G-OH) units between the DMSO soluble and residual lignins. It was interesting to note that the H content was negligible, suggesting that the H units exist predominantly in the condensed component of the lignin. Recent research on the structure of the dimeric fractions solubilised by thioacidolysis again showed no significant H content (Lapierre, personnal communication), casting some doubt on the conventional classification of grass lignins.

SIM of the thioacidolysis samples (Table 3.5.2) provides rather more detail about the lignin structure. Comparison of the terminal units (S/G-OMe) shows that guaiacyl is the more common terminal unit. This has also been found in every species so far studied by this technique (Lapierre *et al.*, 1988).

The most significant result in this analysis is the clear differences in terminal/internal guaiacyl ratios. This suggests that the DMSO soluble lignin from ryegrass is composed of small and/or highly branched molecules, whereas the ryegrass driselase residue and barley straw DMSO soluble lignins would appear to be composed of large and/or straight chained molecules. Unfortunately it was not possible to determine if a similar difference between DMSO soluble and driselase residual lignins exist for barley straw.

Comparison of S-OH/G-OH by conventional thioacidolysis (Table 3.5.1) and SIM (Table 3.5.2) gives comparable results, demonstrating the reliability of the ion selective monitoring. It was therefore thought possible to approximate the total G and S as a percentage of dry matter. Using the dry weight of G and S-OH from conventional thioacidolysis and the percentage of G and S-OH from the total from SIM the approximate total dry weights were calculated (Table 3.5.3). By using the lignin contents presented earlier (Tables 3.2.3.1 and 3.3.1.1) it was also possible to approximate the % condensed lignin content.

Table 3.5.3 Approximate dry matter content of S + G.

	Barley straw DMSO soluble	Ryegrass DMSO soluble	Ryegrass driselase residue
ΣG	2.4	0.7	2.4
ΣS	1.9	0.5	1.2
ΣS/ΣG	0.79	0.71	0.50
ΣS + ΣG	4.3	1.2	3.6
Lignin	28.58	7.67	5.95
% Condensed lignin	84.9	84.9	39.8

These results (Table 3.5.3) clearly show that differences not only exist in total S/G ratios, the DMSO soluble lignins being similar, but significant differences occur in the degree of condensation, the DMSO soluble lignins being identical.

It would appear that, in ryegrass, DMSO extracts a low Mwt and/or highly branched lignin with a high condensed lignin content, whereas the residual lignin is high M_{wt} and/or straight chained lignin with a low condensed lignin content and lower relative syringyl content. A similar comparison for barley straw was not possible although DMSO also solubilises a highly condensed syringyl lignin with a similar high M_{wt} and/or highly branched structure.

4.0 GENERAL DISCUSSION.

Mild acid hydrolysis has been shown to be less selective for the hydrolysis of arabinose than suggested in the literature (Fry, 1988), especially in barley straw, where solubilisation of arabinose was accompanied by a substantial solubilisation of xylose. It is not known whether the incomplete hydrolysis of the arabinose is a function of the hydrolysis conditions or due to the nature of the remaining arabinose residues. It is probable that the remaining arabinose is retained because of covalent linkages to lignin. The high solubilisation of xylose was unexpected and is difficult to explain in terms of the conventional model of arabinoxylan structure.

The higher solubilisation of phenolics in ryegrass may reflect a higher phenolic substitution of arabinose in ryegrass and, possibly, a higher phenolic substitution of xylose in barley straw. The retention of *p*-coumaric acid in both suggests that the major portion this acid is linked to sugars other than arabinose, or may simply reflect *p*-coumaric acid is more closely associated with the insoluble lignin fraction, as has been previously suggested (Shimada *et al.*, 1971; Scalbert *et al.*, 1985). In both barley straw and ryegrass, lignin was solubilised by oxalic acid (19.01% and 60.31% of the original in barley straw and ryegrass respectively). This must supports the view of Chesson *et al.*, (1983) that arabinose (and xylose) are the major sugars involved in lignin-carbohydrate linkage.

DMSO extraction of the oxalate hydrolysed material resulted in the solubilisation of a ß 1-4 xylan from both barley and ryegrass. It would

appear that some mixed-linked ß D-glucan was also solubilised in both and possibly some xyloglucan in ryegrass. Thioacidolysis shows that the lignin components of each fraction differ considerably in terms of their S/G ratios although their degree of branching and/or size are very similar.

Significant differences would appear to exist in the structure of the LCC's from ryegrass and barley as can be seen by their response to alkali hydrolysis. Their relative linkage patterns to the carbohydrate components, however, are rather different, with approximately 50% of the phenolics hydrolysed by alkali in barley straw LCC (similar to whole barley straw) and 76% being hydrolysed in ryegrass LCC. This would therefore indicate be a significant difference between barley straw and ryegrass in terms of alkali labile to acid labile linkages. This difference was also suggested in the driselase residues by solid state NMR.

Using the 2,3,4-*O*-methyl xylose content as a measure of terminal xylose residues, and therefore of chain length, and 2-*O*-methyl and 3-*O*-methyl xylose as measures of branch points, the xylan chain in ryegrass LCC would appear to be much smaller and/or be more highly branched than the barley LCC.

The significant retention of disubstituted xylose after DMSO extraction is difficult to explain. Possibly it is due to the presence of two distinct polymers, the disubstituted xylose being predominantly

located in a xylan polymer 'deep' inside the cell walls, thus being retained.

The great differences in response to driselase hydrolysis may simply reflect the difference in phenolic contents. It would appear that, in ryegrass at least, disubstituted sugars do not significantly inhibit enzymic hydrolysis. Overall, the driselase residues bear a close similarity to rumen incubated material.

Pyrolysis analysis, in general, agreed closely with conventional wet chemical techniques, and has demonstrated its particular value in the rapid analysis of cell wall structure. However, the discrepancies found in phenolic acid content (especially ferulic) by pyrolysis and HPLC analysis gives some cause for concern over the precise assignments of the phenolic acid ions in pyrolysis, although *m/z* 120/150 has previously been shown to have a high correlation with phenolic acid content (Hartley & Haverkamp, 1984).

The time resolved pyrolysis MS technique proved to be particularly useful in the partial separation of cell wall components although the precise physical processes involved in the volatilisation are not well understood. A detailed study of the pyrolysis of model compounds may give some additional insight to the pyrolytic breakdown of cell wall components, especially the lignin component.

Thioacidolysis of the DMSO soluble and driselase residue fractions conclusively showed significant structural differences in their

respective lignin moieties. The DMSO soluble, type 1, lignin is apparently associated with a ß 1-4 xylan, whereas the insoluble, type 2, lignin is apparently associated with a pentose/hexose fraction. Both barley and ryegrass 'type 1' lignins have similar high condensed lignin contents (84.9% in both barley and ryegrass). The ryegrass 'type 2' lignin has a much lower condensed lignin content (39.8%).

Driselase hydrolysis and subsequent molecular sieve chromatography of the barley LCC produced small (<700 daltons) phenolic fragments, suggesting that the thioacidolysis results may indicate small oligomeric lignin molecules rather than highly branched molecules. The pyrolysis lignin fingerprints of the two lignins showed a higher content of the small monomeric pyrolysis fragments in the barley DMSO residue, whereas the DMSO and driselase residues showed a higher concentration of the dimeric markers which may, also suggest that the 'type 1' lignin is composed of small molecules and the 'type 2' lignin is composed of larger molecules.

Over the past decade, the term 'lignin heterogeneity' has become increasingly used (see Monties, 1985). This heterogeneity has previously been thought to be expressed at three levels 1) monomeric composition, 2) intra polymer linkage patterns and 3) interactions with other cell wall components (Monties & Lapierre, 1981; Scalbert *et al.*, 1986). The results presented here clearly demonstrate heterogeneity in the first two levels and suggests heterogeneity in the third. It would also seem likely that heterogeneity also exists in terms of size of lignin molecules.

Thioacidolysis analysis of untreated and rumen incubated barley straw (Chesson & Lapierre, unpublished data) has demonstrated (Table 4.1) that rumen incubated barley straw has a lower condensed lignin content, a lower syringyl content and a lower content of terminal guaiacyl residues than the untreated straw.

Table 4.1 Thioacidolysis analysis of untreated and rumen incubated barley straw.

			% condensed	
	S+G	**S/G**	**lignin**	**G-OMe/G-OTMS**
untreated	6.13	1.05	52.70	0.61
rumen incubated	10.27	1.00	45.21	0.55

Histological examination, using differential staining techniques in angiosperms has suggested that differences between syringyl and guaiacyl contents may exist between secondary and primary cell walls (Akin, 1986, 1989). It is also known that secondary cell walls are generally more easily degraded in the rumen than differentiated primary cell wall region of lignified cell walls (Engels, 1989). The results presented here demonstrate the presence of small Mwt, highly condensed lignin molecules relatively rich in syringyl. It is proposed that these small DMSO soluble lignin molecules originate from secondary

cell walls whereas the higher molecular weight lignin molecules originate from primary cell walls.

The existance of small, oligomeric lignin molecules in the secondary cell wall supports the 'block' model for cell wall digestion described in the introduction. The small lignin molecules associated with the lignin-carbohydrate blocks in the secondary cell wall being physically incapable of forming the lignin 'wall' which is thought to limit degradation, thus explaining the observed greater degradation, in the rumen, of secondary cell walls compared to secondary thickened primary cell walls. The higher syringyl content of the secondary cell wall lignin would also explain the observed preferential solubilisation of syringyl lignin in the rumen.

The differences found in molecular weights, S/G ratios and condensed/uncondensed contents clearly suggest that different control mechanisms must exist in the availability of monomers and polymerisation of lignin at different stages of secondary thickening.

It is proposed that future work should concentrate on further characterisation of the two lignin moieties especially with regards to their molecular weights. It would also be benificial to confirm the location of these lignin types within the cell wall, and their presence in different cell wall types. Further characterisation of the higher molecular weight pyrolysis fragments from the lignin may give a deeper insight to the lignin structure and characterisation of the dimeric and trimeric fragments released by thioacidolysis may also reveal further detail on the condensed regions of the lignin. It is also felt that this

phenomenon should be investigated in other taxonomic groups, to determine the extent of this heterogeneity within the plant kingdom.

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