ISOLATION AND CHARACTERIZATION OF FERULIC ACID CARBOHYDRATE ESTERS FROM CORN HULL (*Zea Mays*)

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Dissertation submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Food Science and Technology

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April, 1988

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(ABSTRACT)

Refined corn hulls (bran) were hydrolyzed with 30 mM oxalic acid in order to release carbohydrate fragments containing ferulic acid. The fragments which were high in ferulic acid also contained high levels of arabinose, xylose, and galactose, which are characteristic of sugars comprising corn hull hemicellulose side chains. Corn hull hydrolysate fragments were fractionated by chromatography to obtain purified feruloyl oligosaccharides. The structure of the major feruloyl compounds were characterized by analysis of the products of acid, alkali, and enzyme hydrolysis, in combination with carbon-13 and 2D proton NMR spectroscopy. Three feruloyl oligosaccharides were identified as 5-O-(*trans*)-feruloyl-L-arabinofuranose (FA), 2-O-β-xylopyranosyl-(5-O-*trans*-feruloyl arabinofuranose) (FXA), and O-(6-O-*trans*-feruloyl-α-D-galactopyranosyl)-(1→4) -O-β-D-xylopyranosyl-(1→2)-L-arabinofuranose (FGXA).

ACKNOWLEDGEMENTS

The author would like to thank Dr. J.K. Palmer for his tutelage, guidance, support, and friendship throughout his graduate study. Appreciation is extended to Dr. K.D. Stanley for his guidance during the period of research at Staley MFG Co. Thanks are also extended to my advisory committee: Dr. W.N. Eigel, Dr. G.J. Flick, Dr. J.C. Johnson, and Dr. M.D. Pierson, for their comments, advice, and suggestions.

Special thanks go to Dr. R.S. McDaniel for his invaluable NMR spectroscopy, and to for his capable help with gas chromatography. Appreciation is expressed to Staley MFG Company Research and Development for funding this research.

Heartfelt appreciation is extended to the author's parents and family for their continued support and encouragement. Above all, the author wishes to express that it was the love, friendship, encouragement, and unselfish sacrifices of his wife, , that made all this possible.

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INTRODUCTION

The corn kernel is comprised of four basic components: starch, gluten, germ, and hull (fiber). The hull (bran) represents approximately 3.5% of the corn kernel on a dry weight basis. The corn hull is primarily composed of non-starch polysaccharides, with hemicellulose being the major fraction (@ 60%). The complex structure of corn hull makes it an important source of dietary fiber (Kies, et al, 1982), ranging from about 65-92% depending on the source and isolation method. The soluble fiber content of corn hull is small (<1%), as compared to other brans such as oat bran, where about 11% of the total fiber is soluble. Corn hull is used in a variety of products (cereals, bakery, snacks, etc) primarily for fiber enrichment or calorie reduction.

Under alkaline conditions, corn hull (bran) develops an off color (green), which can affect its performance in certain food applications. In addition, alkaline hydrolysis of corn hulls (NaOH) dissolves a noncarbohydrate fraction which liberates the hemicellulose fraction (Antrim and Harris, 1977). This noncarbohydrate fraction contains relatively large quantities of ferulic acid (3-methoxy, 4-hydroxy-cinnamic acid) which is usually associated as an intermediate in lignin biosynthesis. However, removal of the noncarbohydrate fraction by alkaline hydrolysis suggests that corn hulls do not contain lignin. Lignin removal typically requires high temperature treatment

under highly alkaline or acid conditions along with certain chlorine and sulfur compounds.

Many plants that do not ordinarily contain high polymer lignin were found to contain ferulic acid as the phenylpropenoid component (Hartley and Harris 1976). Research on various mono- and dicotyledon cell walls has shown ferulic acid to be esterified to cell wall carbohydrates. The role or location of ferulic acid in the corn hull structure has not been investigated. It is possible that corn hull cell wall carbohydrates also contain ferulic acid esters, which may play an important role in the cell wall structure.

The primary goal of this research project was to determine if ferulic acid carbohydrate esters exist in corn hull. Preliminary identification of ferulate esters in corn hull led to subsequent objectives of isolation and structural characterization of feruloyl oligosaccharides.

REVIEW OF THE LITERATURE

Corn

A. Background

Corn is believed to have originated in Mexico. The oldest archaeological corn (7000 years) was found in Mexico's valley of Tehuacan. Corn then spread northward to Canada, and south toward Argentina. After European discovery of the Americas, corn moved to Europe, Africa, and Asia. The modern races of corn derived from prototypes developed by Mexico and Central and South American agriculturists. However, yellow dent corn, the product of postcolonial North America, dominates the U.S. corn belt, Canada, and most of Europe today (Brown et al., 1984).

Corn (*Zea mays*) belongs to the grass family (*Gramineae*). Corn is an annual plant containing a fibrous root system, and an erect stalk with single leaves in two opposite ranks at each node. Corn is a cross-pollinated species, containing separate male (tassel) and female (ear) flowers on the same plant making it monoecious. Being a warm season crop, corn requires temperatures between 10 and 45° C for optimal growth, and about 130-140 days to mature (Benson and Pearce, 1987).

The main function of the plant after fertilization is to develop the corn ears. In the first 10-14 days, the cob is the main area of growth followed by rapid deposition of dry matter in the kernel. About 800 corn kernels are produced on the ear (Kiesselbach, 1949). Kernels are attached to the cob by the

pedicel. The corn kernel is a seed and contains a complete embryo and all of the structural, nutritional,and enzymatic apparatus required to initiate embryo growth and development (Watson, 1987).

B. Corn Kernel Composition

Figure 1 illustrates the corn kernel components discussed in this section. One of the main structural components of the corn kernel is the germ. The germ is composed of the embryo and the scutellum, which functions as a nutritive organ for the embryo (Watson, 1987). The germ accounts for 10-12% of the corn kernel dry weight. Nutrients and hormones are stored in the germ, and are activated by enzymes liberated during the initial stages of germination (Watson,1987). Specific organelles known as oil bodies, or spherosomes, are found in all oil seeds (Gurr, 1980, Watson, 1987). Enzymes necessary for lipid hyrolysis and synthesis were isolated from maize scutellum spherosomes (Semadeni, 1967). Pits and intercellular spaces of the walls of scutellum cell facilitate movement of material among cells (Watson, 1987). Contact between the germ and the endosperm is achieved by a single layer of secretory cells on the outer margin of the scutellum. The germ cell walls contain mainly hemicellulose composed of arabinan, and a little cellulose (Seckinger et al, 1960). The cell walls become thin during germination, and hydrolytic enzymes diffuse into the endosperm to digest starch and protein, thus allowing sugars and amino acids

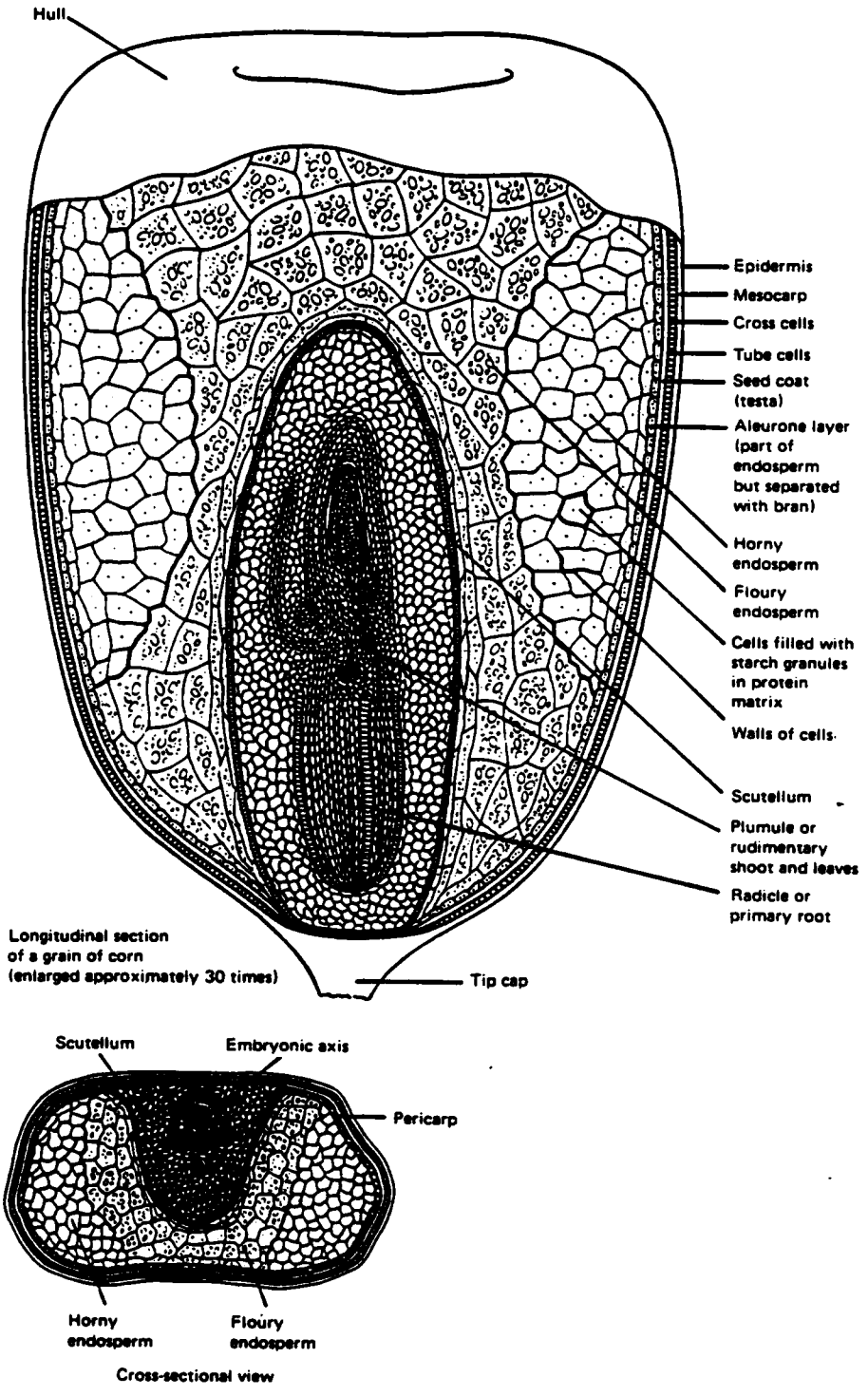


Figure 1. Longitudinal and cross section through a corn kernel (From Pomeranz, 1987).

to translocate through the scutellum to the embryo (Watson, 1987).

The endosperm is the major component of the corn kernel, accounting for 82-84% of the kernel dry weight. About 86-89% of the endosperm is starch. Starch is the major carbohydrate in corn, accounting for 72-73% of the corn kernel (Watson, 1987). The starchy endosperm is of two types, floury and horny. The floury endosperm is opaque to transmitted light due to light refraction by small air pockets around starch granules (Duvick, 1961). During drying, the thin protein matrix of the floury endosperm tears, and no longer completely surrounds the starch granules (round in shape). In contrast, the protein matrix of the horny endosperm is thicker and remains intact upon drying, with plastic starch granules compressed into polyhedral shapes (Rubetti et al, 1974, Watson, 1987).

The protein matrix of corn endosperm contains discrete protein bodies which are embedded into amorphous protein material (Christianson et al, 1969). Zein constitutes the majority of protein bodies, and is a lysine deficient protein fraction. The protein matrix upon isolation is often referred to as corn "gluten". Corn gluten is suitable for food use, but it does not have the viscoelastic properties of wheat gluten.

The outer endosperm contains a single layer of cells called the aleurone layer. The aleurone layer covers the entire starchy endosperm and germ, and can constitute about 2.2% of the kernel dry substance depending on corn variety. Aleurone cells contain

protein granules but no starch, and are also rich in minerals (Pomeranz, 1973). Attached to the outer surface of the aleurone layer is a thin, hyaline, semipermeable membrane called the seed coat. The seed coat is the outermost structure of the seed (Wolf et al, 1952).

All tissues exterior to the seed coat are collectively called the pericarp or hull. The hull extends to the base of the kernel, and unites with the tip cap. The hull is composed of dead cells that are cellulosic tubes arranged in the inner most layer longitudinally against the aleurone layer (Pomeranz, 1973). Next is the cross-cell layer, which is a loose and open area covered by a thick, compact layer known as the mesocarp. The mesocarp is composed of closely packed, empty, elongated cells with numerous pits that provide capillary interconnections between cells. The epidermis is the outer layer of cells which is covered by a waxy cutin layer. The pericarp, or hull, accounts for about 5-6% of the corn kernel dry weight (Watson, 1987). The thickness of the hull can vary from 62-160 micrometers depending on the corn variety (Helm and Zuber, 1969). Genetic differences in the hull can also effect the color of the corn kernels (i.e. white, yellow, orange, red, purple and brown) (Neuffer et al, 1968).

C. Corn Hull Composition

Corn hull (bran) contains a primary and secondary cell wall, which thickens after cell expansion ceases (Esau, 1960). Corn

hull is primarily composed of non-starch polysaccharides with cellulose (fibrous polysaccharides) and hemicellulose (matrix polysaccharides) being the major structural components. Hemicellulose is associated with cellulose in both primary and secondary cell walls. The composition of corn hull varies depending on the method of isolation. Typically, refined corn hulls are composed of about 22% cellulose, 60% hemicellulose, 5% starch, 4% protein, 2% fat, 3% klason lignin, and about 4% moisture. Less pure forms of corn hull usually contain higher amounts of non- fibrous impurities such as protein, starch, germ, tip caps, etc.

The complex structure of corn hull make it an important source of dietary fiber (Kies, et al, 1982). Nearly 80% of the total corn kernel fiber (9.5%) is due to the corn hull. Corn hull has the highest dietary fiber content as compared to other grain related fibers. The total dietary fiber (TDF) content of corn hull ranges from about 65-92%, depending on the source and isolation method. The soluble fiber content of corn hull is small (<1%), as compared to other brans such as oat bran, where about 11% of the total fiber is soluble.

Hemicellulose is the major non-starch polysaccharide accounting for about 60% of the corn hull. The structural characteristics of corn hull hemicellulose (CHH) was investigated around 1955-57, showing it to be a highly branched hetero-polysaccharide. Lime water extraction of corn hulls followed by acid hydrolysis showed CHH is comprised of D-xylose

(48-54%), L-arabinose (33-35%), DL-galactose (7-11%), and some D-glucuronic acid (2-4%). Hydrolysis of fully methylated CHH yielded a variety of products shown in Table 1 (Whistler and BeMiller, 1956). The presence of dimethylxylose, monomethylxylose, and xylose indicate a xylan backbone to which sidebranches may be attached at either the 2 or 3 position. Latter work by Srivistava and Smith (1957) further supported the presence of a (1→4) linked xylan backbone through isolation of 4-O-β-D-xylopyranosyl-D-xylose (xylobiose). About 73% of the arabinose in the CHH molecule occurs as non-reducing end units, as indicated by large amounts of trimethyl-L-arabinose. Partial acid hydrolysis splits off L-arabinofuranose units leaving the xylan nucleus (Whistler 1955). Methylation studies of the CHH shows galactose produces only 2,3,4,6-tetra-O-methyl-D and L-galactose, suggesting that all galactose residues in CHH occupy terminal positions (Whistler et al., 1955; Srivastava and Smith 1957).

Whistler and Corbett (1955) partially hydrolyzed and methylated corn hull hemicellulose to identify types of linkages and order of the sugar units. Mild acid hydrolysis caused preferential liberation of CHH side chains, consisting of L-arabinofuranose units. In addition to L-arabinose, the hydrolysate also contained the disaccharide 3-O-2-D-xylopyranosyl- arabinose, and trisaccharide L-galactopyranosyl-(1→4)-D-xylopyranosyl-(1→2)-L- arabinose. Three disaccharides, 3-O-α-D-xylo-pyranosyl-L- arabinose, 4-O-β

Table 1. Hydrolysis products of methylated corn hull hemicellulose (Whistler and BeMiller, 1956).

Product	Yield
2,3,4,6-tetra-O-methyl-DL-galactose	2 moles
2,3,5-tri-O-methyl-L-arabinofuranose	8 moles
2,5-di-O-methyl-L-arabinofuranose	2 moles
2,3-di-O-methyl-D-xylopyranose	10 moles
3-O-methyl-L-arabopyranose	1 mole
2-O-methyl-D-xylopyranose	8 moles
D-xylopyranose	2 moles

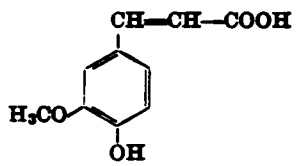
-galactopyranosyl-β-D-xylopyranose (Montgomery et al, 1957), and 5-O-β-D-galactopyranosyl-L-arabinofuranose (Smith, 1957) were isolated and characterized by controlled depolymerization of CHH. It was concluded that the di- and trisaccharides were side chains or branches of the corn hull hemicellulose. No side chains larger than 3 sugar units have been isolated. This was the first time such a combination of sugars had been isolated and identified from a natural product.

In additional studies, CHH was methylated, hydrolyzed, and treated with ion-exchange resin to separate neutral and acidic components. The aldobiouronic fragment 2-O-(2,3,4-tri-O-methyl -D-glucopyranosyluronic acid)-3-O-methyl-D-xylopyranoside was isolated, suggesting that the D-glucuronic acid molecules occupied a terminal position, and were linked directly to the xylan backbone of the polysaccharide (Montgomery et al, 1956).

The protein fraction of corn hull is tightly bound within the cell wall. Boundy et al (1967) isolated a glycopeptide from corn pericarp which contained 17 amino acids, a hexoseamine, and hydroxyproline. They suggest that the glycoprotein is an integral part of the cell wall matrix. It is possible that this molecule may act as a cross-linking agent between the glycopeptide, xylan, and the cellulose structural polysaccharides.

Little information is available concerning the lignin or non-carbo- hydrate fraction of corn hulls. Hooper (1942) first reported the composition of corn hull but made no mention of a

noncarbohydrate fraction. Van Soest et al (1979) suggested that whole corn contained 0.2% lignin while Nyman et al, (1984) reported a value of 1.4%. Sanstead et al (1978) found 0.1% lignin in corn hull by the acid detergent fiber procedure which solubilizes all pentosans and other hemicelluloses, leaving only cellulose and lignin. Antrim and Harris (1977) found that alkaline hydrolysis of corn hulls (NaOH) dissolved a noncarbohydrate fraction which liberated the hemicellulose fraction. This noncarbohydrate fraction contained relatively large quantities of ferulic acid and possibly ferulic acid precursors. Ferulic acid (3-methoxy, 4-hydroxy-cinnamic acid)



is usually associated with lignin as a precursor or a product of lignin degradation. However, removal of the noncarbohydrate fraction by alkaline hydrolysis suggests that corn hulls do not contain lignin. Lignin removal typically requires high temperature treatment under highly alkaline or acid conditions along with certain chlorine and sulfur compounds. The role or location of ferulic acid in the corn hull structure has not been investigated.

Ferulic Acid

A. Role in lignin biosynthesis

Lignin is widely distributed in plants and is associated with mature plant cells (Dreher, 1987). In secondary cells walls, lignin replaces water and prevents further growth (Northcote, 1972). Lignin does not appear to occur in primary cell walls, but the occurrence of phenols is widespread (Harris and Hartley, 1980). Structurally, lignin is a polyphenolic macromolecule, formed by condensation of coniferyl, sinapyl, and p-coumaryl phenolic alcohols (Figure 2).

There are three categories of lignin that are classified according to source: gymnosperm or softwood lignins; angiosperms or hardwood lignins, and monocotyldoneous angiosperms or grass lignins. These lignins are best differentiated on the basis of nitrobenzene oxidation products. Gymnosperms yield mainly vanillin with some p-hydroxybenzaldehyde; angiosperm lignins mainly produce both syringaldehyde and vanillin and some p-hydroxybenzaldehyde, and significant amounts of all three aldehydes are obtained from grass lignin (Sarkanen, 1971).

Cinnamic acids are widely distributed in plants, and play a central role in the biosynthesis of lignin, flavonoids, and related compounds (Bate-Smith, 1956, 1962; Kremers, 1957). These acids do not usually occur in the free state, but rather as esters. Biosynthesis of lignin in plants is thought to occur via conversion of cinnamic acids (including ferulic acid and p-coumaric acid) to the corresponding alcohols (e.g. coniferyl

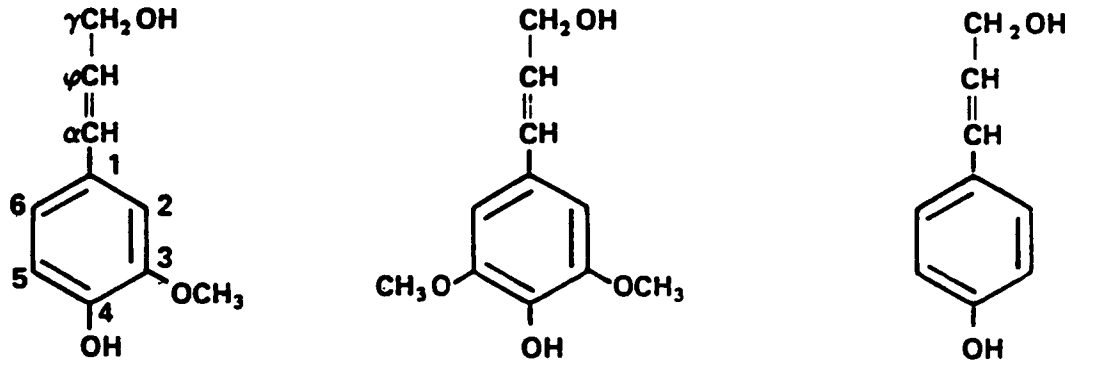


Figure 2. Structure of p-hydroxycinnamyl alcohols.

alcohol) followed by oxidative coupling reactions (Neish, 1961).

Extensive studies by Freudenberg and co-workers in the early 1940's suggested that coniferyl alcohol was involved in the polymerization reactions of lignification. Evidence suggested that phenylpyruvic and p-hydroxyphenylpyruvic acids were the first aromatic phenylpropenoids formed in plants, possibly through conversion to coniferyl alcohol. Early attempts by researchers (Higuchi et al., 1954; Ishikawa and Takaichi, 1955; Ishikawa and Okubo, 1958) showed that shikimic acid and phenylpropenoid compounds were the most effective in stimulating lignin formation in plant tissues during feeding studies. Brown and Neish (1956) found that in heading wheat plants, ferulic acid was efficientlly converted to coniferyl lignin. They later found in *Acer negundo* that p-hydroxycinnamic acid and caffeic acid were comparable to phenylalanine as lignin precursors (Brown and Neish, 1956). Freudenberg (1956) found that phenylpropenoid ketones were obtained (by ethanolysis) after labelled ferulic acid was fed to spruce: Higuchi (1962) found that L-phenylalanine, p-hydroxycinnamic and ferulic acids were good precursors of coniferyl lignin in tissue cultures of *Pinus strobus*.

Phenylalanine, an essential amino acid, is abundant in plants. The conversion of phenylalanine to trans-cinnamic acid catalyzed by phenylalanine ammonia lyase (PAL) was found to play a key role in initiating phenolic metabolism in plants cells (Koukal and Conn, 1961; Camm and Towers, 1973). Phenylalanine

was found as a good precursor of caffeic acid in tobacco plants, where much of it was bound as chlorogenic acid (Geissmann and Swain, 1957; Reid, 1958). McCalla and Neish (1959) found that feeding labelled C-14 -phenylalanine to cuttings of *Salvia splendens* produced labelled cinnamic acids: caffeic, ferulic, sinapic, and p-coumaric.

All plants can convert phenylalanine to lignin, however, many plants are unable to convert tyrosine for this purpose. Grasses are unique in that they are able to convert tyrosine directly to p-coumaric acid. Brown and Neish (1956) suggested that grasses possess a tyrosine metabolizing enzyme, tyrosine ammonia lyase (TAL) not present in the other families studied. Corn is a member of the grass family (Gramineae) and can utilize both phenyalanine and tyrosine with equal efficiency. Gymnosperms and angiosperms can only synthesize lignin from L-phenylalanine.

Figure 3 shows the proposed biosynthetic pathway for lignin biosynthesis. Lignification is believed to occur through random, dehydrogenative polymerization of three *trans* (E) monolignols, p-coumaryl, coniferyl and sinapyl alcohols. Cinnamic acid or p-hydroxy cinnamic acid is formed from corresponding amino acids depending on the plant source. Specific hydroxylases convert cinnamic acid to p-coumaric acid and then caffeic acid. O-Methyltransferase then converts caffeic acid to ferulic acid, which is hydroxylated to 5-hydroxyferulic acid. All of the enzymes leading to trans monolignols have been recently found,

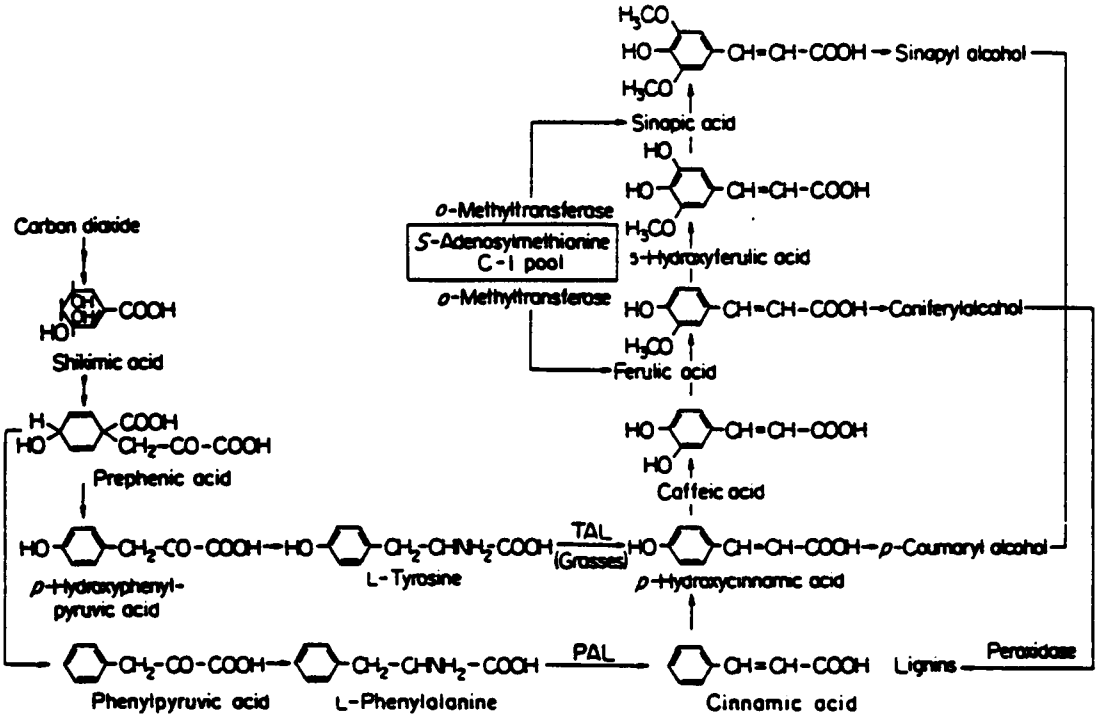


Figure 3. Proposed biosynthetic pathway of lignin from carbon dioxide. (From Higuchi et al., 1977).

including the isolation of ferulic acid-5- hydroxylase (Grand, 1984). The formation of sinapic acid therefore appears to be via methylation of 5-hydroxyferulic acid and not 3,4,5-trihydroxy-cinnamic acid. Ohashi et al. (1987) have isolated E-5-hydroxyferulic acid (esterified) and esterified E-sinapic acid from the cell walls of young *Zea mays* and *Hordeum vulgare*.

Enhanced lignin synthesis is often associated with a concerted increase in lignin synthesis enzyme activity (Vance et al., 1980). Several enzymes characterized by different specificities toward cinnamic acids have been shown to esterify cinnamic acids into thioesters of CoA, precursurs of lignin monomers (Ranjeva et al., 1976; Knobloch et al., 1975; Wallis et al., 1977). This enzymatic step could play a role in controlling the lignification process (Grand and Rossignol, 1983). Stafford (1974) proposed a membrane-associated multicomplex in which ferulic acid is reduced to the corresponding cinnamyl alcohols by successive mediation of three enzymes: hydroxycinnamate:CoA ligase, hydroxy- cinnamyl-CoA reductase, and hydroxycinnamyl alcohol reductase (Fig 4).

B. Ferulic acid in non-lignified cell walls

Many plants that do not ordinarily contain high polymer lignin were found to contain ferulic acid as the phenylpropenoid component. Evidence suggests that primary cell walls may contain phenolic compounds even when such walls fail to show a classic lignin histochemical test (Hartley and Harris 1976).

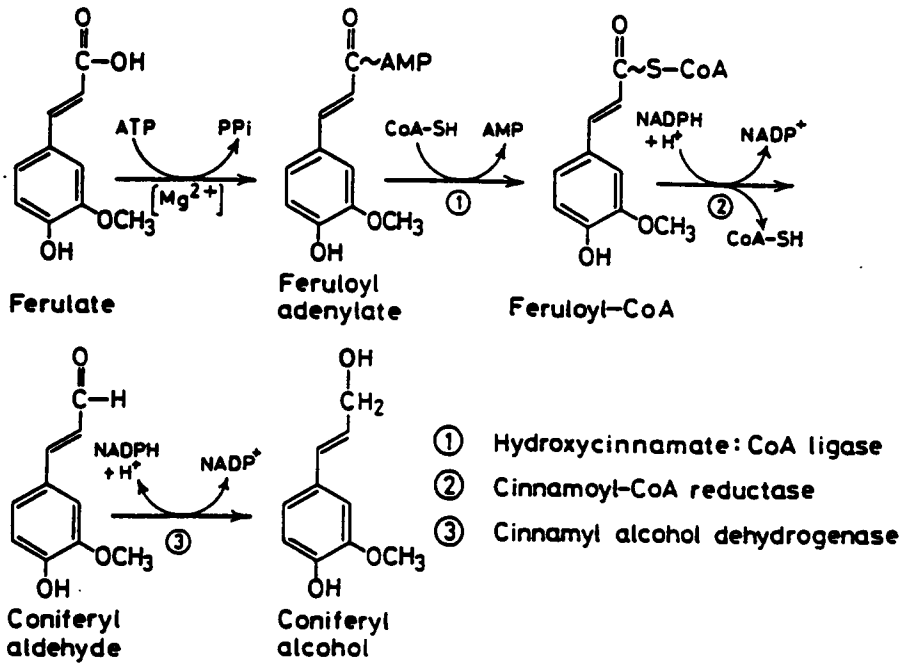


Figure 4. Reduction of ferulic acid to coniferyl alcohol (Stafford, 1974).

Ferulic acid is widely distributed in plant cell walls, but its function within the cell walls is not known. Research during the early 1960's suggested that hydroxycinnamic acids, particularly p-coumaric and ferulic acids may occur in plants as soluble esters and glycosides (Towers et al, 1984). These hydroxycinnamic acids were first identified in the hydrolysate of native lignins of plants including wheat and sugar-cane.

Fausch et al. (1963) originally detected ferulic acid in extracts from wheat flour. Latter work by Painter et al (1968) showed that ferulic acid was esterified to arabinoxylan molecules in wheat flour. Whitmore (1974) found that wheat coleoptile cell walls did not contain ordinary high polymer lignin, instead ferulic acid was found as the most abundant phenylpropanoid component. He indicated that ferulic acid was probably bound by an ester linkage to carbohydrate. Ferulic acid esters were found in wheat root cell walls with lesser amounts of other components including p-coumaric and diferulic acid (Smith and O'Brien, 1979). Recent findings by Scalbert et al. (1985) show lignin fractions isolated from wheat straw contain ether linked phenolic acids.

Sosulski et al., (1982) fractionated the phenolic components of cereal flours into free, soluble, and insoluble forms. Ferulic, p-coumaric, and syringic acids were present in the soluble fractions, while ferulic acid was the major phenolic acid in the insoluble residues. Ferulic acid was found in barley endosperm cell walls (Fincher, 1987), and in barley seedling cell

walls (Yamomoto et al, 1985). In both cases ferulic acid was shown to be ester linked with high molecular weight cell wall carbohydrates. Shibuya (1984) detected ferulic acid, p-coumaric acid, and diferulic acid in alkaline extracts of rice endosperm cell walls. A series of ferulic acid esters were isolated from enzymatic digests of these extracts. Ferulic acid esters may help explain the alkali extractability of various cell wall arabinoxylans (Mares and Stone, 1973)

Grass coleoptile cell walls also contained ferulic acid and p-coumaric acid carbohydrate esters (Towers et al, 1984). The structure of the phenolic-carbohydrate complex in coleoptile cell walls is similar to that found in the glycoproteins of wheat flour. Arabinoxylan is the primary hemicellulose fraction of flour glycoprotein, and it is estimated that one pentose residue in 50 contains a ferulic acid ester. Harris and Hartley (1980) found 39 of 104 species of monocotyledons in 52 families contained ester linked ferulic acid. These were from families *Commelinidea*, *Palmae* (part of *Arecidae)*, *Philydraceae*, *Pontederiaceae, and Haemodoraceae* (all part of *Liliidae*). The occurrence of di- and triferuloylsucrose was reported in anther of 157 di- and monocotyledons, and most predominantly in the *Liliacea* (Meurer et al, 1984).

Pectin is an important structural polysaccharide found in the primary cell walls and intercellular layers in land plants such as citrus fruits, apples, and sugar beet pulp. The binding of ferulic acid to pectins of unlignified plant cell walls

appears to be limited to the *Caryophyllales (Centrospermae)*, to which both spinach and sugar beet belong (Rombouts and Thibault, 1986). Sugar beet pectins were found to contain 1-2% phenols, of which feruloyl groups are covalently bound. Feruloyl groups were found to be absent from pectins from other plant sources including potato, apple, citrus, apricots, and cherry (Rombouts and Thibault, 1986).

C. Characterization of feruloyl polysaccharides

In order to understand the role of cell-wall bound phenolics, it is important to determine where the phenolics are linked in the cell walls. The carbohydrate to which ferulic acid is esterified appears to be different for monocots and dicots. Ferulic acid is esterified to arabinoglucuronoxylan in monocots as opposed to arabinan and galactan portions in dicots (Kato and Nevins, 1985).

Fry (1982) treated cell walls from growing suspension cultures of spinach (*Spinacia oleracea L.*) with a fungal hydrolase to liberate low moleular weight feruloyl esters. Two feruloyl oligosaccharides, 4-O-(6-feruloyl-b -D-galactopyranosyl) -D-galactose and 3-O-(3-O-feruloyl- a -L-arabinopyranosyl) -L-arabinose were isolated. At least 60% of the cell-wall ferulate occurred in the two esters. Fry feels that the large proportion of cell ferulate linked to arabinose units implies that addition of phenols to cell-walls is not a random process. Feruloyl-tranferases with high specificity for the feruloyl

acceptor may exist. Feruloyl-coenzyme A may be a likely donor.

Phenolic carbohydrate complexes of ryegrass were isolated using cellulase and mild acid hydrolysis. Complexes contained D-glucose, D-xylose, D-arabinose, and D-galactose residues in a ratio of 16.7:10.0:4.0:1.7, and also ferulic and p-coumaric acid linked by ester linkages. The phenolic complexes were based on (1→4)-β-D-xylan chains with arabinofuranose and D-galactopyranose side chains. Phenolic complexes based on galactoarabinoxylan associated with (1→3), (1→4)-β-D-glucans were also isolated. It was estimated that there was one phenolic residue for every 80 neutral sugars (Tanner and Morrison, 1983).

A ferulated tetrasaccharide was isolated from pectinated milled sugar-cane baggase with the primary structure being O-β -D-xylopyranosyl-(1→4)-O-(5-O-feruloyl-α-L-arabinofuranosyl -(1→3))-O-β-D-xylopyranosyl-(1-4)-D-xylopyranose. This structure is the same as that isolated from various other plants in the family *Gramineae* (Kato et al., 1987).

Ferulic acid was identified as the major phenolic acid in endosperm cell wall of five spring and four winter barley varieties. The arabinoxylan fraction appeared to carry all the ferulate groups while the beta-glucan fraction was free of ferulate. The two major ester were identified as 3-O-(3-O-feruloyl-α-L-arabinopyranosyl)-L-arabinose and 4-O-(6-O-feruloyl-β-D-galactopyranosyl)-D-galactose. The degree of feruloylation was estimated at about 1 in 138 arabinose

residues (Ahluwalia and Fry 1986).

An arabinoglucuronoxylan fraction liberated from *Zea* shoot cell walls by mild acid and enzyme treatment contained ferulic acid. Ferulic acid was found to occur at 3-O-arabinosyl branch points of the linear (1→4) linked xylan backbone by isolation of O-(5-O-feruloyl-α-L-arabinofuranosyl)-(1→3)-O-β -D-xylopyranosyl-(1→4)-D-xylopyranose (Kato and Nevins 1985).

Feruloyl oligosaccharides were isolated from cell walls of wheat bran with a mixture of polysaccharide hydrolyases. The major feruloyl compound released was identified as 2-O-(5-O-*trans*-feruloyl-β-L-arabinofuranosyl)-D-xylopyranose (FAX). Cell walls of other graminaceous plants: barley straw, wheat leaves, Italian ryegrass, and wheat endosperm were also found to contain FAX at different levels. The amount of ferulic acid in cell walls is probably dependent on genotype, cell type, component polysaccharides, and growth conditions (Smith and Hartley, 1983).

Mueller-Harvey et al., (1986) used similar methodology (acid and enzyme hydrolysis) to isolate feruloyl carbohydrates from barley straw cell-walls. Two feruloyl trisaccharides were isolated and identified as O-(5-O-feruloyl-α-L-arabinofuranosyl) -(1→3)-O-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FAXX), and O-(5-O-coumaroyl -α-L-arabinofuranosyl)-(1→3)-O-b-xylopyranosyl -(1→4)-D-xylopyranose (PAXX). They estimated that 1 in every 121 pentose units was feruloylated, and about 1 in every 243 pentose units was p-coumaroylated.

D. Feruloylation Mechanism

The location in the cell where feruloylation occurs is not known. The deposition of monomeric ferulic acid in cell walls is associated with a sequence of concerted developmental processes as exemplified in the lignification process (Wardrop, 1971). There is some question as to whether sugar residues are first feruloylated and then incorporated into the cell wall, or whether the feruloyl groups are transferred directly onto a preformed polysaccharide (Fry, 1987). In healthy, actively lignifying tissue of soybean, it was found that lignin precursors were formed in the cytoplasm and then transported to the cell wall were polymerization took place (Stafford, 1974). Yamomoto and Towers (1985) suggested that polysaccharides were feruloylated extracellularly after being deposited in the cell wall. However, Fry (1985) feels that in old coleoptiles, the feruloylpolysaccharides are synthesized intracellularly de novo, and secreted, at relatively high rates after the deposition of total (non-feruloylated) polysaccharides has slowed down and perhaps been exceeded by turnover. Hartley and Jones (1976) suggest lignin biosynthesis in *Graminae* involves oxidative coupling of phenolic acids linked to cell wall carbohydrates forming dimers and higher polymers. Soluble sugar esters of phenolic acids may be transported to the cell walls and participate in the coupling process.

Spinach cell walls were found to contain feruloyl groups on arabinose and galactose residues (Fry, 1987). Tests were conducted to determine whether arabinose residues were feruloylated intra- or extracellularly. Incorporation of labeled arabinose into cultured spinach cells showed polysaccharide bound labeled feruloylarabinose accumulated radioactivity at a linear rate in 4-5 min. Radioactive polysaccharides and extensin did not appear outside the plasmalemma until about 25 min. Results suggest that feruloylation of arabinose residues of polysaccharides began while still intracellular. Additional extracellular feruloylation of polysaccharides was relatively slow. Fry (1985) suggests that feruloylation was either a post-synthetic modification of the polysaccharide, or possibly UDP-arabinose was feruloylated prior to polymerization.

Alkaline hydrolysis of plant cell walls have shown ferulic acid to be esterified with arabinose residues of arabinoxylans (Smith and Hartley, 1983 and Kato and Nevins, 1983). If ferulic acid is transferred to the cell walls as an ester of arabinose or arbinoxylan, then the incorporation of ferulic acid and arabinose into the hemicellulose fraction should have similar kinetics. Compositional analysis data obtained for phenolic carbohydrate complexes of rice endosperm shows increased ferulic acid content of isolated fractions correlated with increased arabinose content (Shibuya, 1984). However, Yamomoto et. al. (1985) showed that wall bound ferulic acid in barley coleoptile

increased after there was no measurable gain in cell wall dry weight. Barley coleoptile cell wall growth is well correlated with the increase of cell wall arabinose (Sakurai and Masuda, 1978). The difference in incorporation of arabinose and ferulic acid suggests that ferulic binds to arabinose residues of preformed polysaccharides in these cell walls (Yamomoto et al., 1985).

Functional Characteristics of Feruloyl Polysaccharides

A. Ferulic acid dimerization

Cell wall bound hydroxycinnamic acids are believed to have various roles/functions. Ester-linked ferulic acid may provide an alternative means of cross-linking cell wall polysaccharides. When low concentrations of oxidizing agents are applied to cereal grain arabinoxylans, gels form (Geissmann and Neukom, 1973a, 1973b). It was suggested that gels were formed by oxidative dimerization of ferulic acid residues in the arabinoxylan, thus forming cross-links or bridging between chains (figure 5A).

Diferulic acid residues were detected in the rice endosperm cell walls; about one diferulic acid/2600 sugar residues of arabinoxylans. This level of diferulic acid is consistent with levels which are necessary for the gelation of arabinoxylans (Fry, 1979). Towers et al., (1984) found diferulic acid in the insoluble cell wall fractions as esters of carbohydrates. Geissman and Neukom (1973a, b) have identified diferulic acid in the insoluble pentosan fractions of wheat flour. Crosslinking of

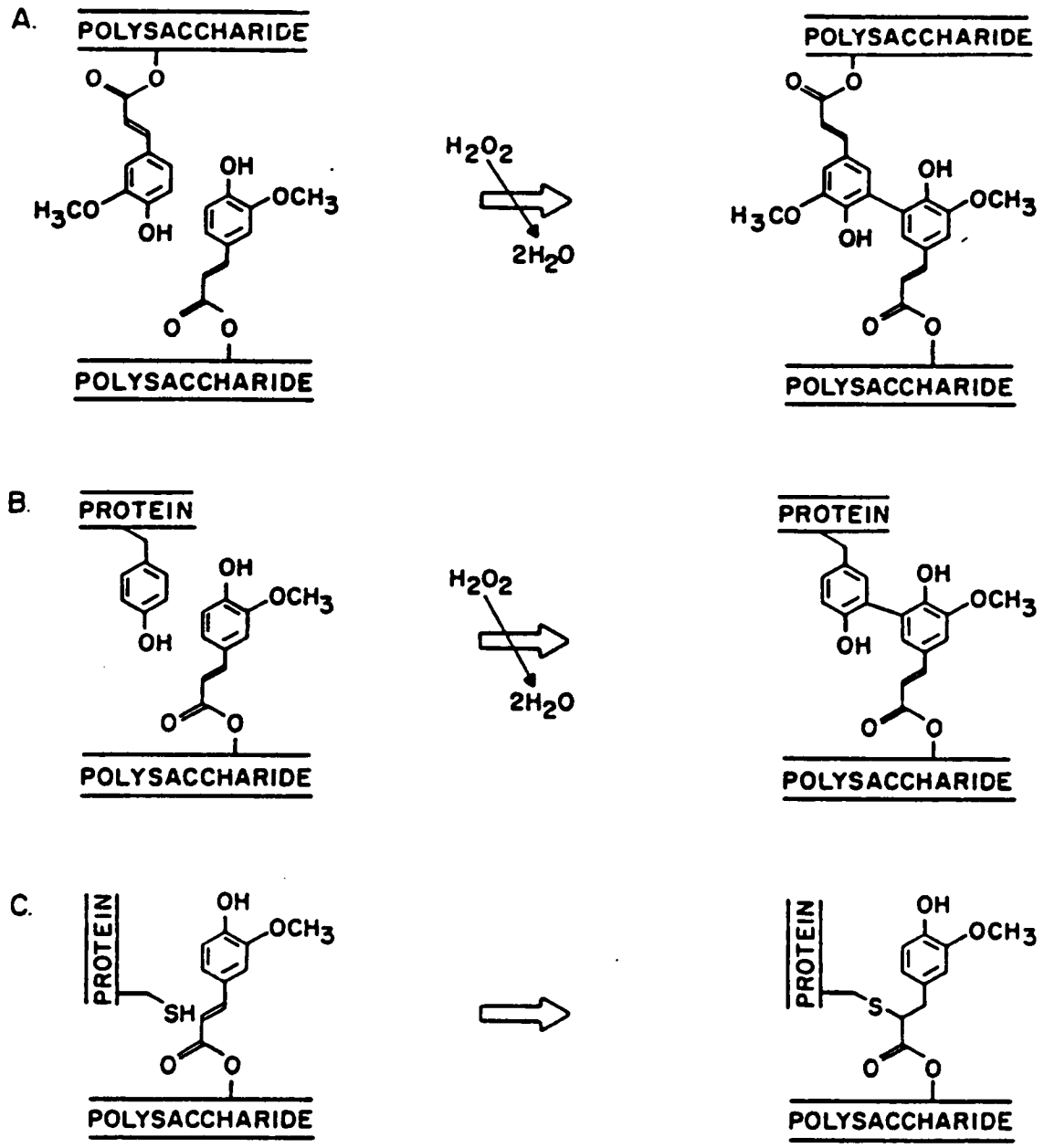


Figure 5. Proposed mechanism for ferulic acid dimerization (Fincher and Stone, 1986).

cell wall polysaccharide chains through oxidative dimerization of ferulic acid residues may render the crosslinked chains insoluble (Geissman and Neukom 1973a, b), which may be important in maintaining plant cell wall structure. Ferulic acid dimerization may also play a key role in the gelling of wheat flours to form doughs.

Feruloylation of polysaccharides, secretion of peroxidase, or the production of an oxidant may effect crosslinking. Hartley and Jones (1976) found that diferulic acid was released from cell walls of *Lolium multiforum* by treatment with NaOH. The isomers were believed to be bound via ester linkages to the cell wall carbohydrates involving one or both of their carboxyl groups. They feel that oxidative coupling reactions involving peroxidases may form diferulic acid *in situ* in the growing plant. Peroxidase and peroxide treatment of *L. multiforum* did not increase the number of diferulic acid units, suggesting that ferulic acid units of the cell walls are not suitably oriented to allow coupling to occur.

Phenolics crosslinks between polysaccharides may play a vital role in stabilizing the cell wall architecture. The mechanical regulation of cell growth through cell wall conformational changes may be due to wall bound ferulic acid dimerization (Fry, 1983). Whitmore suggests that ferulic acid dimerization may limit the extension of the cell particularly in the latter stages of growth. Supporting evidence was found when suppression of peroxidase by gibberellic acid favored cell expansion in spinach cultures (Fry, 1982; 1985). Cell wall bound

phenolic esters have been proposed to be involved in the lignification process. Phenolic esters linked to carbohydrates may be oxidized to hydroquinones which can then chelate with group IIA cations forming crosslinks between cell wall polysaccharides.

Phenolic esters and dimerization in seed hulls may effect seed germination. Seeds can survive in the dormant state for many years, however, the basic mechanism of wild seed dormancy is not well understood (Chen et al., 1982). Evidence suggests that seed hulls or certain seed hull components may influence seed germination (Black 1959). Phenolic acid dimerization in seed hulls may inhibit germination by restricting embryo expansion, acting as a barrier to gas and water uptake, or acting as a source/ or barrier of germination inhibitors. Koeves (1957) found a phenolic compound in mustard seed which suppressed germination. Chen et al., (1982) detected phenolic compounds in wild oat hulls which included ferulic and p-coumaric acid. However, the concentration of phenolics in the oat hulls did not account for suppression of seed germination.

Ferulic acid may also interact with cell wall associated proteins to form cross-links. Neukum (1976) has shown that proteolytic enzyme treatment of wheat flour arabinoxylans adversely effects gelation. Oxidative cross-links between tyrosine residues of cell wall proteins and feruloyl arabinoxylans (figure 5B) may occur (Neukom, 1976; Hosney and Faubion, 1981). The gelation of flour extracts suggested that

sulfhdryl groups of cysteine residues may reduce double bonds in phenolic acids, forming cross-links between feruloyl polysaccharides and proteins (figure 5C; Sidhu et al, 1980; Hosney and Faubion, 1981). Such interactions may explain the increase in mixed dough viscosity when oxidants (dough improvers) are applied (Durham, 1925; Baker et al, 1943; Patil et al, 1975). Ciacco and D'appolonia (1982) showed that wheat fractions containing the higher amounts of ferulic acid gave higher viscosity upon oxidation.

B. Effect on cell wall digestion

The effect of feruloylation and crosslinking in plant cell walls is not understood, but is of interest in growth studies, defense, digestability and decay. Hartley (1974) showed that increased phenolic content of grass cell walls decreased ruminant digestion of the grass cell wall carbohydrates. Feruloyl polysaccharides and oxidative coupling of phenolic acids may hinder the attack of cell wall carbohyrates by carbohydrases. This is supported by evidence of alkali pretreatment of cell walls normally nondegradable by ruminants renders the cell wall degradable. This is possibly due to the rupturing of ester bonds between phenolic acids and cell wall carbohydrates allowing for degradation by carbohydrases (Hartley and Jones, 1976).

The physical effect of low degree phenolic substitution on plant cell walls is not understood. Smith and O'Brien (1979) found that the inner portion of the outer epidermal wall which

had high intensity autofluorescence was resistant to fungal degradation. They feel this may be due to the differences in the degree of phenolic substitution and/or the type of linkage and phenolic entities between the inner and outer layers of the epidermal walls. However, certain anaerobic fungi were found which can attack tissues containing phenolic compounds (Akin et al, 1983). Fulcher at al. (1972) showed that wheat aleurone cell walls rich in phenolics were partially resistant to autodigestion during germination, and remained long after the endosperm walls had disintegrated. Similarly, resistance to cellulolytic digestion of oat mesophyll cell walls was due to wound induced lignification. Wounding stimulated phenylalanine ammonia lyase and peroxidase activity (caused by wound-induced ethylene synthesis) causing subsequent lignification (Gabelle and Galston, 1983).

Forages were found to contain phenolic levels of over 1% of the plant cell wall content (Akin et al., 1985). Phenolic acid can inhibit forage digestion by binding to structural carbohydrates. Free phenolic acids were also found to have a toxic affect on rumen bacteria and protozoa, thus inhibiting fiber digestion (Chesson et al., 1982). Sawai et al. (1983) found that the degradability of forage fibers by commercial cellulases decreased significantly with an increase of cell wall esterified phenolic acids. Apparently, two adjacent glucosyl units with ferulates or p-coumarates, esterified at C-6, inhibited hydrolysis of the linkage in cellulose.

C. Antimicrobial activity

Feruloyl polysaccharides and dimerization may protect plants against invasion of pathogens by inhibiting hydrolytic enzymes or by acting as growth inhibitors (Hartley and Jones, 1976). Grand and Rossignol (1983) studied the lignification process of muskmelon shoots in response against *Colletotrichum langarium* fungus inoculation. They found labeled phenylalanine was incorporated into lignin monomers of the protected seedlings. In addition, it was found that ferulate:CoA ligase and p-coumarate:CoA ligase were stimulated in the protected plants. They believe that hydroxycinnamate:CoA ligases could play a role in controlling the lignification process by regulating the monomeric composition of lignin.

Lignified cell walls are considered to be effective barriers to the progression of plant pathogens since lignin is highly resistant to attack by most microorganisms (Kirk, 1971). Increased lignification at the point of attack is a common plant response to parasites (Vance, 1980). However, the lignin formed in response to pathogen attack differs from healthy tissue by the change in the amounts of p-coumaryl, coniferyl, and sinapyl alcohols. The primary precursors of lignin are the alcohols of p-coumaric, ferulic and sinapic acid (Sarkenin, 1971). The lignin formed in wheat leaves after inoculation with fungi is composed mainly of guaiacyl units (containing mainly p-coumaryl and feruloyl residues), while lignin from healthy tissue/leaves has a higher content of syringyl propane units (consisting mainly

of sinapyl and ferulyl residues; Vance, 1980).

The formation of a polyphenolic layer in cell walls surrounding an infection site of ripe fruits may play an important role in inhibiting the spread of infection after the main defense mechanisms weaken (Glazener, 1982). Ferulic acid and p-coumaric acid increased in young tomato fruits during the first days after inoculation with conidia of *Botrytis cinerea*. The activity of phenylalanine ammonia lyase also increased during this period, indicating de novo synthesis of these compounds. Ride (1975) found that wound inoculation of wheat leaves produced a more rapid formation of lignin. Henderson et. al. (1975) showed that resistance towards *Phytophthora infestans* in potatoes was accompanied by rapid deposition of lignin-like material in tuber slices.

The presence of phenolic acids in plant extracts was found to be succesful for inhibiting aflatoxin production by *Aspergillus parasiticus*. In further study, three common cereals: rice, wheat,and maize, and two oil seeds: groundnut and mustard, were soaked in 500ppm aqueous ferulic acid to measure the effect on aflatoxin production. Ferulic acid inhibited aflatoxin production on all seeds by more than 56% (Bilgrami et al., 1981). Aqueous and methanolic extractions of the herbaceous plant, *Heum athamanticum*, show a platelet anti-aggregant acivity believed to be due to cinnamic acid esters which include methyl ferulate (Barron et al, 1984).

Bound and free forms of p-hydroxybenzoic acid, vanillic,

p-coumaric and ferulic acids were found in water extracts of soils, plant roots and leaf litter. It is possible that phenolic acids in soils might exert allelopathic effects against microbial activity (Whitehead, 1983). This affect was thought to be limited to free forms of phenolic compounds. However, work by Tai et al (1981) on *Lilium longiflorum* has shown bound phenolic compounds to have allelopathic activity as well.

D. Ferulic acid esters and cell wall fluorescence

The cell walls of grass coleoptiles are fluorescent, largely due to ferulate and/or p-coumarate present in ester linkages (Towers et al, 1984). Ferulic acid may be detected by its blue fluorescence in UV light and is generally associated with unlignified cell walls of many tissues, such as the immature internodes of cereals and leaf laminae of grasses. Upon alkaline treatment, a color change from fluorescent blue to green occurs. Species that showed a color change were found to contain bound ferulic and p-coumaric acids. This spectral shift and color change upon alkaline treatment may be indicative of saponification of ferulic acid in ester linkage with carbohydrate (Fulcher et al, 1972).

Alkali extraction of phenolic acid carbohydrate complexes of wheat root cell walls coincided with a reduction in blue auto-fluorescence of the cell walls. However, due to the various types of cells, the distribution of phenolic acids in any one cell wall type was not identified (Smith and O'Brian, 1979).

Harris and Hartley (1980) examined the phenolic constituents of 104 species of monocotyledons in 52 families by UV fluorescence. Samples were divided into two groups depending on ability to change fluoresced blue to green upon treatment with ammonia. Fincher (1987) confirmed the presence of ferulic acid in barley endosperm cell walls using ultraviolet fluorescence microscopy and microspectofluorimetry. Alkali treatment of feruloyl-glucose also showed a change in fluorescence color from blue to green (Meurer et al, 1984).

E. Photoisomerization

Hydroxycinnamic acids are susceptible to photoisomerization, and are known to produce E and Z isomers upon UV irradiation *in vitro* (Smith and Hartley, 1983). Both E and Z isomers are also known to be present in plants (Hartley and Jones, 1977, and Engelsma , 1974). When phenylalanine ammonia-lyase acts on L-phenylalanine, E cinnamic acid is the product. Therefore, the natural occurrence of the Z isomer must be the result of either photoisomerism or the activity of an E/Z isomerase (Yamamoto et.al., 1985).

The photoisomerism is a reversible phenomenon, and the final proportion of E to Z isomers is dependant on the irradiation wavelength (Smith and Hartley, 1983). Towers et al (1984) believe that changes in the geometry of bound hydroxycinnamic acids/esters would be expected to alter the cell wall structure due to the displacement of macromolecules brought

about by photoisomerization. Such changes may affect balances in cell turgor pressure and water reflux. Research using barley seedlings showed that when light was removed there was a gradual increase in the ratio of E to Z isomers of cell wall bound ferulate (Yomamoto, 1985). Towers et al, (1984) suggested that this effect may be due an isomerase, or the continuous production of E-ferulate. These changes may help to explain other ultraviolet absorbance and blue light responses such as solar tracking and the opening and closing of flowers or of stomates (Towers et al, 1984).

F. Effect on food systems

Ferulic acid and related phenolic acids may contribute certain effects when applied to food systems. Although most of the phenolics in food ingredients are cell wall bound, the conditions of food processing may liberate bound phenolics. Phenolic acids (including ferulic acid) have been shown to produce off flavors in foods at levels of 40-90 ppm (Maga and Lorenz, 1973). Interestingly, corn was found to be more susceptible to off flavor and color development than other cereal flours. Curcumin (coloring compound) was shown to decompose in alkaline solution to ferulic acid and feruloylmethane producing yellow and brownish off colors. Fincher (1987) suggests that feruloyl carbohydrates from barley cell walls may contribute to phenomena important to the brewing industry such as foam stability , haze formation, and possibly wort separation.

MATERIALS AND METHODS

A. Plant Material

Corn hulls were obtained from shelled corn as a co-product of starch and sweetener production (A.E. Staley MFG Co.) as outlined in Figure 6. Briefly, shelled corn is cleaned and then undergoes steeping to loosen the components for processing. After steeping, the germ is removed and processed for corn oil and the remaining corn kernel is ground and screened to separate the starch slurry from the hull and gluten. After separation on washing screens, the hull fraction may be diverted from the feed driers to the Refined Corn Bran process. The hull is then co-refined (hot water extractions) in a separate facility (Figure 7) to remove excess starch, protein, and fat to yield a high dietary fiber (90% by total dietary fiber method, Prosky et al., 1984) food ingredient. For this study, refined corn hulls were ground to Ultrafine particle size (90% through 200 mesh screens) using a Rectch Mill (Brinkman).

B. Isolation of Feruloyl Oligosaccharides

Feruloyl oligosaccharides were isolated from corn hulls according to the methods of Kato and Nevins (1985). This method involves mild acid hydrolysis and a series of chromatographic separations as outlined in Figure 8. Details of the procedures used are given below:

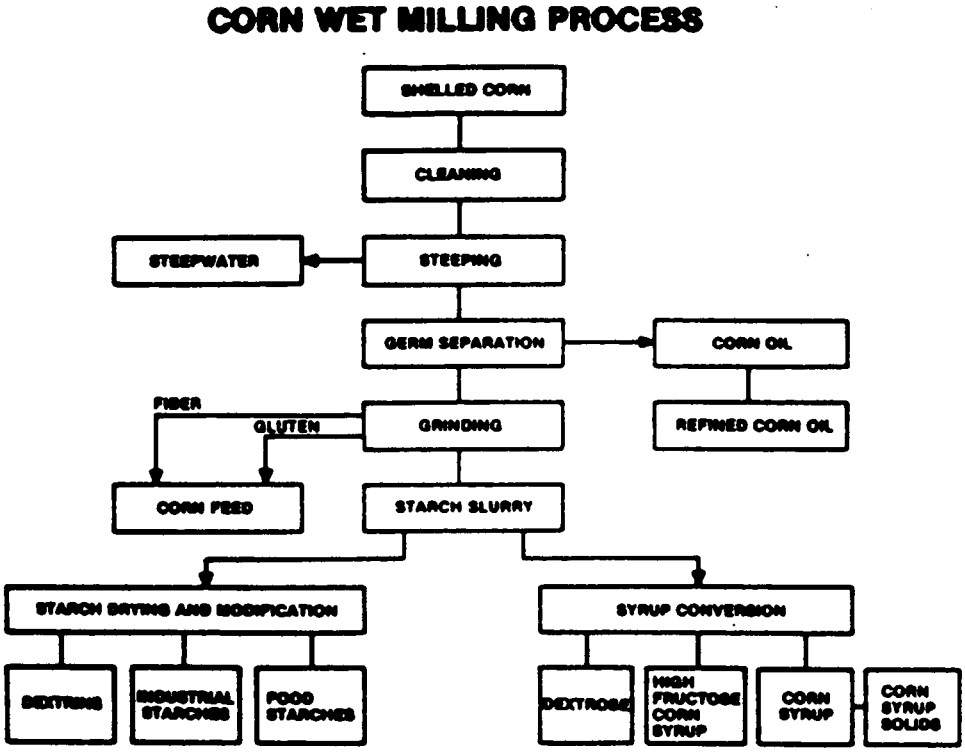


Figure 6. Flow diagram of the corn wet milling process (A.E. Staley Mfg. Co., Decatur, IL).

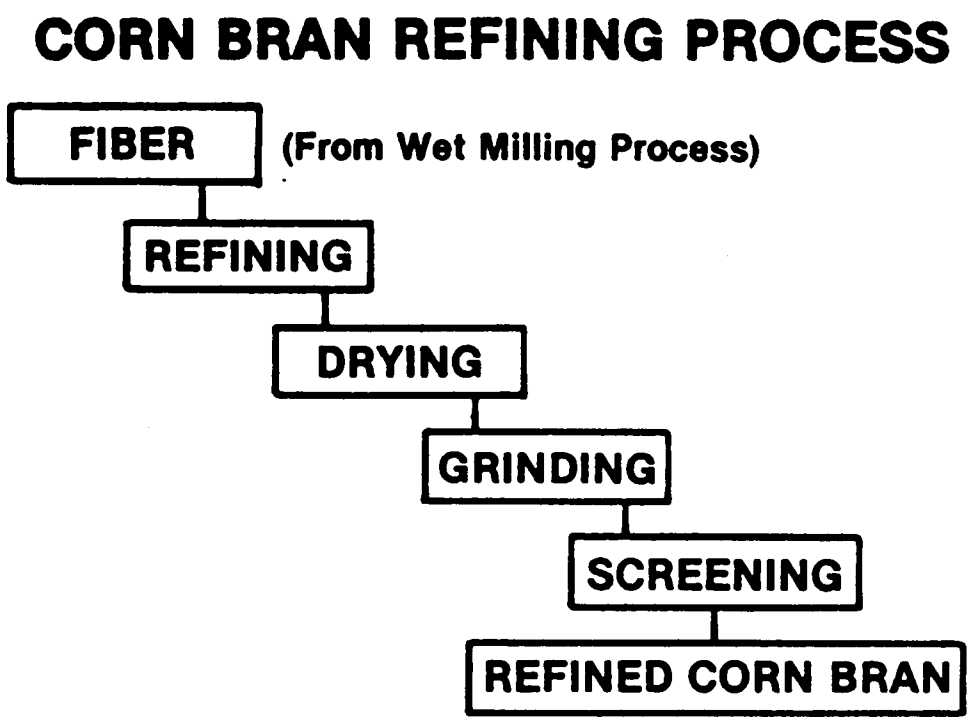


Figure 7. Flow diagram of the corn bran refining process.

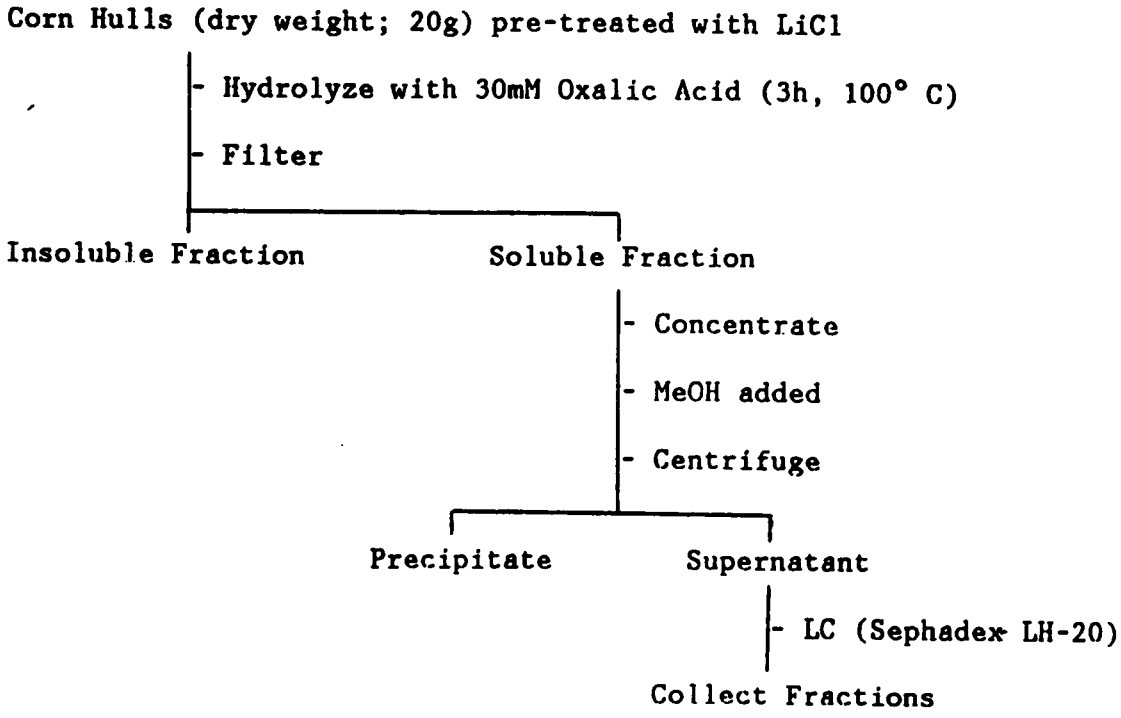


Figure 8. Corn hull hydrolysis and separation scheme to obtain feruloyl oligosaccharides.

1. Hydrolysis of Corn Hull cell walls

Ultrafine ground corn hulls (60 g) were pretreated with LiCl (1500 ml, 3M) to remove cell wall associated proteins. LiCl-pretreated corn hulls (20 g dry wt) were suspended in 1 L of 30mM oxalic acid and hydrolyzed under reflux in a boiling water bath for 3 h. The hydrolysis suspension was filtered (Whatman #1), and the residue was washed three times with 100ml of water. The (oxalic acid) insoluble residue was dried, weighed, and saved for further analysis. The filtrate and washings were combined, neutralized with 0.1M NaOH and concentrated to 40ml by rotary evaporation under diminished pressure in a 35-40° C water bath. Methanol (40ml) was then added to the concentrate with stirring, and the mixture was stored at room temperature overnight to allow for complete precipitation. The mixture was centrifuged (8,000 x g, 15 min) to separate insoluble and soluble fractions.

2. Chromatography of the 50% MeOH soluble fraction

A model K 26/100 chromatography column (2.6 x 100cm) containing approximately 70 ml bed volume of Sephadex LH-20 (Phamacia Inc., Piscataway, NJ) was equilibrated with 500 ml of MeOH:water (1:1, v:v). Sephadex LH-20 has an affinity for aromatic compounds in addition to acting as a molecular sieve. Sample (10 ml) was loaded onto the column by gravitational force, and then eluted through the column with 50% MeOH using a model 2132 peristaltic pump (LKB instruments, Rockville, MD). Flow rate was 60 ml/h and 9 ml fractions were collected using a model 2070 Ultrarac II fraction collector (LKB instruments, Rockville,

MD). Fractions assayed for carbohydrate (phenol-sulfuric assay) and UV absorbance at 325 nm (maximum abs. for ferulic acid). Tubes corresponding to eluted peaks I (15-28), II (28-44), III (45-67), IV (68-82), and V (83-102) were separately combined and concentrated to dryness. This procedure was repeated eight times, and peaks were pooled yielding fractions I-V.

3. Purification of fractions III, IV, and V

Fractions III-V were separately re-chromatographed using the LH-20 column as already described. Sub-fractions obtained by rechromatography were isolated and analyzed by reverse phase high performance liquid chromatography (RP-HPLC). The sub-fractions which produced a major peak by RP-HPLC, accounting for >70% of the total, were collected. These isolated sub-fractions were then rechromatographed and recollected until only a single peak was obtained on both Sephadex LH-20 and RP-HPLC. The purified fractions were separately concentrated, freeze dried, and stored in a desiccator until used.

C. Characterization of Feruloyl Oligosaccharides

1. Alkaline hydrolysis

A portion (1 mg) of isolated fractions was dissolved in 1 ml of NaOH (1N) and maintained for 24 h with stirring at room temperature. The solutions were acidified (dropwise addition of 50% HCl to pH 1.0) and then extracted with ether (10 ml). The ether phase was washed with water and evaporated to dryness. The water phase was treated with mixed bed resin and evaporated to

dryness. The ether phase was subjected to RP-HPLC chromatography and eluting components tentatively identified by comparison of retention times to known standards: ferulic, p-coumaric, caffeic, and sinnapic acids. The water phase was analyzed for saccharide distribution, monosaccharide composition, and chemical shifts by C-13 and proton NMR.

2. Hydrolysis with carboxyl esterase

Purified feruloyl oligosaccharides (2 mg) were dissolved in 1 ml of sodium phosphate buffer (pH 7.7) and incubated with carboxyl esterase (100 µl; Sigma) at 37° C for 24 h. Aliquots of the incubation mixture were taken at selected time intervals and were directly analyzed by RP-HPLC to monitor release of ferulic acid. The resultant hydrolysis mixture was then subjected to liquid chromatography (Biogel P-2) to separate released oligosaccharides from ferulic acid and enzyme. A model K 16/70 (1.6 x 70 cm) chromatography column (Pharmacia Inc., Piscataway, NJ) containing approximately 35 ml bed volume of Biogel P-2 (Bio-Rad, Richmond, CA) was equilibrated with 300 ml of 0.1 N acetic acid. Hydrolysis mixture (2 ml) was loaded onto the column by gravitational force. Sample was eluted through the column with 0.1N acetic acid using a peristaltic pump. Flow rate was 30 ml/hr, and 5 ml fractions were collected as described. Protein and ferulic acid were monitored by measuring absorbancy at 280 nm using a model UV-1 single path monitor with a 280 nm filter (Pharmacia Inc., Piscataway, NJ). Carbohydrate was monitored by refractive index using a differential refractometer

(Waters Assoc., Milford, MA). The carbohydrate and ferulic acid fractions were collected, concentrated, deionized, and freeze dried.

3. Monosaccharides composition

Samples (1 mg) were placed in a reactivial (Pierce Chem. Co., Rockford, IL) and hydrolyzed with 1N H_2SO_4 (2 ml) for 3 h at 100° C. After cooling, $Ba(OH)_2$ was added until neutral pH was achieved. The solution was then filtered (Acrodisc 0.45 um, Gelman) and concentrated to dryness with a stream of nitrogen. The residue was then freeze dried, and solutions analyzed for monosaccharide composition by HPLC and GC against known standards: arabinose, xylose, glucose, galactose, mannose. Unused portions were stored in a desiccator.

4. Galactosidase treatment of feruloyl trisaccharide

Purified feruloyl trisaccharide (FGXA) and deferuloylated trisaccharide (GXA, 3 mg) were treated with equivalent activity units (0.5 units) of alpha- and beta- galactosidase (*A. niger*, Sigma) at pH 4.5 (sodium acetate buffer) for 16 h at 25° C. After incubation, samples were deionized (mixed bed resin) and freeze dried. Samples were then derivatized (TMS) and subjected to GC analysis.

5. Reduction of feruloyl oligosaccharides

For GC monosaccharide analysis - Sample (4.5 mg) was placed into a culture tube (10 ml) and 1 ml of sodium borohydride solution (1.5 mg /ml made up in 1M ammonium hydroxide solution). The solution was held at room temperature for 2 h. The reaction

mixture was neutralized by the drop wise addition of glacial acetic acid until bubbling has stopped. One ml of methanol was added and the mixture was evaporated to dryness using dry nitrogen and a 50-55° C water bath. An additional 1 ml of methanol was added followed by 3 ml of HCl / MeOH solution (10 drops of conc. HCL / 100 ml MeOH), and the mixture was evaporated to dryness. The mixture was silylated for GC analysis.

For NMR analysis - Reduction procedure was modified to prevent saponification of ferulic acid ester. Sample (15 mg) was placed into a culture tube and 2 ml of cold MeOH was added. To this mixture 5 mg of sodium borohydride was added, stirred, and placed into an ice bath for 30 min. The reaction was quenched by adding 10 drops of water, the mixture was deionized with mixed bed resin, filtered, and freeze dried. The sample was analyzed by RP-HPLC to check for release of ferulic acid.

6. Neutral Carbohydrate Analysis - (Phenol Sulfuric)

Neutral carbohydrate was determined using the Phenol sulfuric method of Dubois et al (1956). To 0.25 ml of sample, 0.5 ml of phenol reagent (5%) was added and mixed. Concentrated sulfuric acid (2.5 ml) was added with an automatic syringe and mixed. Mixtures were held at room temperature for 10 min. Samples were mixed, placed in a water bath (25-30° C) for 20 min, and aborbancies were read at 490 µm in a model DU-7 UV-VIS spectrophotometer (Beckman Instruments, Irvine, TX) relative to standard solutions of arabinose, xylose, and galactose (separate

standard curves). A water blank and ferulic acid control were tested in parallel with each set of assays.

7. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra was measured with KBr disks. The disks were prepared using standard KBr pellet technique. Sample (1 mg) was combined with 200 mg of KBr, ground, and pressed into a disk. The sample was analyzed using a Beckman model FT1300 FTIR spectrometer. The sample was scanned from 4000.93 wavenumber to 401.12, with 50 scans.

8. UV absorbance

Sample (1 mg) was suspended in 3 ml of distilled water, and then diluted 300 fold. Phenolic acids were measured spectrophotometrically using a Beckman DU-7 UV-VIS spectrophotometer. The sample was scanned from 670 nm to 200 nm. A water blank and ferulic acid control were run in parallel with each set of UV-VIS scans.

D. High performance liquid chromatography

1. Equipment and columns

Monosaccharide composition and feruloyl oligosaccharides were examined by HPLC. The chromatograph was equiped with a model PM-30A solvent delivery system (Bioanalytical Systems, Inc., Lafayette, IN), a model R-403 refractive index detector, a lambda max 480 LC spectrophotometer (Waters Assoc., Milford, MA), a model 7125 injector system (Rheodyne, Berkeley, CA), and a SP4100 calculating integrator (Spectrophysics).

A Lichrosorb NH_2 column, 25cm x 4.6mm, 10 µm, (Alltech, Deerfield, IL) was used for separating monosaccharides and short chain oligosaccharides. Samples were eluted with 80% acetonitrile / 30% water mixtures at a flow rate of 1.0 ml/min (1200-1500 psig). Eluting sugars were detected with the refractive index detector and identified from their retention times as compared to standards: arabinose, xylose, galactose, glucose, mannose, maltose, and maltotriose (Sigma Chem. Co., St Louis, MO).

A Biophase ODS - 5µm column (Bioanalytical Systems Inc., Lafayette, IN) was used to separate phenolic acids and feruloyl oligosaccharides. Samples were eluted with 89% water / 10% acetonitrile / 1% acetic acid mixtures at a flow rate of 0.5 ml/min (2400-2500 psig). Eluting phenolic acids and feruloyl oligosaccharides were detected by absorbance at 325 nm and were identified from their retention times relative to known standards, and quantified using standard curves for: ferulic acid, p-coumaric acid, caffeic acid, and sinapic acid (Sigma).

All elution solvents were filtered through 0.45 or 0.50 µm pore size filters (Millipore Corp., Bedford, MA) and degassed under vacuum before use.

2. Preparation of samples and standards

Samples and standards were prepared by dissolving 2 mg quantities in 2 ml of the appropriate eluent mixture. Mixtures were diluted to the appropriate concentration and filtered through a 0.45 µm filter (Gelman, Ann Arbor, MI). All

mixtures were kept frozen when not in use and were refiltered after thawing.

E. Gas Chromatography

1. Equipment and columns

Monosaccharide composition of feruloyl oligosaccharides before and after reduction were analyzed by gas chromatography (GC). Derivatized samples were chromatographed on a Varian 3400 chromatograph equiped with an on-column injector (OCI), a 30 meter SPB-5 (.32 µm ID, .25 µm film) capillary column, and a flame-ionization detector (FID). A 1 µl sample was injected at an initial column temperature of 115° C with a hold time of 5 minutes, temperature was increased to 128° C (8° C/min) with a hold time of 40 min (pentose region), temperature was increased to 210° C (2° C/min) with a hold time of 5 min (hexose region), and the final tmperature increase was to 315° C (20° C/min) with a hold time of 10 min (disaccharide and above region). Response factors for samples and standards were determined using a SP4100 calculating integrator (Spectrophysics).

2. TMS Derivitization

Hydrolyzed samples (2-5 mg) were weighed into cleaned, oven-dried teflon-capped reaction vials (3.0 ml, Wheaton). Silylation grade pyridine (0.5 ml) and bis(trimethylsilyl)-trifluoroacetamide (BSTFA) were added (Sigma Chemical Co., St. Louis, MO). The mixture was heated at 70° C for 20-30 min, or until clear. Standard arabinose, xylose, glucose, and galactose

(Sigma Chemical Co.) were dereivatized as described above and analyzed to determine response factors compared to internal standard phenyl-β-D-glucopyranoside. Response factors were linear over the ranges evaluated.

F. Nuclear Magnetic Resonance

1. Equipment and sample preparation

Proton and carbon-13 n.m.r. spectra were recorded with a Varian VXR 400 spectrometer (Palo Alto, CA), using standard Varian software for sophisticated experiments (i.e. difference spectroscopy, relaxation measurements, two-dimensional spectroscopy, resolution enhancement, etc.). Samples (15 mg) were dissolved in DMSO (3 ml) and placed in a 5 mm diameter n.m.r tube. All spectra were run under ambient conditions.

2. Carbon-13 n.m.r.

Carbon-13 n.m.r. was recorded at 100 MHz, spectral width was 25000 Hz, acquisition time was 0.501 sec, and pulse width was 90 degrees. The number of repetitions was 12800 for FA, 6400 for FXA, and 16053 for FGXA. Alternating pulse technique (APT) C-13 pulse sequence spectra was recorded under the same conditions utilizing 90 degree and 180 degree pulses. The number of repetitions was 3200 for feruloyl monosaccharide (FA), 4000 for feruloyl disaccharide (FXA), and 4884 for feruloyl trisaccharide (FGXA). Chemical shifts are reported in p.p.m. relative to the center of the DMSO peak at 39.5 p.p.m.

3. Proton n.m.r

Proton n.m.r. spectra were recorded at 400 MHz, with a deuterium lock, and resolution enhancement. Chemical shifts are reported in p.p.m. relative to the center of the DMSO peak at 2.49 p.p.m.

Homonuclear H,H, J-correlated spectroscopy was preformed with the COSY (N-type) programme (Bax and Freeman, 1981) utilizing standard Varian software. COSY utilized pulses of 45 and 90 degrees. The 2-D spectral width was 1053 Hz for FGXA (2142.7 Hz for FXA) with an acquisition time of 0.486 sec (0.239 for FXA), and a relaxation delay of 2.0 sec (1.0 sec for FXA). The number of repetitions was 32 for FGXA and 16 for FXA, with 256 increments using double precision acquisition. Data was pseudo-echo shaped and fourier transformed to 2K x 2K.

RESULTS

A. Isolation of Feruloylated Oligosaccharides from Corn Hull

1. Oxalic acid hydrolysis of corn hull

Three major fractions were obtained after oxalic acid hydrolysis: oxalic acid insoluble; 50% MeOH insoluble; and 50% MeOH soluble fractions. Analysis of ferulic acid content (alkaline hydrolysis and RP-HPLC analysis) and sugar composition (monosaccharide analysis) showed (Table 2) that 51.5% of the corn hull was unaffected by the mild acid hydrolysis and contained 1.26% ferulic acid. The 50% methanol insoluble fraction accounted for 26.5% of corn hull and contained 2.14% ferulic acid. The 50% MeOH soluble fraction, which represented lower MW material, had the lowest yield but contained the largest amount of ferulic acid, accounting for 57% of the total ferulic acid content. Ferulic acid content also appeared to correlate with increased amounts of arabinose and galactose which is indicative of sugars which comprise corn hull hemicellulose side chains.

2. Fractionation of 50% MeOH soluble fraction

The 50% MeOH soluble fraction was resolved into five fractions upon chromatography on Sephadex LH-20 (Fig 9). Table 3 shows the monosaccharide composition of fractions I-V. Fractions I and II contained the highest concentration of carbohydrate and also accounted for most of the 325nm UV absorbing material (Fig 9). Fraction I contained mainly xylose while fraction II

Table 2. Ferulic acid content and sugar composition of 30mM oxalic acid hydrolyzed fractions.

Fraction	% Recovery	% Ferulic	Sugar Comp (mole %)			
			Xyl	Ara	Glc	Gal
Oxalic Insol	51.5	1.26	(ND)[a]			
50% MeOH Insol	26.5	2.14	66.7	11.3	18.3	3.7
50% MeOH Sol	22.0	8.61	51.4	34.5	8.7	5.4

[a]Not determined

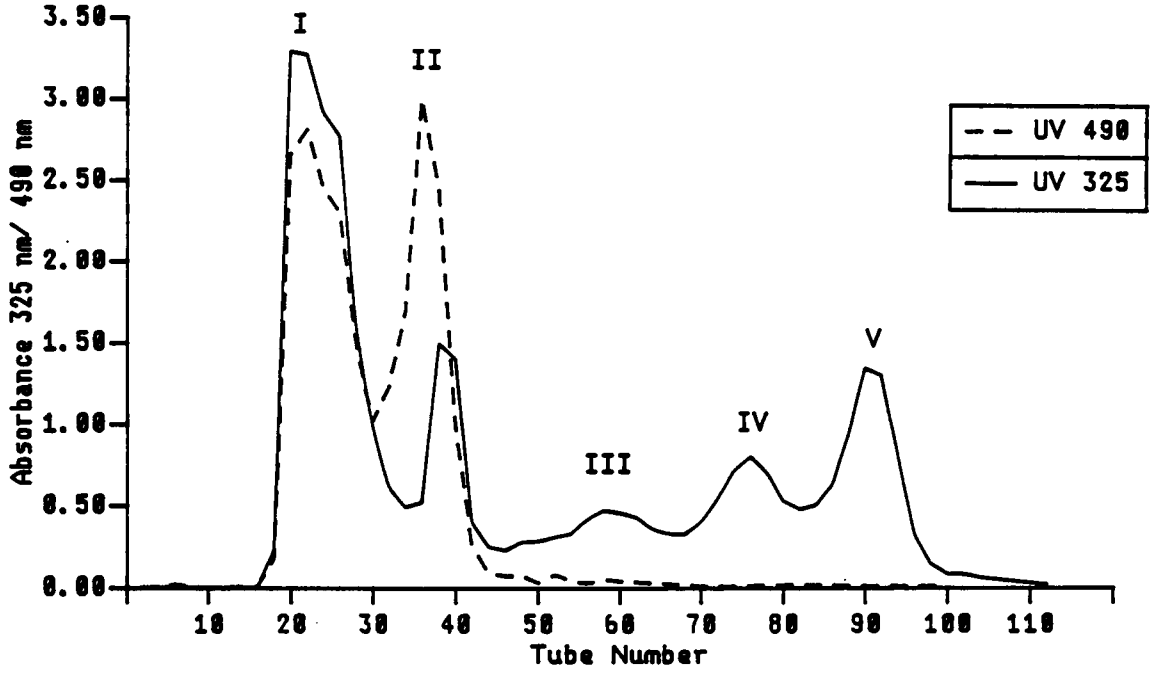


Figure 9. Elution profile of corn bran hydrolyzate 50% MeOH soluble fraction by chromatography on Sephadex LH-20. Sample, 10ml; column dimension, 2.6 x 100cm; bed volume, 70 ml; flow rate; 60 ml/h; eluent, 50% MeOH. Solid line indicates absorbance at 325 nm; dashed line indicates neutral carbohydrate measured by phenol sulfuric acid method.

Table 3. Yields and properties of fractions I - V recovered from chromatography (Sephadex LH-20) of 50% MeOH soluble fraction of oxalic acid hydrolyzed corn hull.

Fraction	Yield (mg)[a]	Total CHO (mg)[b]	Total Phenolic Compounds (mg)[c]	Sugar Comp (mole%)			
				Ara	Xyl	Gal	Glc
I	-[d]	1850.0	334.0	14.6	67.2	10.7	7.5
II	-[d]	947.0	113.0	54.4	36.4	8.2	1.0
III	166.9	58.3	87.7	37.9	39.6	19.8	2.7
IV	151.2	22.5	117.9	54.1	45.9	-	-
V	195.4	23.75	135.7	89.4	10.6	-	-

[a] Yield from 20g starting material
[b] Determined by the phenol-sulfuric acid method (as xylose)
[c] Absorbance at 325 nm against ferulic acid standard
[d] Not determined

contained a high amount of arabinose and xylose. Alkaline hydrolysis and subsequent saccharide distribution analysis (HPLC) of fraction I and II showed fraction I to contain high MW saccharides of DP 10 and above. Fraction II contained some (<20%) high MW saccharides, but was mainly composed of mono- and di-saccharides. The short retention time of these mono and di-saccharides as compared to feruloyl mono- and disaccharides suggests they do not contain phenolic components.

Fractions III, IV, and V accounted for about 45% of the total UV absorbance (50% MeOH soluble fraction), but contained the lowest amount of carbohydrate (< 100 mg combined). The monosaccharide composition varied between the three fractions, with fraction V being nearly all arabinose. In addition, UV absorbance of fractions III - V showed a correlated increase with arabinose content. The low recoveries of fraction III - V (as compared to fractions I and II) prevented alkaline hydrolysis and saccharide distribution analysis.

Reverse phase chromatograms (RP-HPLC) of fractions III , IV, and V showed lack of homogeneity (Figs 11A, 19A, and 29A), indicating all three fractions were not of pure form. However, upon RP-HPLC analysis fractions III - V did yield a major peak which accounted for about 40% of the total fraction. The components responsible for the major peaks were subjected to further purification to obtain feruloyl oligosaccharides for structural characterization.

B. Characterization of Fraction V

1. Purification of Fraction V

Re-chromatography of fraction V on Sephadex LH-20 yielded 3 sub-fractions labeled V-a to V-c (Fig 10). Reverse-phase HPLC analysis found fractions Va and Vb to be heterogeneous, while fraction V-c yielded a major peak accounting for >70% of the total fraction. Fraction Vc was collected and re-chromatographed on sephadex LH-20 (3X), producing a single fraction (Fig 10) that was essentially homogenous upon RP-HPLC analysis (Fig 11). Purification yielded approximately 80 mg of fraction V-c.

2. Compositional analysis of Fraction V-c

Acid hydrolysis and monosaccharide analysis (HPLC) of fraction V-c showed only the presence of arabinose (Fig 12). Fraction V-c showed a maximum UV absorption of 325 nm (Fig 13) which was indicative of a ferulate ester (Fry, 1983; Smith and Hartley, 1983; Kato and Nevins, 1985). Infrared spectroscopy of fraction V-c showed an i.r. spectrum with major bands: 3700-3200 (OH), 2920 (CH), 1694 (C=O), 1630 and 1597 (aliphatic and aromatic C=C), and 1516 (aromatic C=C). Comparison to the i.r. finger-print spectrum of ferulic acid showed some similarities, but carbohydrate bands (1400 - 900) in the UV spectrum masked some of the more distinctive ferulic acid bands, making positive ID difficult. Alkaline hydrolysis of fraction V-c followed by RP-HPLC showed the presence of ferulic acid as compared to retention times of phenolic acid standards. The results indicated fraction V-c contained arabinose and ferulic acid, and V-c was

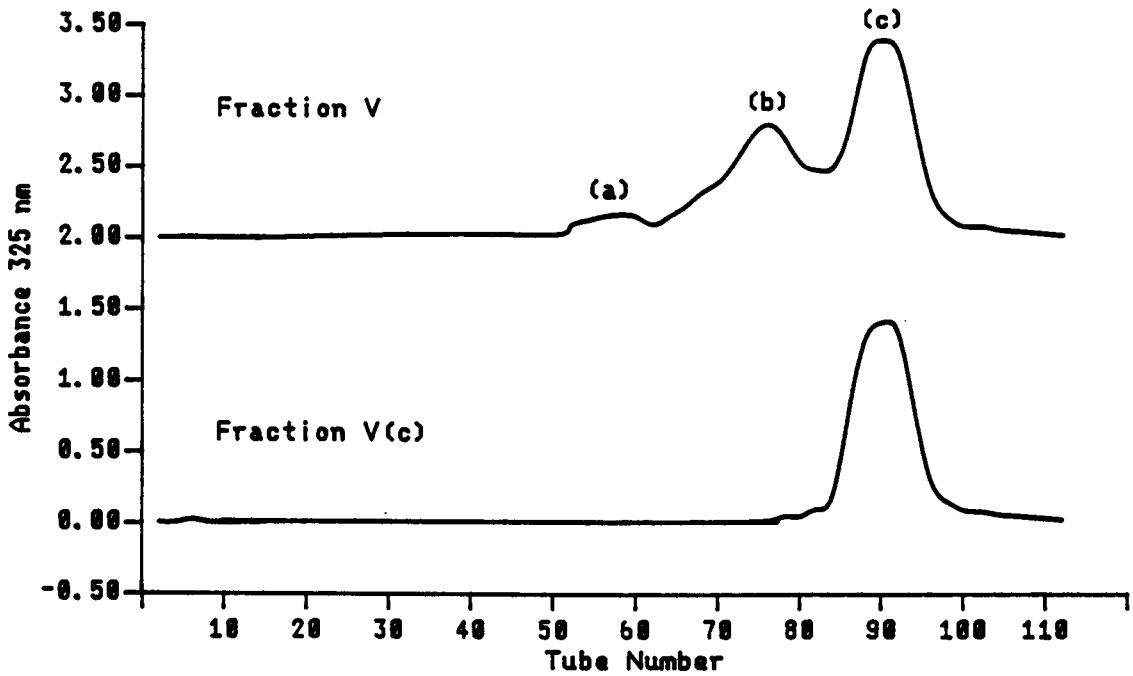


Figure 10. Elution profile of fraction V and V-c by chromatography on Sephadex LH-20. Conditions as in figure 9.

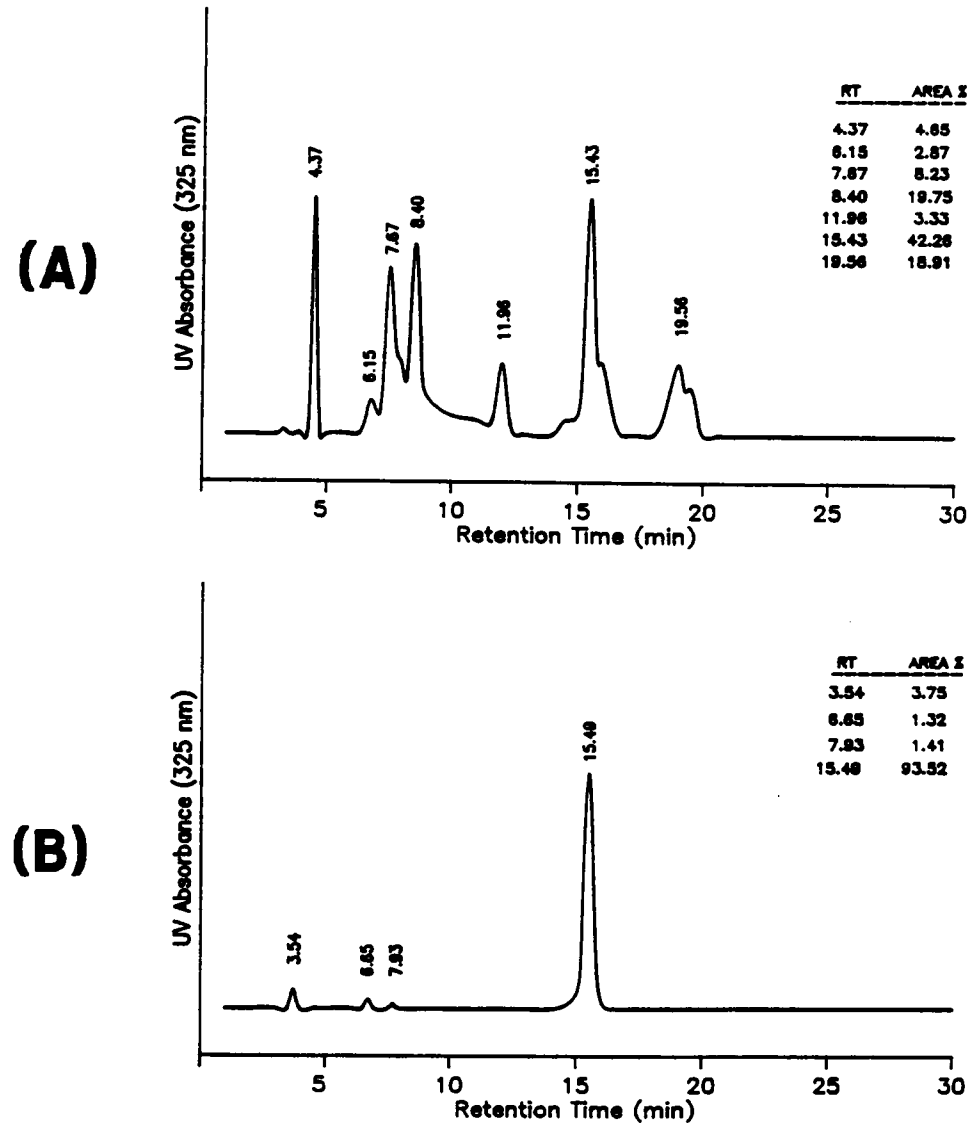


Figure 11. Reverse-phase HPLC chromatogram of (A) - crude fraction V; (B) - purified fraction V-c. Eluent, 89% water/10% acetonitrile / 1% acetic acid; flow rate, 0.5 ml/min; column, Biophase ODS - 5µm; UV detector (325mn).

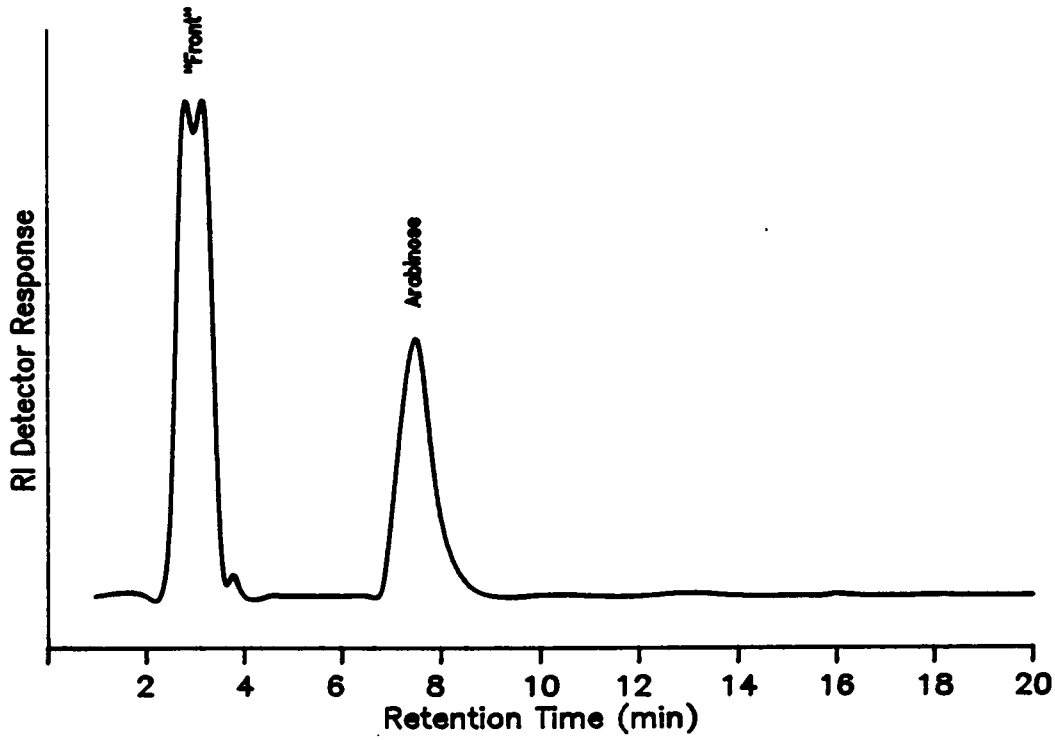


Figure 12. HPLC chromatogram of hydrolyzed fraction V-c. Eluent, 80% acetonitrile/20% water; Flow rate, 1 ml/min; column, Lichrosorb NH_2; refractive index detector.

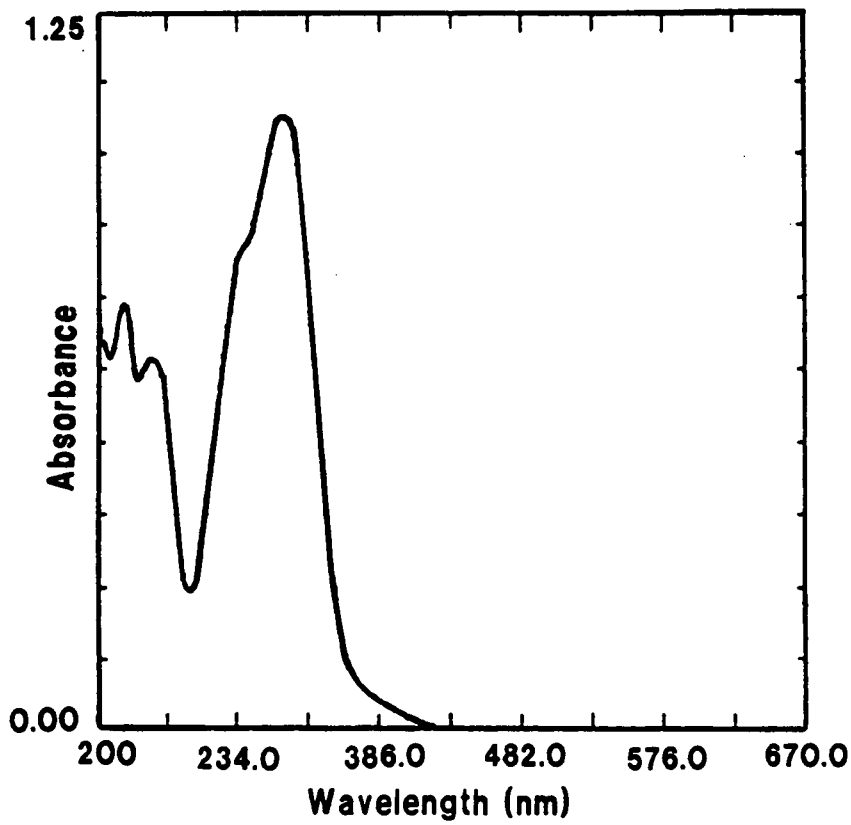


Figure 13. Absorbance spectra of purified fraction V-c.

labeled FA for ferulic acid and arabinose.

3. C-13 NMR Spectroscopy of FA

NMR spectroscopy (proton and C-13) was employed to elucidate ring form, number of carbons, anomeric configuration of the arabinose, and point of attachment of the non-carbohydrate fraction. Figure 14 shows an APT (alternating pulse technique) C-13 NMR spectrum for FA. Briefly, this technique provides a spectrum in which carbons having an even number of hydrogens (0,2,4,) give an upward signal, while carbons with an odd number of hydrogens (1,3,) give a downward signal. Nine distinctive signals were obtained in the non-carbohydrate region of the spectrum (110 and above) plus a methyl signal at 56.19, accounting for all ten carbon signals and thus confirming the presense of a ferulate ester moiety. Signals were assigned for ferulate ester (Table 4) and agreed with published values (Himmelsbach and Barton, 1980; Smith and Hartley, 1983; Kato and Nevins, 1985; Mueller-Harvey et al., 1986).

Ten carbon signals were obtained for the carbohydrate portion (105-62 ppm) of the spectrum (Fig 15). Signal assignments are listed in table 4, and are consistent with carbon signals for alpha and beta-arabinofuranose (Bock and Pederson, 1983; Joseleau et al., 1984). The absence of signals at 108-110 indicates the lack of a glycosidic linkage at C-1 which would give a 7-9 ppm downfield shift. A furan ring form was supported by signals for C-2, C-3, and C-4 which occur between 77 - 84 ppm, as opposed to signals at 68-74 ppm for the pyranose form. The

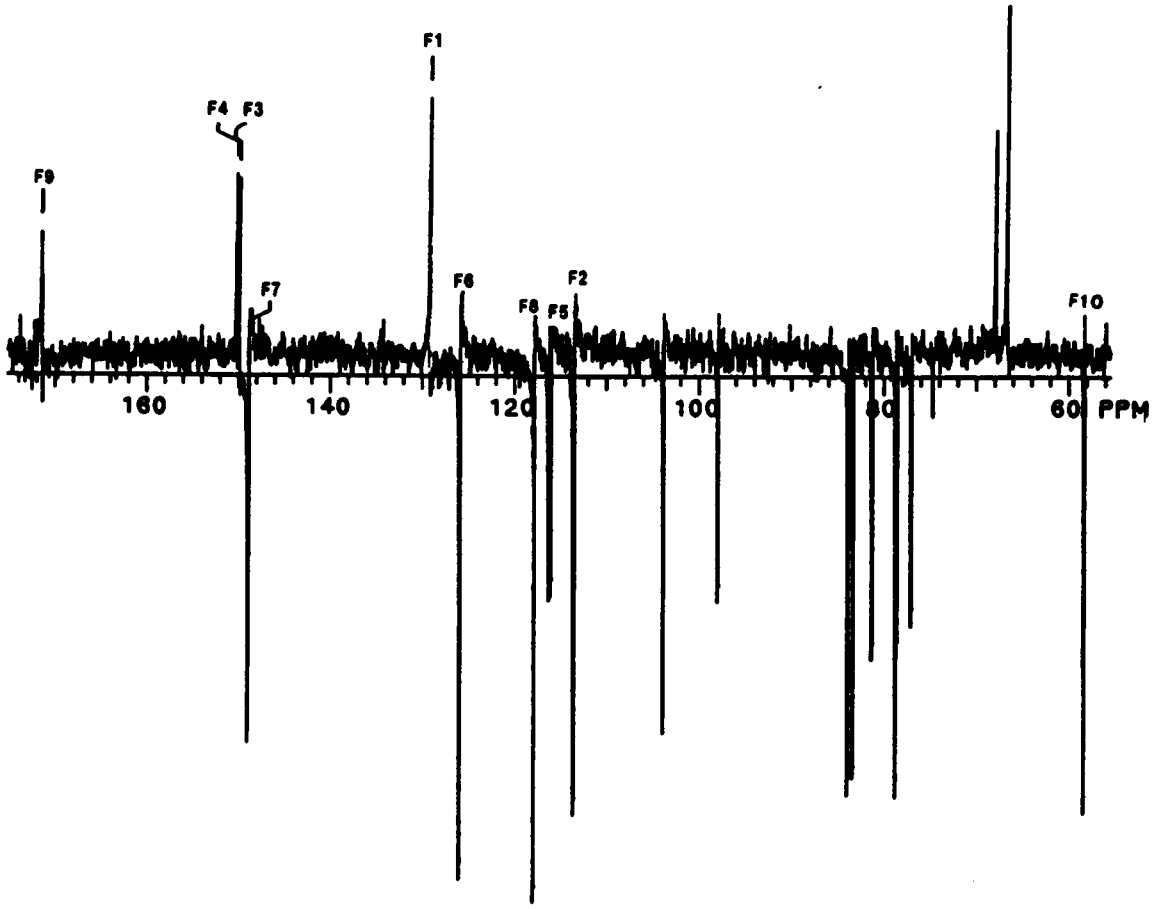


Figure 14. APT carbon-13 (100 MHz) NMR spectrum of FA recorded in D_2O at room temperature (F = ferulate ester carbon signals, see table 4 for assignments)

Table 4. Assignments of C-13 NMR spectra for FA.[a]

Assignment	Ferulic acid	α -Ara*f*	β-Ara*f*
C-1	129.18	103.82	97.91
C-2	113.57	81.24	78.67
C-3	150.23	78.53	76.99
C-4	149.89	83.88	83.29
C-5	116.12	66.65	67.88
C-6	125.87		
C-7	148.83		
C-8	117.86		
C-9	171.52		
C-10	58.26		

[a] Structure shown in Figure 17.

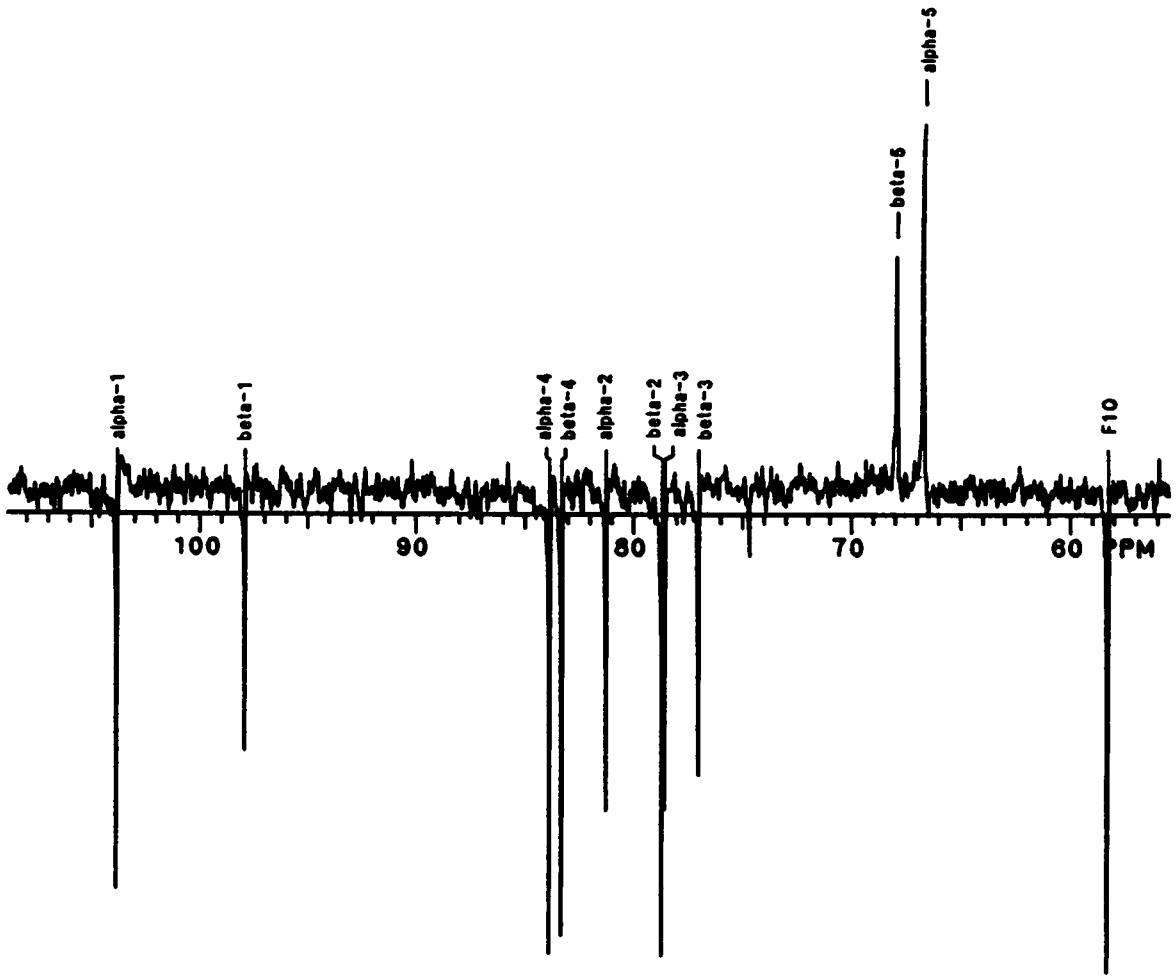


Figure 15. APT carbon-13 (100 MHz) NMR spectrum focusing on the carbohydrate region for FA. Carbon signals are labelled for alpha- and beta- arabinofuranose (table 4).

C-5 signals for arabinose are easily detected in the APT NMR spectrum (upward signals) at 66-67 ppm. Literature values for C-5 of arabinofuranose are 61-62 ppm, indicating a 4-5 ppm down field shift. This shift is indicative of the deshielding affect of an ester linkage (Smith and Hartley, 1983; Kato and Nevins, 1985), and that ferulic acid is attached to arabinose at C-5 position.

4. Proton NMR of FA

The proton NMR spectrum for FA is shown in Figure 16. Proton NMR is a useful tool in determining anomeric configuration of carbohydrates for structural analysis. The chemical shift of anomeric protons (4.4 - 5.5 ppm) make them easily distinguishable from the broad signals of non-anomeric sugar-skeleton protons (3.2 - 4.1 ppm). Alpha and beta anomeric protons of pyran ring forms are also easily resolved from each other since beta forms are more shielded (due to H-1 proton being in the axial position) and reside further upfield (4.4 - 4.7 ppm) than less shielded alpha anomers (4.9 -5.4 ppm Vliegenthart et al., 1983). The anomeric proton (H-1) signals for alpha- and beta-arabinofuranose are nearly identical (4.9 and 5.0 ppm) due to the furan ring form. Non-anomeric proton signals for arabinofuranose occur at about 3.5 -4.0 ppm . The presence of signals at 3.6 - 4.3 ppm for FA non-anomeric protons is due to (and consistent with) the deshielding affect of ferulic acid ester linked at C-5 of arabinofuranose (Smith and Hartley, 1983; Mueller-Harvey et al., 1986). All five proton signals (H-2, H-5, H-6, H-7, H-8) for

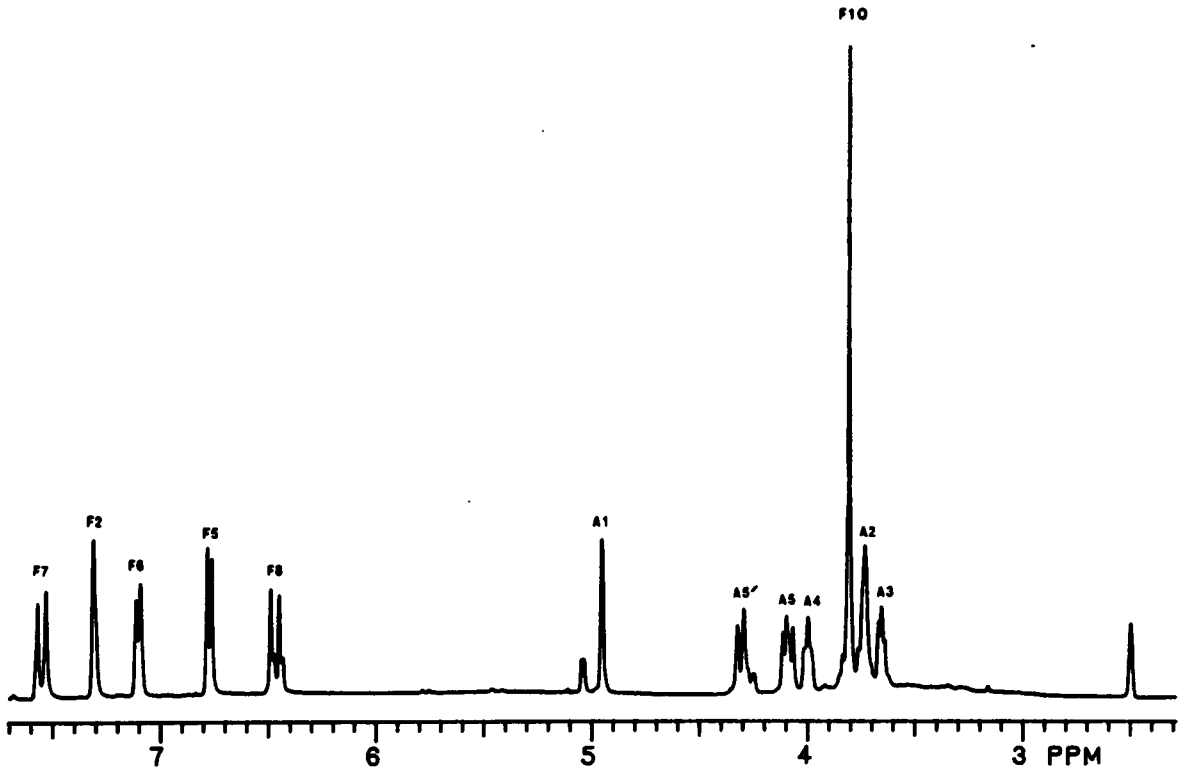


Figure 16. Proton (400 MHz) NMR spectrum of FA recorded in DMSO at room temperature (F = ferulate ester, A = arabinose, see table 5 for assignments and coupling constants).

ferulic acid (6.4 -7.5 ppm) plus the methyl group (H-10, 3.8 ppm) are present. Chemical shifts and coupling constants for FA are listed in Table 5. The coupling constants (Table 5) of 16 Hz for H-7 and H-8 indicated that the ferulate ester occurs as the *trans* isomer (Jackman and Sternhell, 1972). Also, the relative intensities of the proton NMR signals showed about a 1:1 ratio of ferulic acid to arabinose.

5. Proposed structure of FA

Results showed that FA is 5-O-(*trans*)-feruloyl-L-arabinofuranose. The proposed structure of FA is shown in Figure 17.

C. Characterization of Fraction IV

1. Purification of Fraction IV

Fraction IV was re-chromatographed on Sephadex LH-20 and yielded 4 sub-fractions labeled IVa - d (Fig 18). Upon analysis by reverse-phase (RP) HPLC, fraction IVc yielded a major peak which accounted for >70% of the total fraction. Fraction IV-c was collected and re-chromatographed on Sephadex LH-20 (repeated three times) to obtain one major peak (Fig 18) which was found to be >94% pure upon RP-HPLC analysis (Fig 19). Purification yielded approximately 62 mg of fraction IV-c.

2. Compositional analysis of Fraction IV-c

Fraction IV-c showed a maximum UV absorption of 325 nm, and infrared bands with similarities to ferulic acid and FA. Alkaline hydrolysis followed by RP-HPLC indicated the presence of ferulic acid as compared to retention times of phenolic acid

Table 5. Proton NMR chemical shifts and coupling constants (Hz, in parentheses) for FA.[a]

Assignment	Ferulic acid	L-Ara*f*
H-1		4.94 (2.4)
H-2	7.30 (1.6)	3.72 (4.8)
H-3		3.65 (6.4)
H-4		4.00 (6.4)
H-5	6.76 (8.4)	4.10 (2.4)
H-5'		4.30 (11.6)
H-6	7.10 (8.4)	
H-7	7.54 (16.0)	
H-8	6.46 (16.0)	

[a] Structure shown in Figure 17.

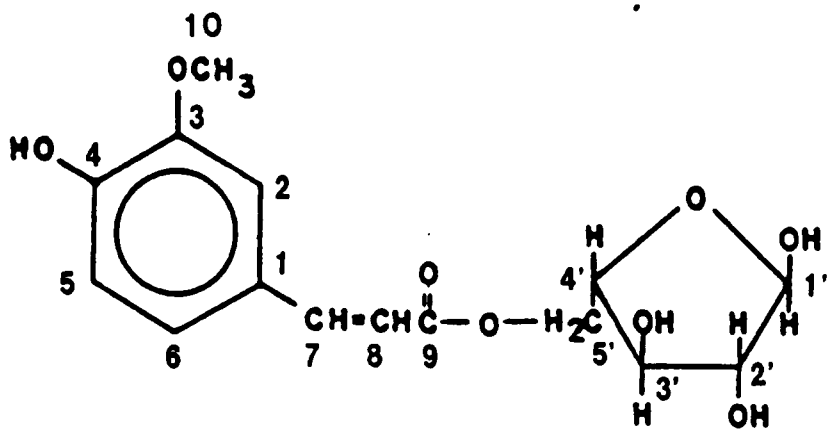


Figure 17. Proposed structure of FA: 5-O-(*trans*)-feruloyl-L-arabinofuranose.

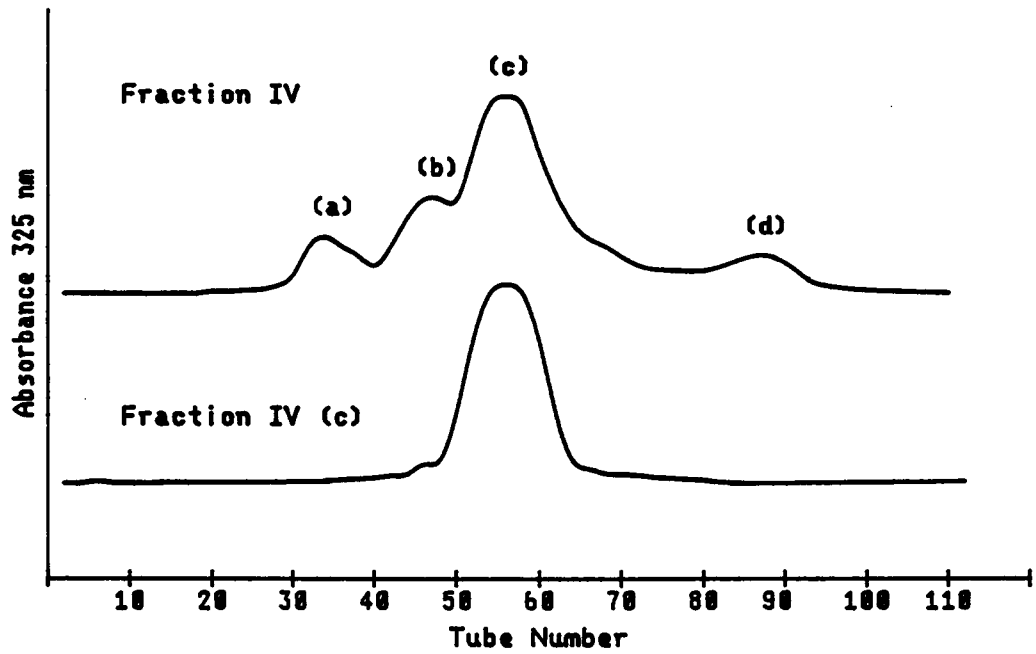


Figure 18. Elution profile of fraction IV and IV-c by chromatography on Sephadex LH-20. Conditions as in figure 9.

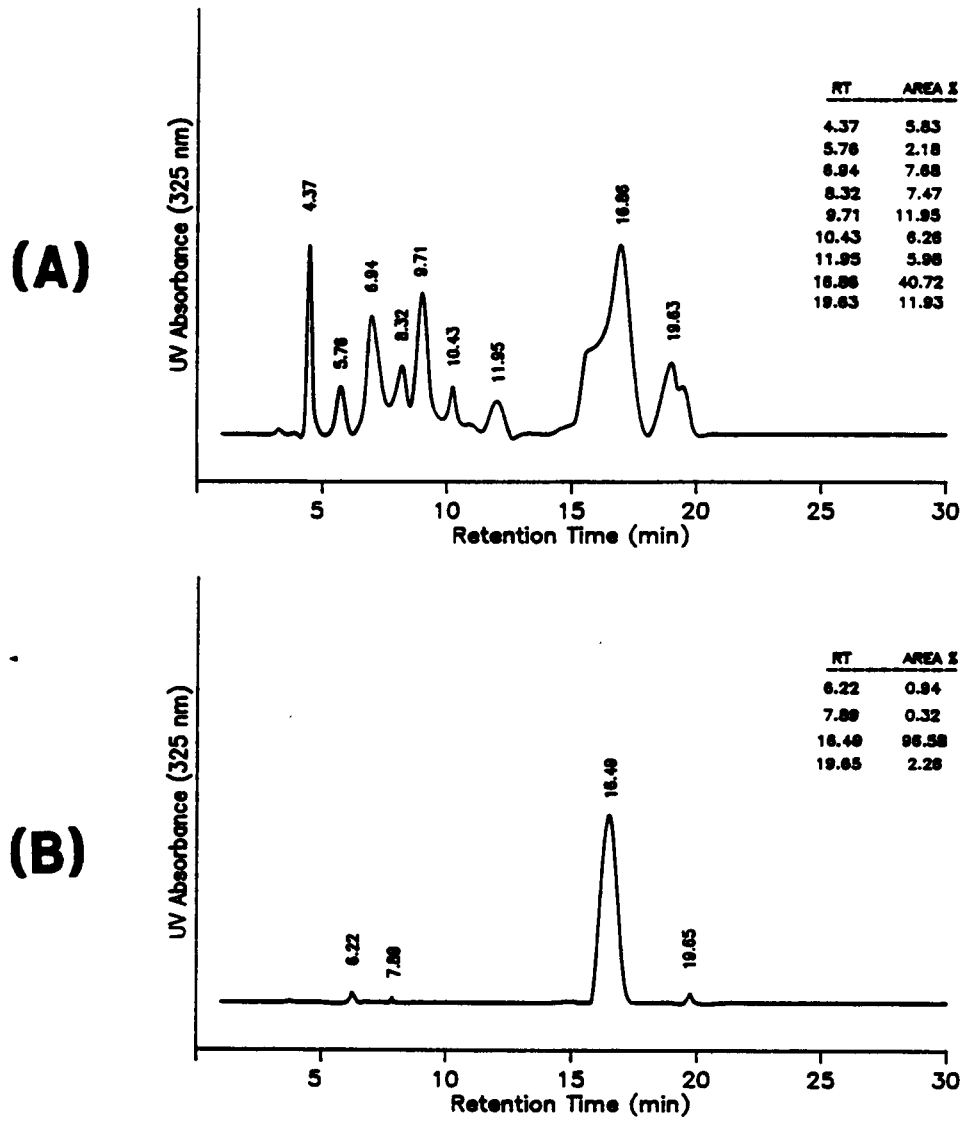


Figure 19. Reverse-phase HPLC chromatogram of (A) - crude fraction IV; (B) - purified fraction IV-c. Conditions as in figure 11.

standards. Monosaccharide composition of hydrolyzed fraction IV-c showed equal molar concentration of xylose and arabinose by HPLC (Fig 20), suggesting the presence of a feruloyl disaccharide. Gas chromatograph of non-reduced hydrolyzed fraction IV-c yielded both alpha- and beta- anomers of arabinose and xylose (Fig 21). Upon reduction followed by hydrolysis, arabinose is lost with the appearance of arabinitol (Fig 21). This indicated that the carbohydrate portion of fraction IV-c is a disaccharide with arabinose at the reducing end. Fraction IV-c was labeled FXA for ferulic acid, xylose, and arabinose. It is interesting to note that FXA (feruloyl disaccharide) has a longer retention time (16.5 min) on a C-18 RP-HPLC column than does FA (15.5 min). This is suprising since an additional sugar unit should make a feruloyl disaccharide more polar than a feruloyl monosaccharide. Apparently, some unique orientation of the disaccharide affects its polarity.

3. C-13 NMR Spectroscopy of FXA

The C-13 NMR spectrum of FXA showed 19 carbon signals (2 overlapping at 76 ppm) accounting for ferulic acid and 2 pentose sugars (Fig 22). The non-carbohydrate portion of the C-13 NMR spectrum for FXA was similar to that obtained for FA (Fig 14). All ten carbon signals were assigned and labeled thus confirming the presence of ferulate ester. Signal assignments are listed in Table 6 and were similar to published values for ferulic acid esters (Himmelsbach and Barton, 1980; Smith and Hartley, 1983; Kato and Nevins, 1985; Mueller-Harvey et al., 1986).

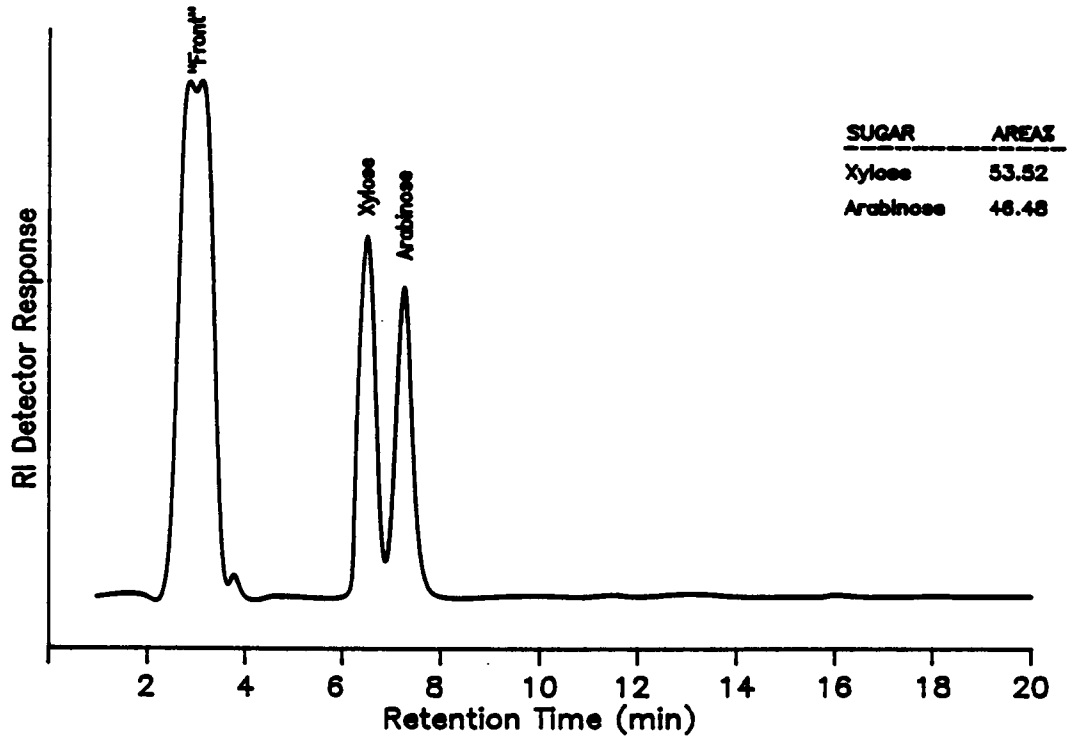


Figure 20. HPLC chromatogram of hydrolyzed fraction IV-c. Eluent, 80% acetonitrile/20% water; Flow rate, 1 ml/min; column, Lichrosorb NH_2; refractive index detector.

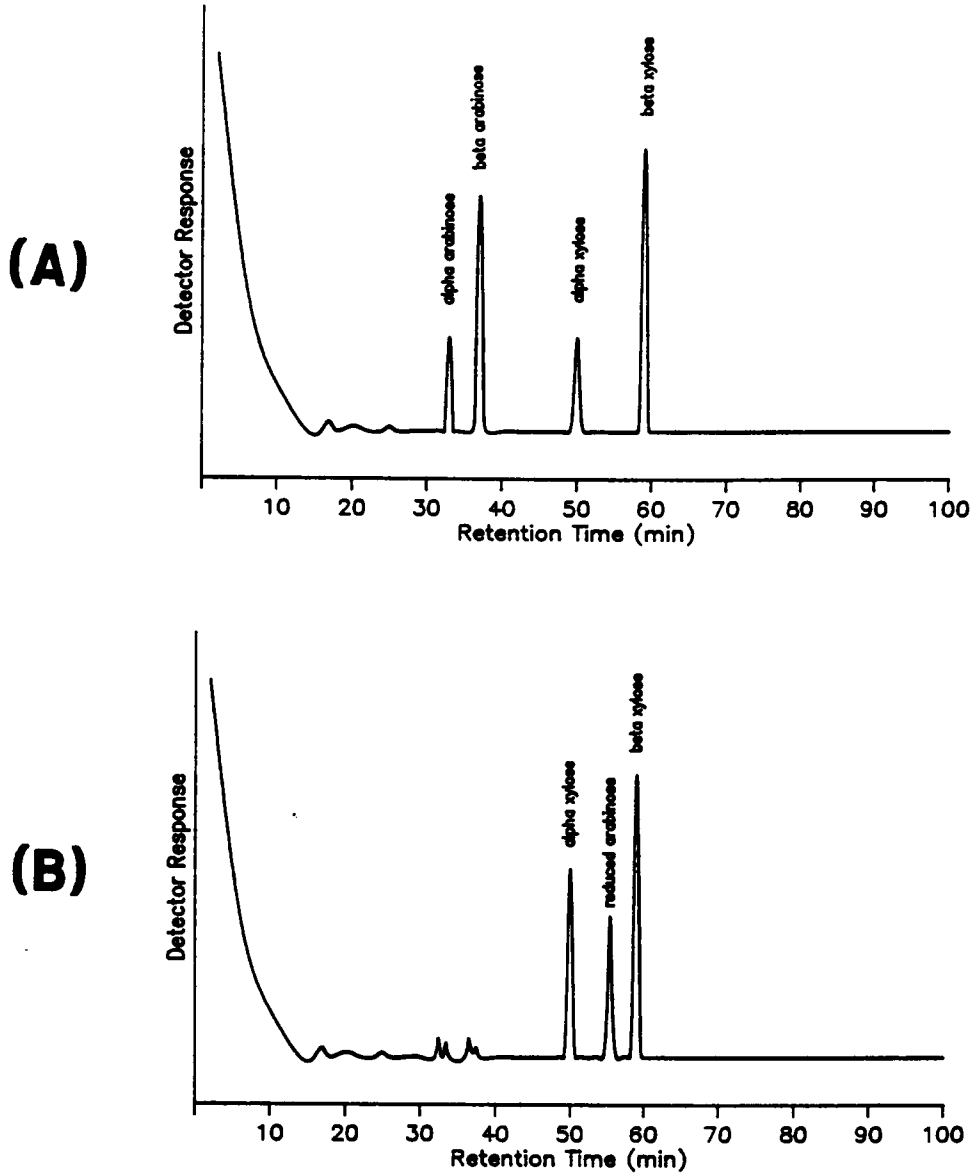


Figure 21. Gas chromatograph separation of TMS derivatives of: A - non-reduced hydrolyzed IV(c); B - reduced followed by hydrolysis. Sample, 1 µl (on-column injector); column, 30 m SPB-5 capillary (0.32 µm, 0.25 µm film); flame ionization detector.

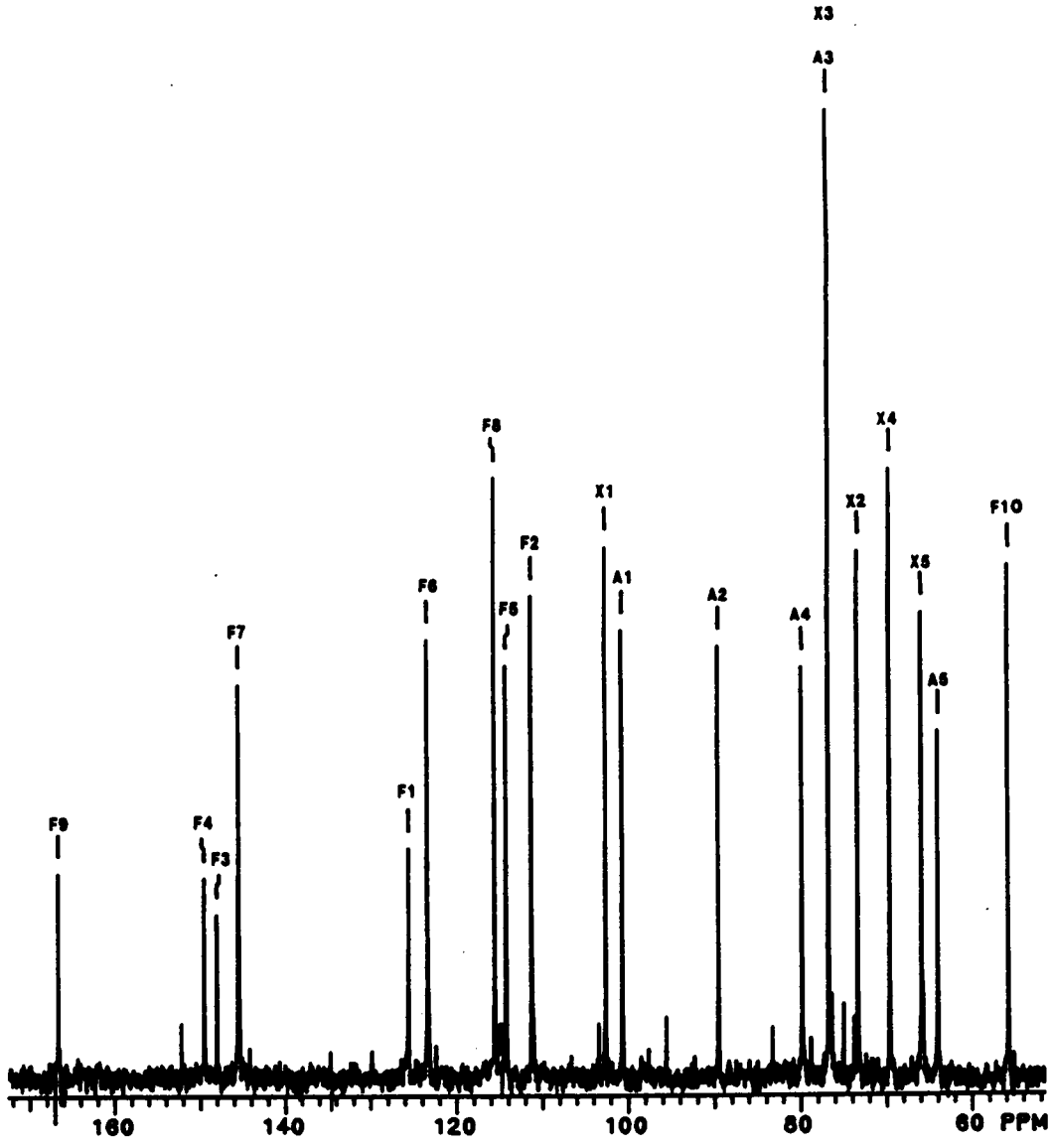


Figure 22. Carbon-13 (100 MHz) NMR spectrum of FXA recorded in DMSO at room temperature (F = ferulate ester, X = xylose, A = arabinose (see table 6 for signal assignments).

Table 6. Assignments of C-13 NMR sectra for FXA.[a]

Assignment	Ferulic acid	Ara*f*	β-Xyl*p*
C-1	125.50	100.61	102.54
C-2	111.16	89.46	73.25
C-3	147.95	76.62	76.62
C-4	149.43	79.75	69.53
C-5	114.14	63.90	65.80
C-6	123.79		
C-7	145.39		
C-8	115.48		
C-9	166.55		
C-10	55.70		

[a] Structure shown in Figure 27.

Figure 23 shows an APT C-13 NMR spectrum for the carbohydrate portion of FXA. Signals were assigned (Table 6) and labeled, accounting for two pentoses sugars. Signals in the high 70's - low 80's again indicated that arabinose is in the furanose form. Two C-5 carbon signals (upward signals) were detected at 65.8 and 63.9 ppm. The absence of signals at 60-61 ppm suggested that the C-5 signal of arabinofuranose is downfield shifted (63.9 ppm) due to the effect of an ester linkage. The C-5 signal of xylose (65.8 ppm) suggested that it is beta configuration (McEwan, et al 1982; Patrakova, 1983; Bock et al., 1984).

The C-13 NMR spectrum of FXA also provides information to help identify glycosidic linkage positions. Carbons which carry glycosidically linked oxygens show downfield shifts compared to carbons carrying hydroxyls (Bock et al., 1984). Depending on the sugar, ring form, and anomeric configuration of the glycosidic linkage, carbon signals can be downfield shifted from 3-9 ppm. The absence of signals at 107-108 ppm indicates the lack of a glycosidic linkage at C-1 of arabinofuranose which would give a 7-9 ppm downfield shift. The glycosidic linkage position of the disaccharide is easily detected since only one carbon signal is downfield shifted. The signal at 89 ppm is the C-2 signal of arabinofuranose downfield shifted due to the effect of a glycosidic linkage. This would suggest that xylose is attached to arabinose via a beta linkage (@ 6-7 ppm shift) and is consistent with the xylose C-5 signal indicating

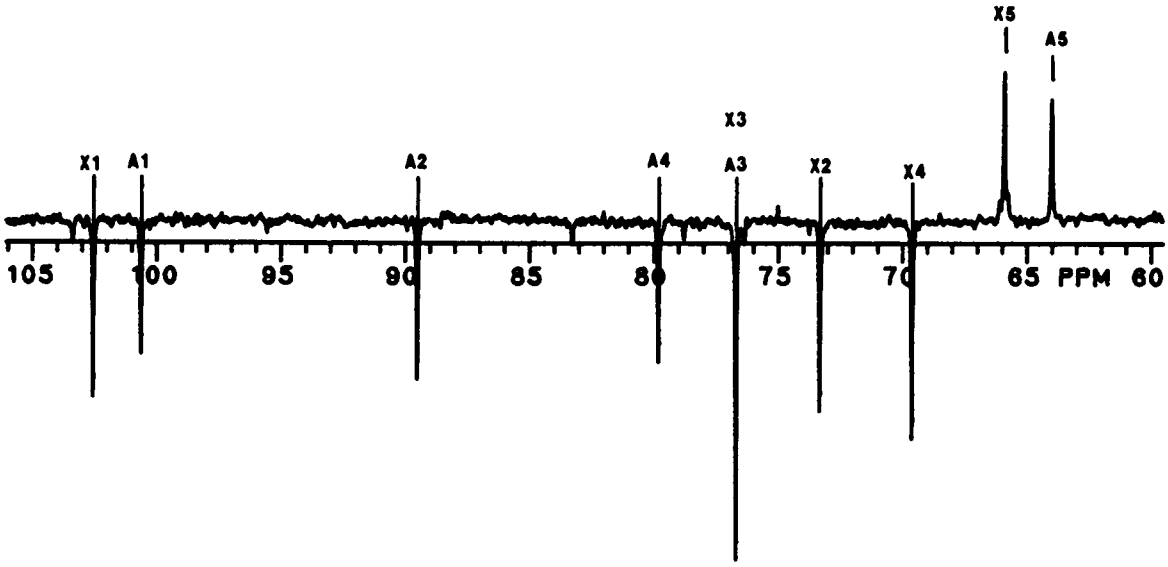


Figure 23. APT carbon-13 (100 MHz) NMR spectrum focusing on the carbohydrate region for FXA (X = xylose, A = arabinose). See table 6 for signal assignments.

beta-configuration. The absence of signals at 83-86 ppm suggest that xylose is not linked to the C-3 position of arabinose.

4. Proton NMR of FXA

Two anomeric peaks were detected in the proton spectrum (Fig 24): a beta proton (4.34 ppm), and an alpha proton or an anomeric proton of a furanose ring (5.1 ppm). This supported C-13 NMR data which suggests that xylose is in the beta anomeric configuration, and arabinose is in a furan ring form. The signals between 4.0 and 4.4 ppm suggests that ferulic acid is attached at arabinose C-5, and is consistent with the proton spectrum obtained for FA (Fig 19). The non-carbohydrate portion of the proton NMR spectra for FXA (5.5 - 7.5 ppm) showed close agreement with FA, and literature spectrums (Mueller-Harvey et al., 1986) for ferulic acid / carbohydrate esters. Again, coupling constants of 16 Hz for H-7 and H-8 (Table 7) indicates that the ferulate ester occurs as the *trans* isomer (Jackman and Sternhell, 1972).

Figure 25 shows the two-dimensional, H homonuclear, J-correlated spectroscopy (COSY) for the carbohydrate portion of FXA. Correlated proton signal assignments for arabinofuranose and xylose are illustrated at the top of the figure. A 2-DJ proton NMR spectrum of FXA is shown in Figure 26. This spectrum provides information about proton signal splitting (i.e. singlet, doublet, triplet, double doublet, etc.,), and coupling constants for the analyzed component. This information helps to assign proton signals, particularly in complex spectra. Chemical

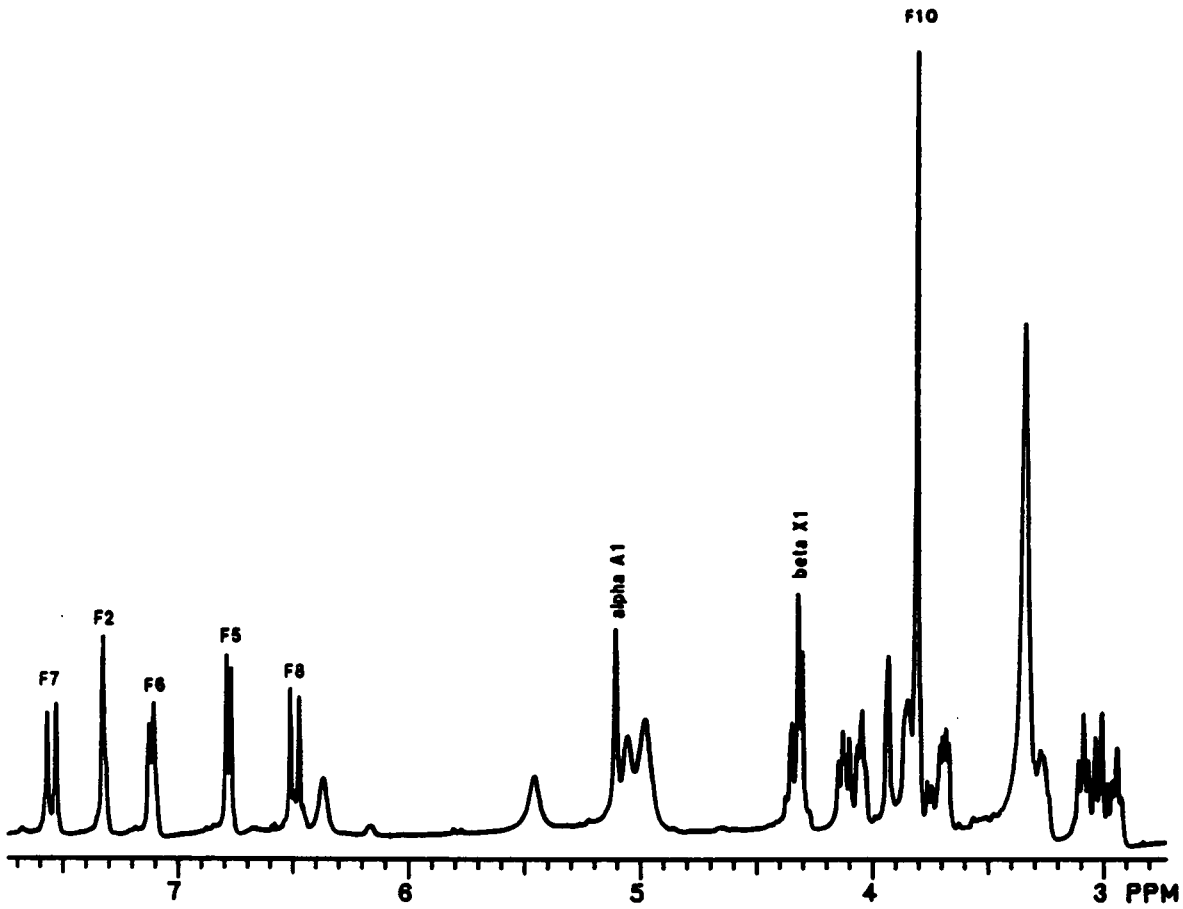


Figure 24. Proton (400 MHz) NMR spectrum of FXA recorded in DMSO at room temperature (F = ferulate ester, A = arabinose, X = xylose).

Table 7. Proton NMR chemical shifts and coupling constants (Hz, in parentheses) for FXA.[a]

Assignment	Ferulic acid	L-Ara*f*	β-D-Xyl*p*
H-1		5.10 (1.6)	4.33 (7.6)
H-2	7.33 (2.0)	3.95 (3.6)	2.95 (8.4)
H-3		3.86 (7.6)	3.12 (8.8)
H-4		4.05 (7.6)	3.27 (10.8)
H-5	6.78 (6.4)	4.14 (2.4)	3.60 (5.2)
H-5'		4.36 (11.6)	3.04 (10.8)
H-6	7.22 (6.4)		
H-7	7.55 (16.0)		
H-8	6.50 (16.0)		

[a] Structure shown in Figure 27.

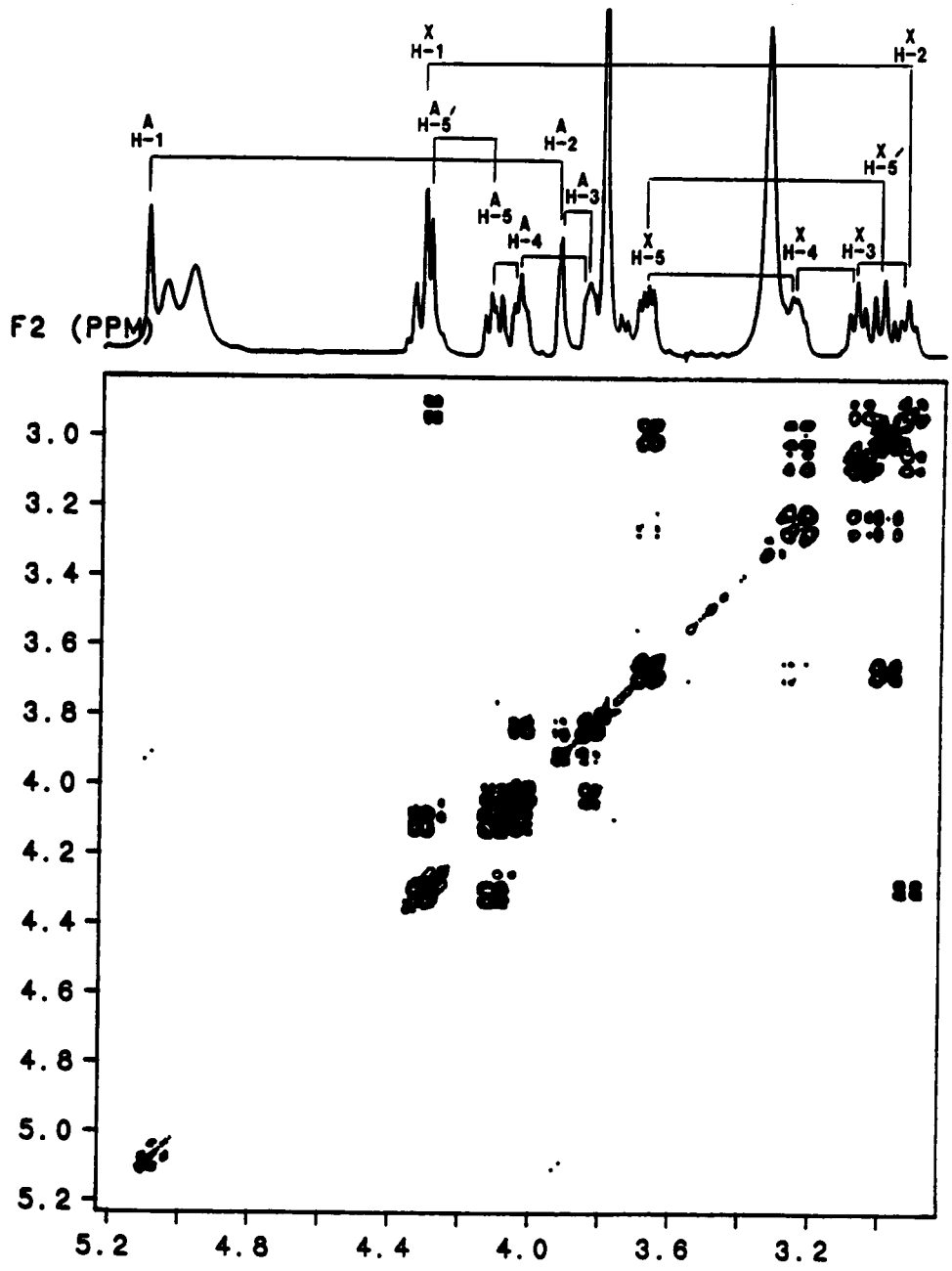


Figure 25. 2D-COSY proton (400MHz) NMR spectrum of FXA focusing on the carbohydrate spectral region. Connective lines illustrate correlated proton signals (X = xylose, A = arabinose). See table 7 for signal assignments and coupling constants.

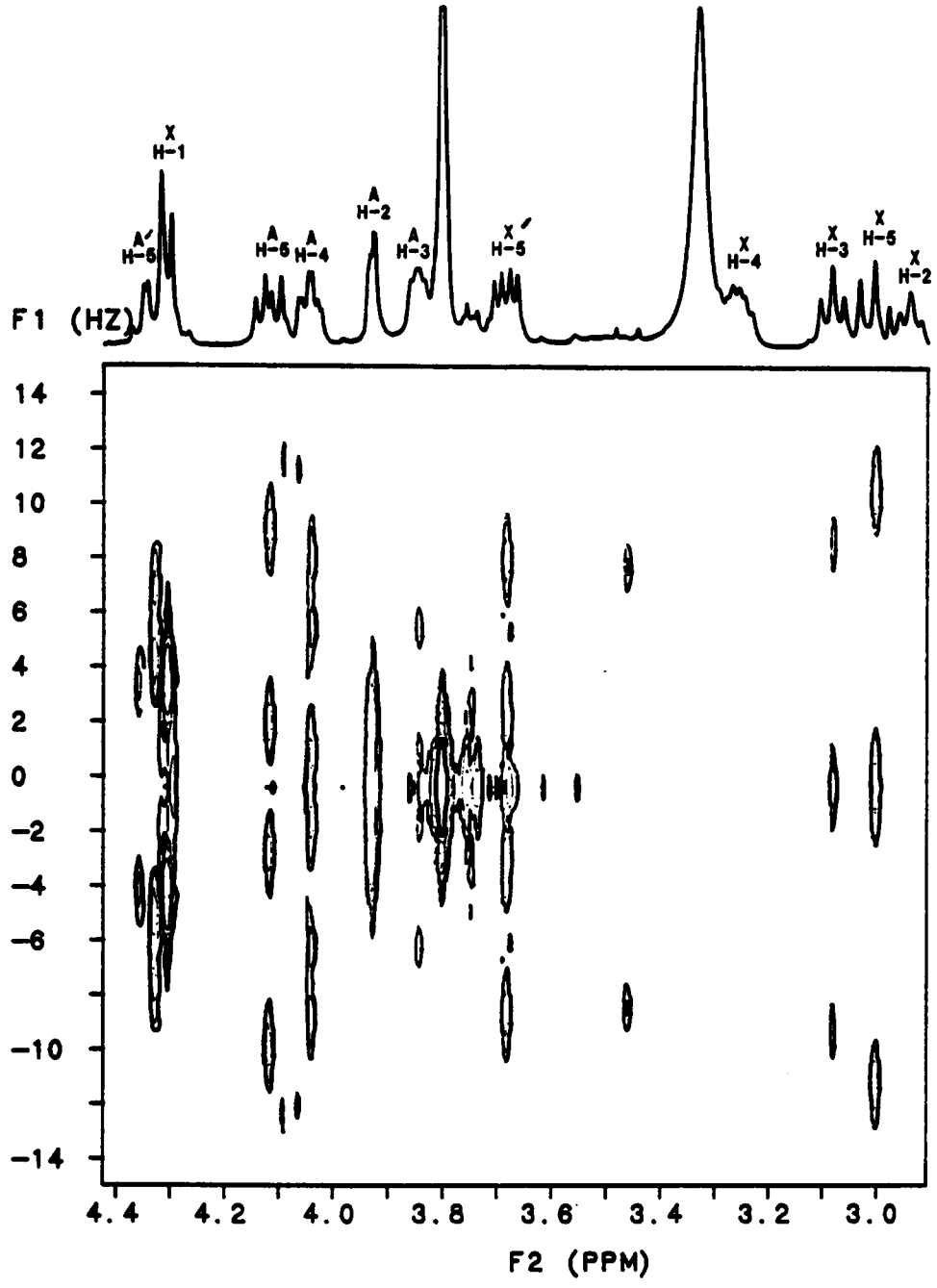


Figure 26. 2D-J proton (400 MHz) NMR spectrum of FXA (X = xylose, A = arabinose). See table 7 for signal assignments and coupling constants.

shifts and coupling constants for FXA are shown in Table 7.

5. Proposed structure of FXA

Combined results indicate that FXA is 2-O-β-xylopyranosyl-(5-O-*trans*-feruloyl arabinofuranose). The proposed structure of FXA is shown in Figure 27.

D. Characterization of Fraction III

1. Purification of Fraction III

Fraction III (Fig 9) was purified under the same conditions as fractions IV and V. Rechromatography of fraction III on Sephadex LH-20 yielded 5 sub-fractions labelled IIIa - IIIe (Fig 28). Isolation and RP-HPLC analysis of each fraction showed fraction III-d to be the cleanest fraction yielding a major peak which accounted for >80% of the total fraction. Fraction III-d was collected and re-chromatographed on Sephadex LH-20 (4x) to obtain one major peak (Fig 28) found to be >94% pure upon RP-HPLC analysis (Fig 29). Purification yielded approximately 46 mg of fraction III-d.

2. Compositional analysis of Fraction III-d

Fraction III-d showed a maximum UV absorption of 325 nm, and infrared bands with similarities to ferulate ester, FA, and FXA. Alkaline hydrolysis followed by RP-HPLC indicated the presence of ferulic acid as compared to retention times of phenolic acid standards. Monosaccharide composition of hydrolyzed fraction III-d showed equal molar concentration of xylose, arabinose, and galactose by HPLC, suggesting the presence of a trisaccharide

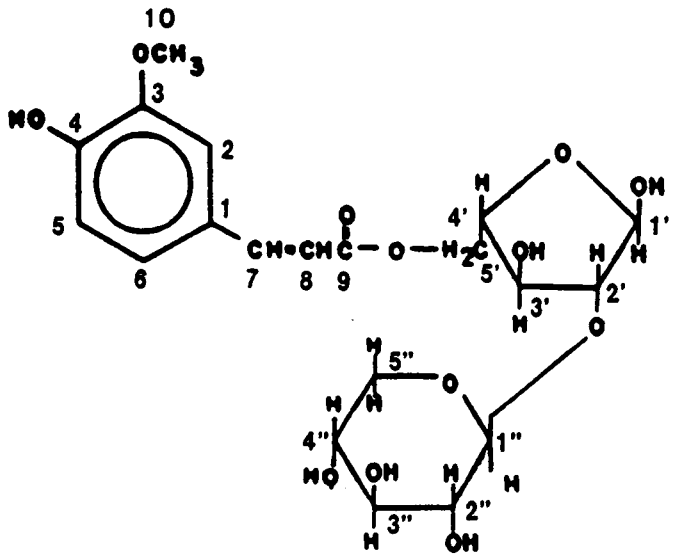


Figure 27. Proposed structure of FXA: 2-O-β-xylopyranosyl-(5-O-*trans*-feruloyl arabinofuranose).

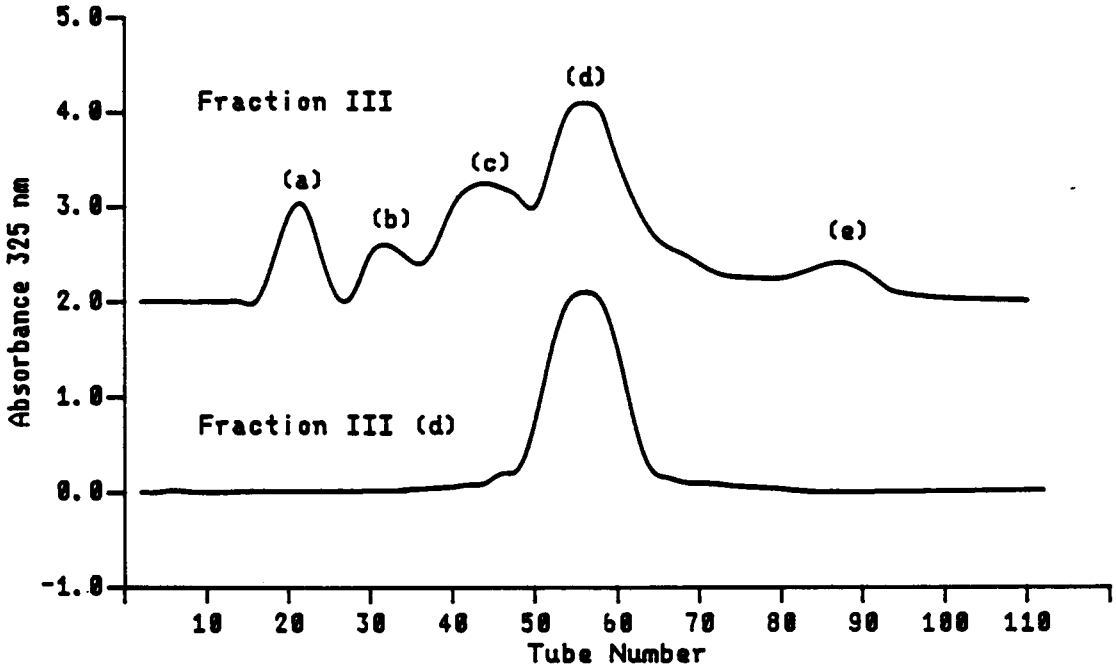


Figure 28. Elution profile of fraction III and III-d by chromatography on Sephadex LH-20. Conditions as in figure 9.

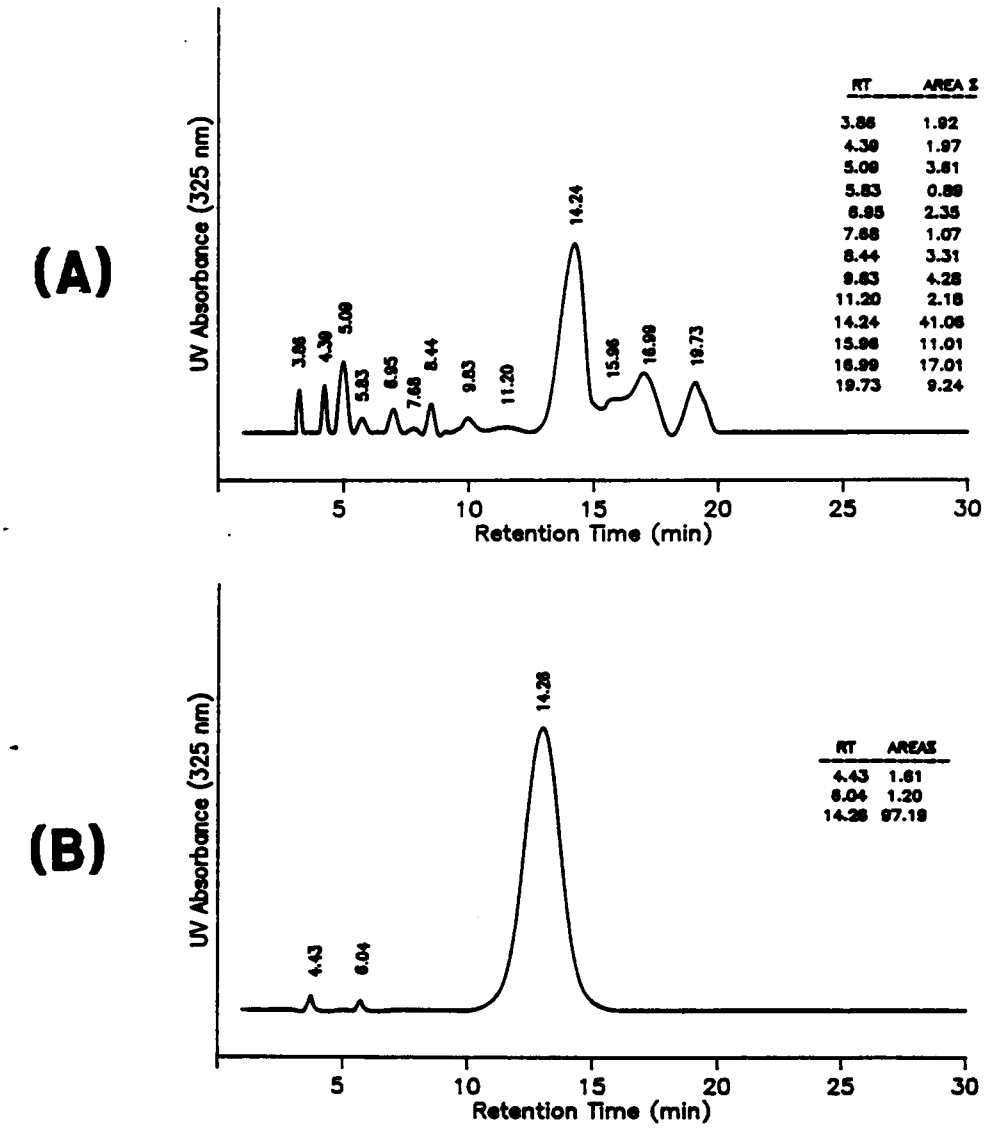


Figure 29. Reverse-phase HPLC chromatogram of (A) - crude fraction III; (B) - purified fraction III-d. Conditions as in figure 11.

(Fig 30). Upon reduction of fraction III-d followed by hydrolysis, arabinose is lost with the appearance of arabinitol as compared to non-reduced sample (Fig 31). This indicated that the carbohydrate portion of fraction III is a trisaccharide and arabinose is the reducing end sugar. Past work involving methylation analysis of corn hull hemicellulose (Table 1) showed all galactose occupied terminal positions (Whistler and BeMiller, 1956), tentetively suggesting galactose in fraction III-d is probably at the non-reducing end. Fraction III-d was labeled FGXA for ferulic acid, galactose, xylose, arabinose.

3. Enzyme Treatment of FGXA

FGXA was treated with alpha- and beta-galactosidase in attempt to remove the non-reducing terminus sugar and aid in the confirmation of the trisaccharide sugar sequence. After 24h incubation, both alpha- and beta-galactosidase treatments resulted in a slight decrease in FGXA, with the appearance of a new peak with a retention time of 20 min (Fig 32a, b). Comparison of retention times against known standards (ferulic acid, feruloyl mono, di, trisaccharides) showed the new hydrolysate peak to have a retention time identical with the peak for ferulic acid (Fig 32b). It appears both galactosidases contain a contaminating esterase that removes some of the ferulic acid ester. GC analysis (TMS) of FGXA after galactosidase treatment showed little appearance of galactose indicating possible steric hindrance of the enzyme due to the ferulate ester.

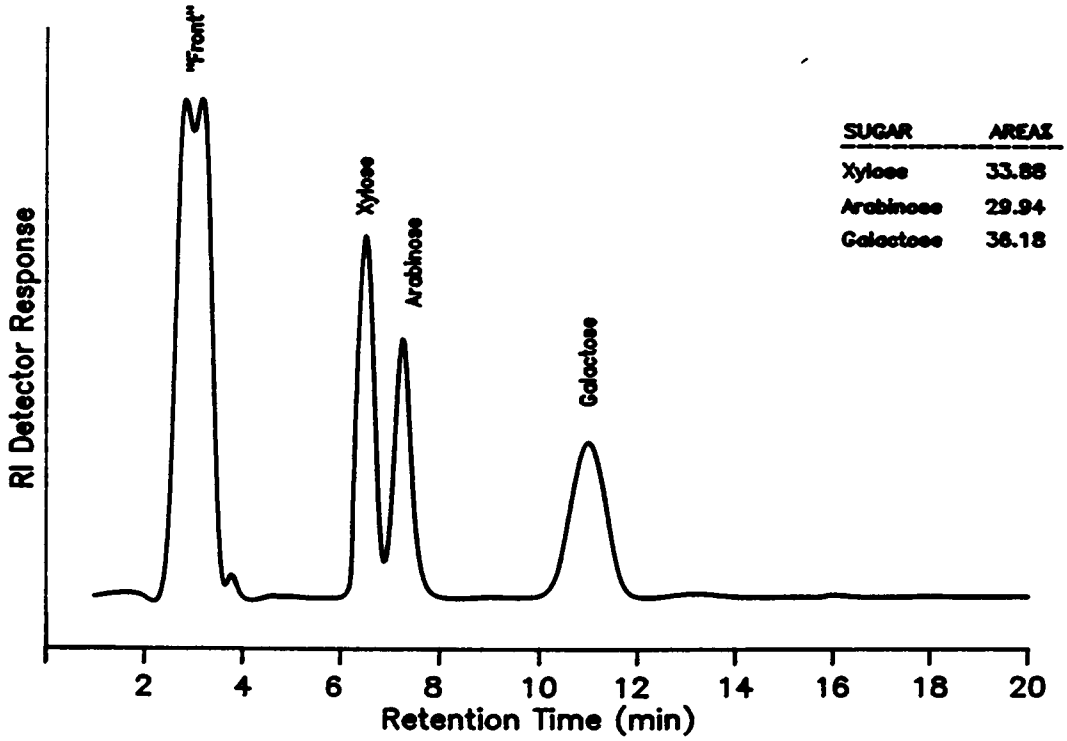


Figure 30. HPLC chromatogram of hydrolyzed fraction III-d. Eluent, 80% acetonitrile/20% water; Flow rate, 1 ml/min; column, Lichrosorb NH_2; refractive index detector.

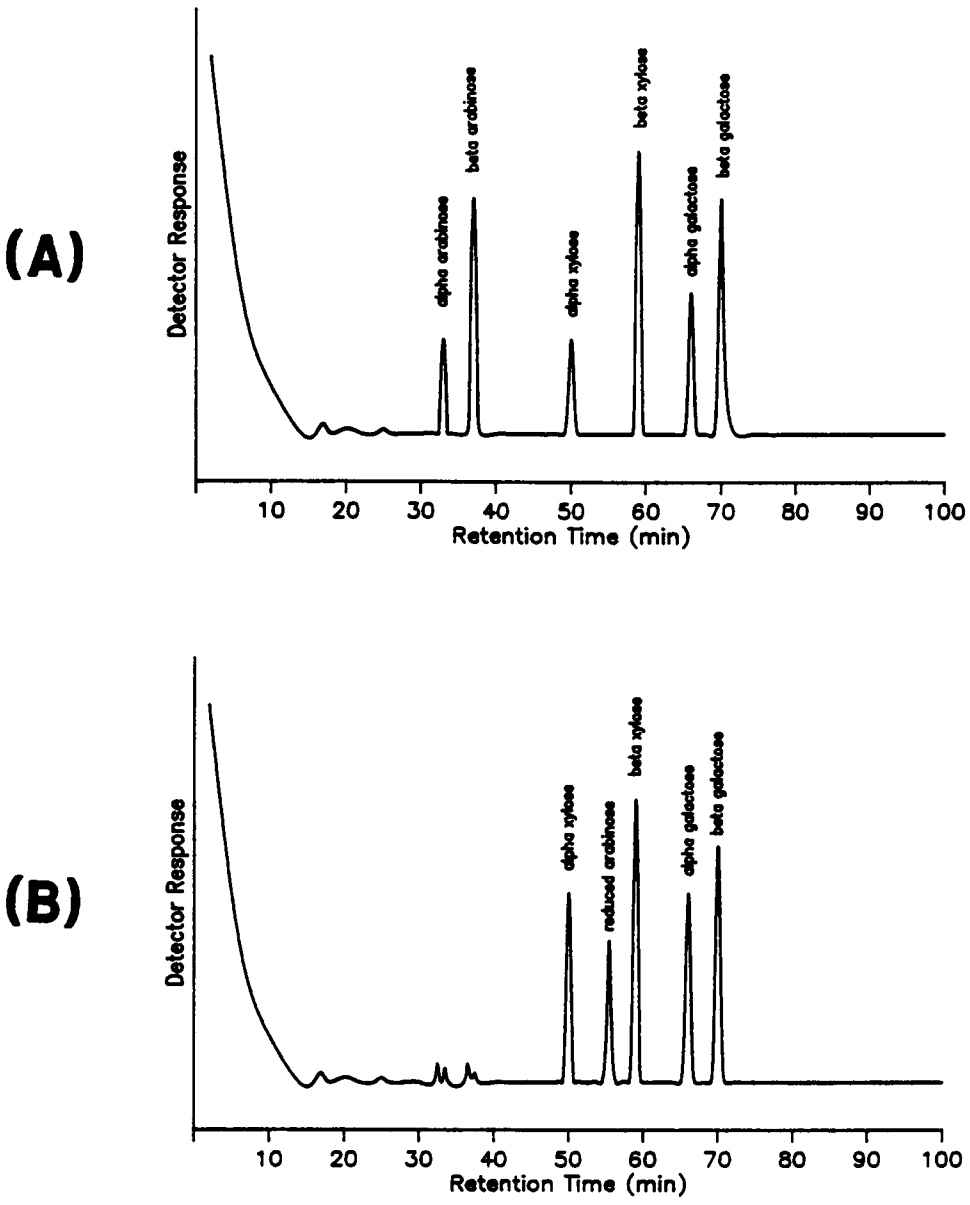


Figure 31. Gas chromatograph separation of TMS derivatives of: A - non-reduced hydrolyzed III-d; B - reduced followed by hydrolysis. Conditions as in figure 21.

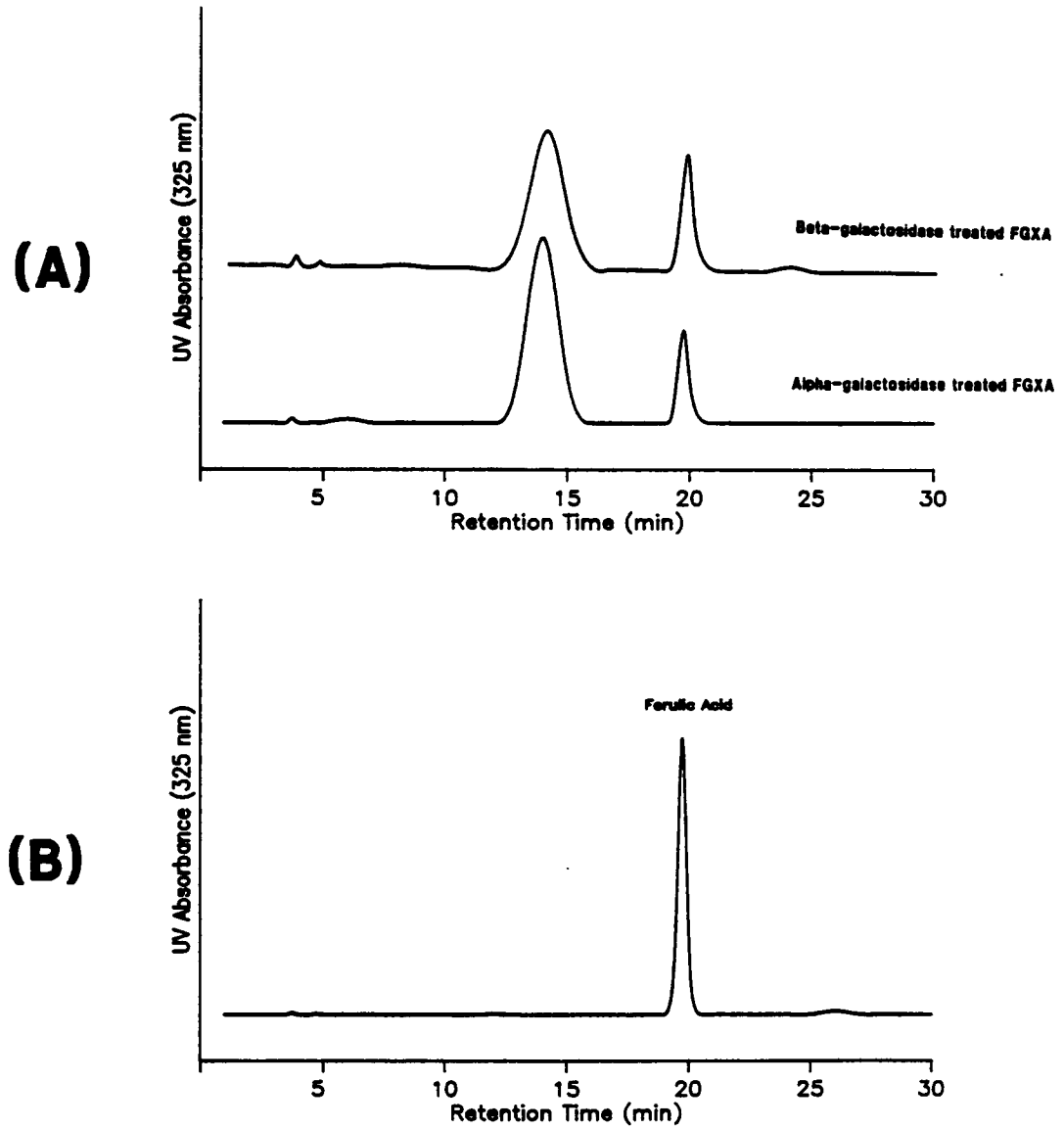


Figure 32. RP-HPLC analysis of: (A top) beta-galactosidase treated FGXA (16 h); (A bottom) alpha-galactosidase treated FGXA (16 h); (B) ferulic acid standard. Conditions as in figure 11.

4. Isolation of GXA

Ferulic acid was removed from FGXA by using either alkaline treatment to saponify the ester, or by hydrolysis using an esterase enzyme. Alkaline treatment was very effective in removing ferulic acid, however, such treatment also effected the reducing end sugar producing a variety of isomerization type products. Alteration of the isolated trisaccharide made comparisons to the feruloyl trisaccharide difficult. Carboxyl esterase treatment of FGXA was effective in removing ferulic acid (Fig 33) without altering the trisaccharide. Liquid chromatography of the hydrolysate mixture (Biogel P-2) was effective in separating ferulic acid, enzyme, and trisaccharide (Figure 34). HPLC analysis of the trisaccharide yielded one peak, and monosaccharide analysis showed equal proportions of arabinose, xylose, and galactose indicating high purity of the isolated trisaccharide.

5. Galactosidase treatment of GXA

Purified GXA was subjected to alpha- and beta-galactosidase treatment to confirm anomeric configuration and sequential position of galactose in the trisaccharide. GC analysis (TMS) of the hydrolysis mixtures showed that beta-galactosidase had little effect on the trisaccharide (Fig 35-a), while alpha-galactosidase showed release of galactose (Fig 35-b). These results indicated that galactose is in the alpha anomeric configuration, linked to xylose at the non-reducing end of the trisaccharide. Also, the increase in enzyme activity after ferulic acid removal suggests

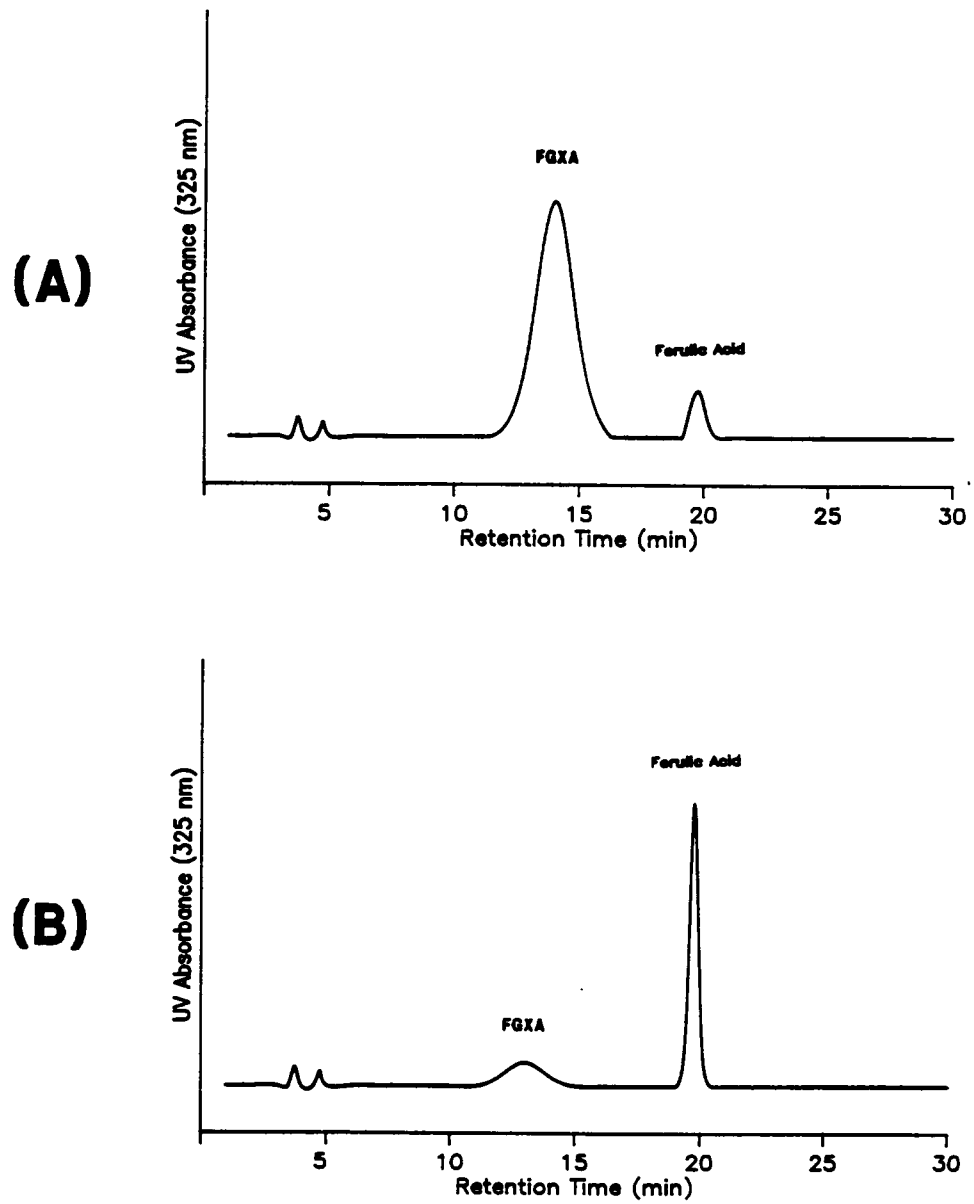


Figure 33. Time course analysis via RP-HPLC of ferulate ester removal from FGXA by carboxyl esterase at (A) 3 h, (B) 18 h. Conditions as in figure 11.

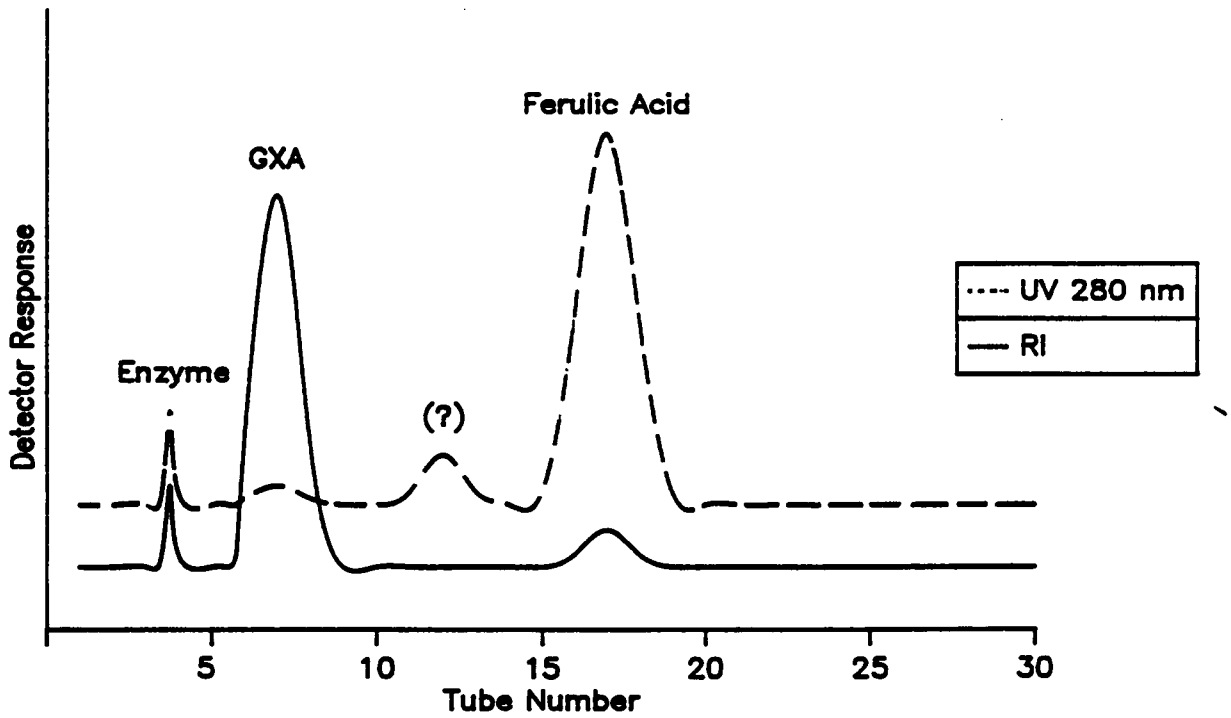


Figure 34. Elution profile of carboxyl esterase treated FGXA by chromatography on Biogel P-2. Sample, 2 ml; bed volume, 35 ml; flow rate, 30 ml/h; eluent, 0.1N acetic acid. Dashed line indicates absorbance at 280 nm; solid line indicates refractive index detection.

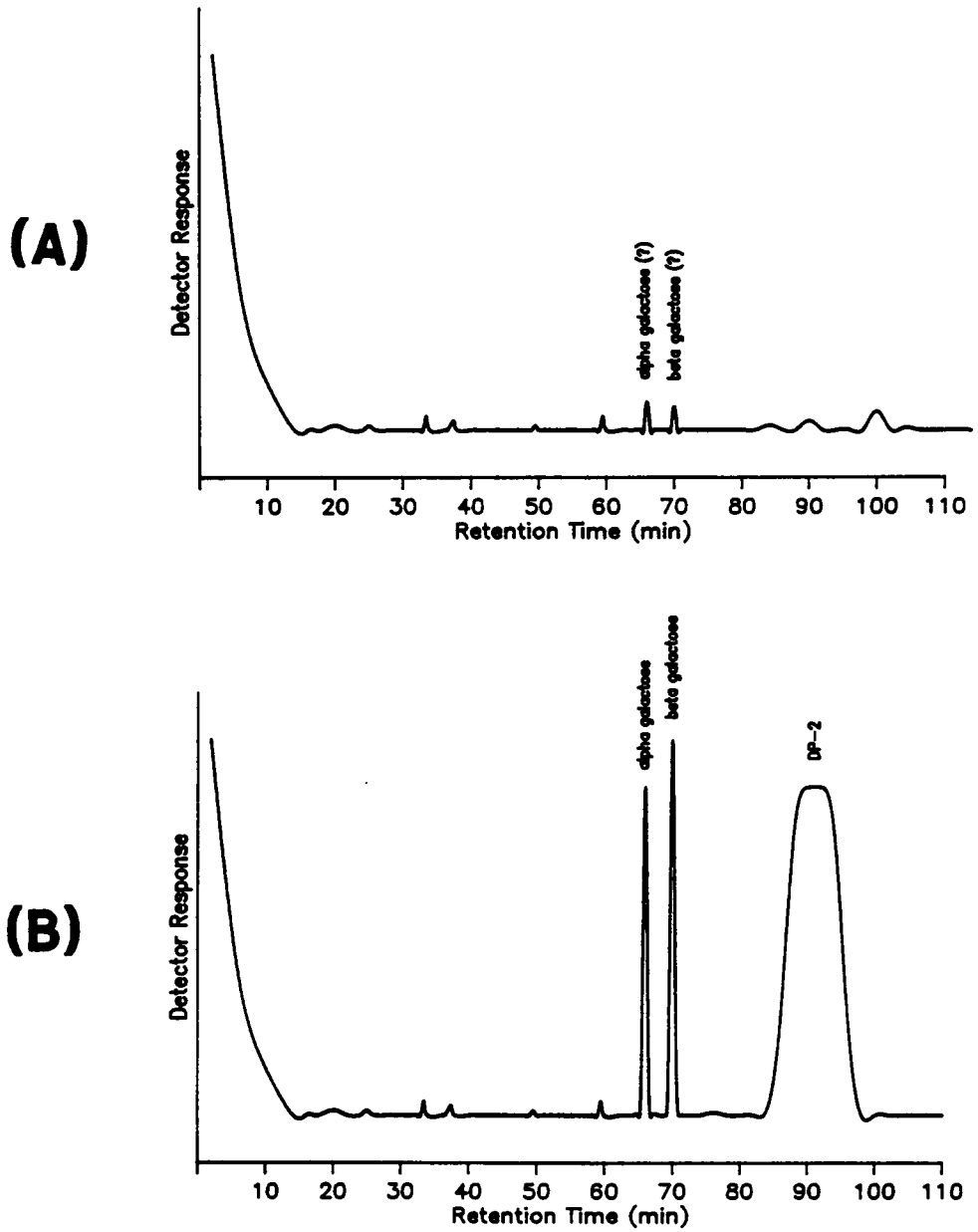


Figure 35. Gas chromatograph separation of TMS derivatives of: A - beta- galactosidase treated GXA; (B) alpha-galactosidase treated GXA. Conditions as in figure 21.

that ferulic acid is attached to galactose.

6. C-13 NMR analysis of FGXA

NMR spectroscopy was employed to aid in structural elucidation of FGXA. The non-carbohydrate portion of the C-13 NMR spectrum for FGXA is similar to that obtained for FA and FXA (Fig 36). All ten carbon signals were assigned and labeled for ferulate esters (Table 8) and agreed with published values (Himmelsbach and Barton, 1980; Smith and Hartley, 1983; Kato and Nevins, 1985; Mueller-Harvey et al., 1986) confirming the presence of ferulic acid.

Figure 37 shows an APT C-13 NMR spectrum for the carbohydrate portion of FGXA. Sixteen carbon signals plus a methyl signal (56 ppm) were detected accounting for two pentoses and one hexose sugar. The anomeric carbon signals (100 -105 ppm) were well resolved from skeletal carbons as are the C-5 and C-6 carbons (60-67 ppm). Signals in the high 70's - low 80's indicated that arabinose is in the furan ring form.

The C-5 and C-6 carbon signals (upward signals) are unequivocally detected in the APT spectrum, aiding in signal assignment. Literature values of C-13 NMR signals for both C-5 of arabinofuranose and C-6 of galactose reside at about 61-62 ppm (Bock et al, 1984; Jansson et al., 1987). The APT C-13 spectrum of FGXA clearly showed only one signal at 61 ppm indicating that either arabinose C-5 or galactose C-6 is downfield shifted due to the effect of an ester linkage. Upon reduction of the feruloyl trisaccharide the signal at 61 ppm is lost (Fig 38) with the

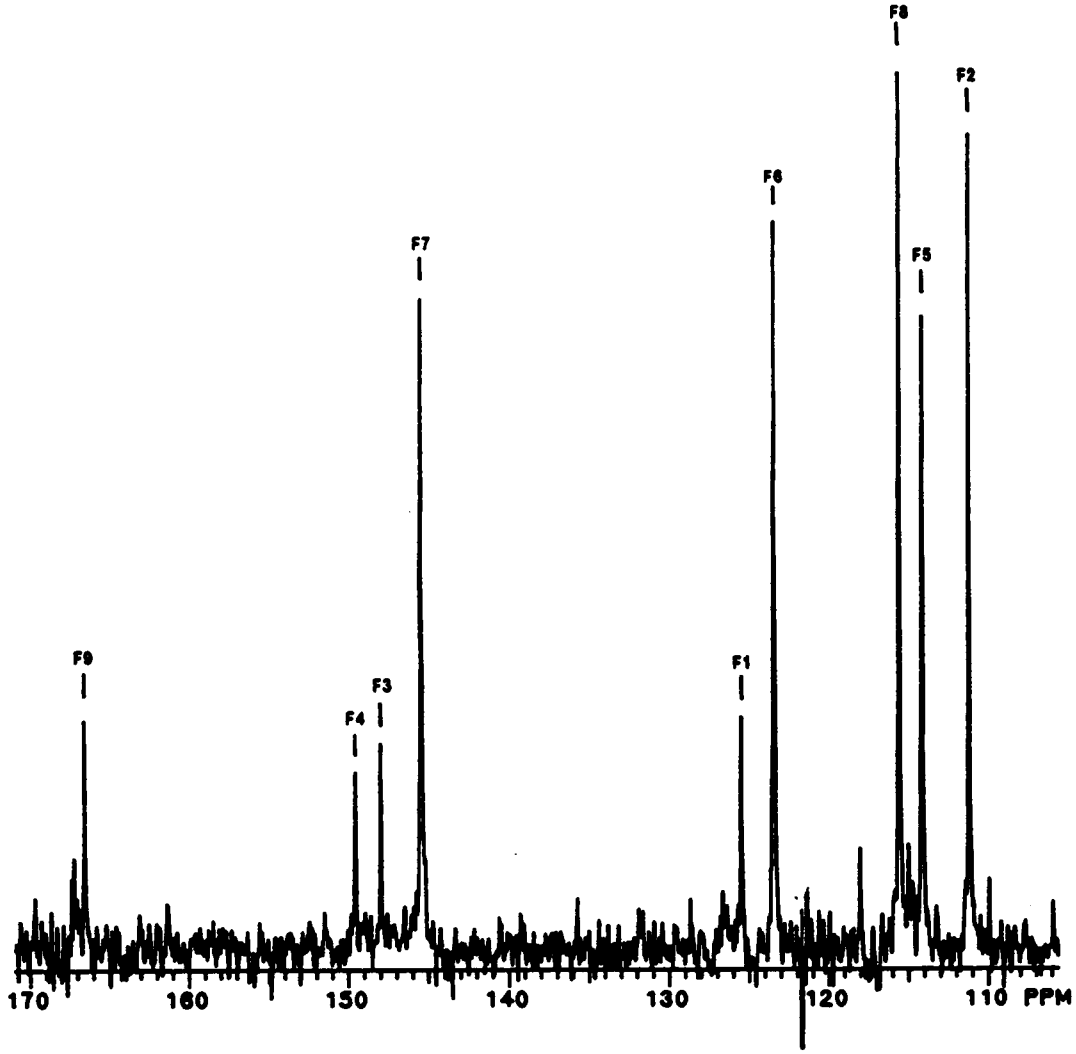


Figure 36. Carbon-13 (100 MHz) NMR spectum of FGXA recorded in DMSO (room temperature) focusing on the non-carbohydrate spectral region (F = ferulate ester). See table 8 for signal assignments.

Table 8. Assignments of C-13 NMR sectra for FGXA.[a]

Assignment	Ferulic acid	Araf	β-Xylp	α-Galp
C-1	125.34	100.26	101.17	100.26
C-2	111.11	89.84	71.17	68.79
C-3	147.96	76.71	76.48	69.63
C-4	149.55	80.06	79.11	69.34
C-5	114.03	60.54	65.55	69.18
C-6	123.34			64.20
C-7	145.41			
C-8	115.48			
C-9	166.54			
C-10	55.67			

[a] Structure shown in Figure 43.

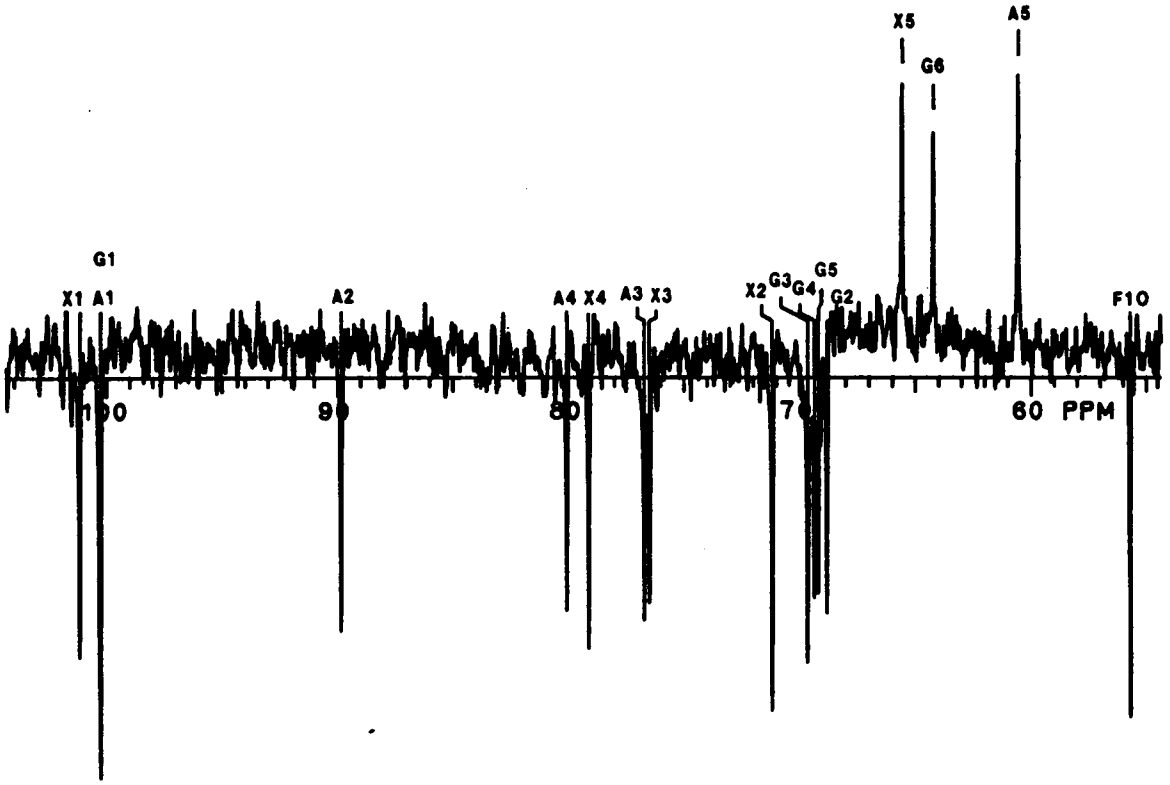


Figure 37. APT carbon-13 (100 MHz) NMR spectrum focusing on the carbohydrate region for FGXA (X = xylose, A = arabinose, G = galactose; see table 8 for signal assignments.

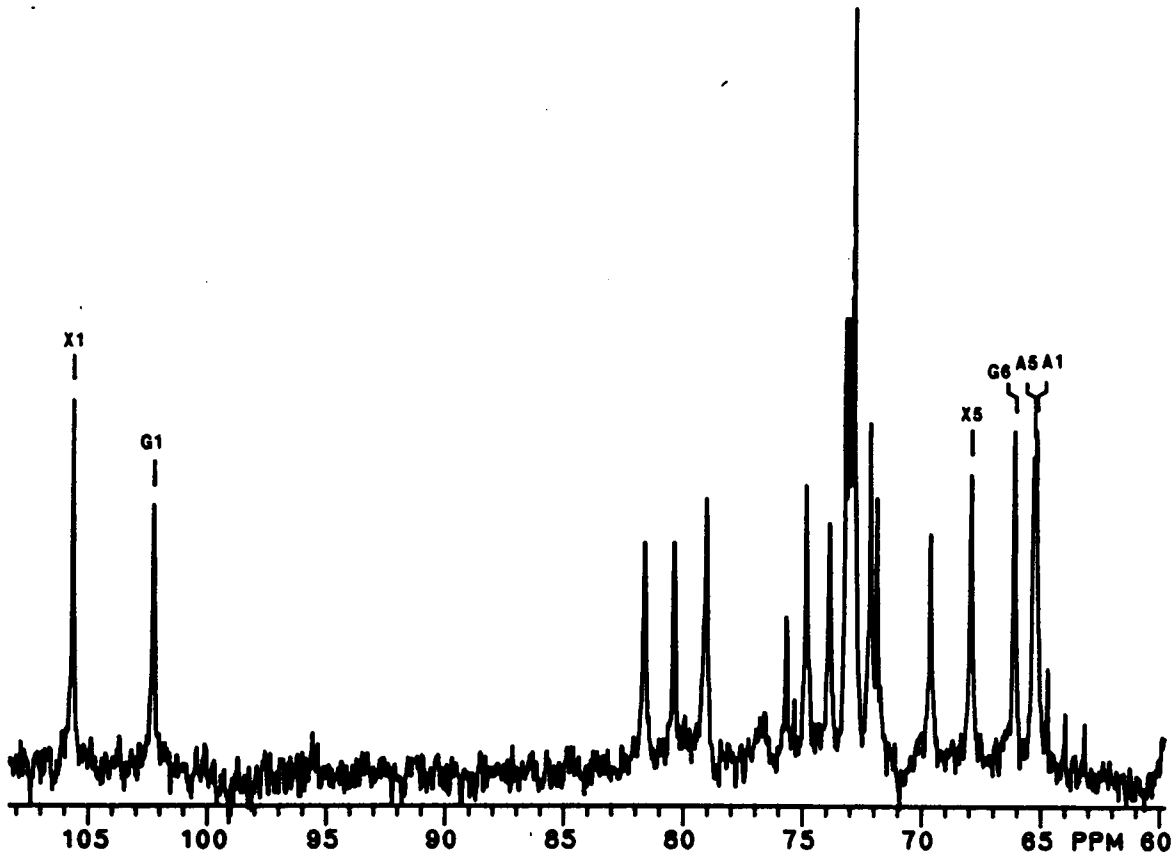


Figure 38. Carbon-13 (100 MHz) NMR spectrum focusing on the carbohydrate region for reduced FGXA (X = xylose, A = arabinose, G = galactose).

appearance of overlapping signals at 65 ppm. This is indicative of C-1 and C-5 signals of arabinitol (Bock et al., 1984) since both are equivalent. This indicated ferulic acid was ester linked to the C-6 position of galactose due to the downfield shift and absence of signals at 61-62 ppm. A C-5 substitution of arabinitol would have a 3-6 ppm downfield shift and not overlap with C-1. The position of the C-5 xylose signal suggested it is in the beta configuration.

The signal at 89 ppm (Fig 37) is the the C-2 signal of arabinofuranose downfield shifted due to the effect of a glycosidic linkage, and is identical to the C-2 glycosidic linkage of FXA (Fig 23). This indicated that xylose is attached to arabinose via a beta linkage (i.e. 6-7 ppm shift) and agreed with the xylose C-5 signal indicating beta-configuration.

The carbon signal at 79 ppm (Fig 37) was assigned for C-4 of xylose and is consistent with a 9-10 ppm downfield shift due to an alpha glycosidic linkage (Bock et al., 1984). The C-13 signals supported results of enzyme treatment which indicate galactose is glycosidically linked alpha-(1-4) to xylose. The alpha configuration of galactose was further supported by the four skeletal carbon signals at 69-70 ppm, and the absence of beta galactose skeletal carbon signals at 72-75 ppm.

7. Proton NMR of FGXA

Figure 39 shows the proton spectrum for FGXA. Proton signals for the non-carbohydrate portion of the FGXA NMR spectra (5.5 - 7.5 ppm) are consistent with ferulate ester signals

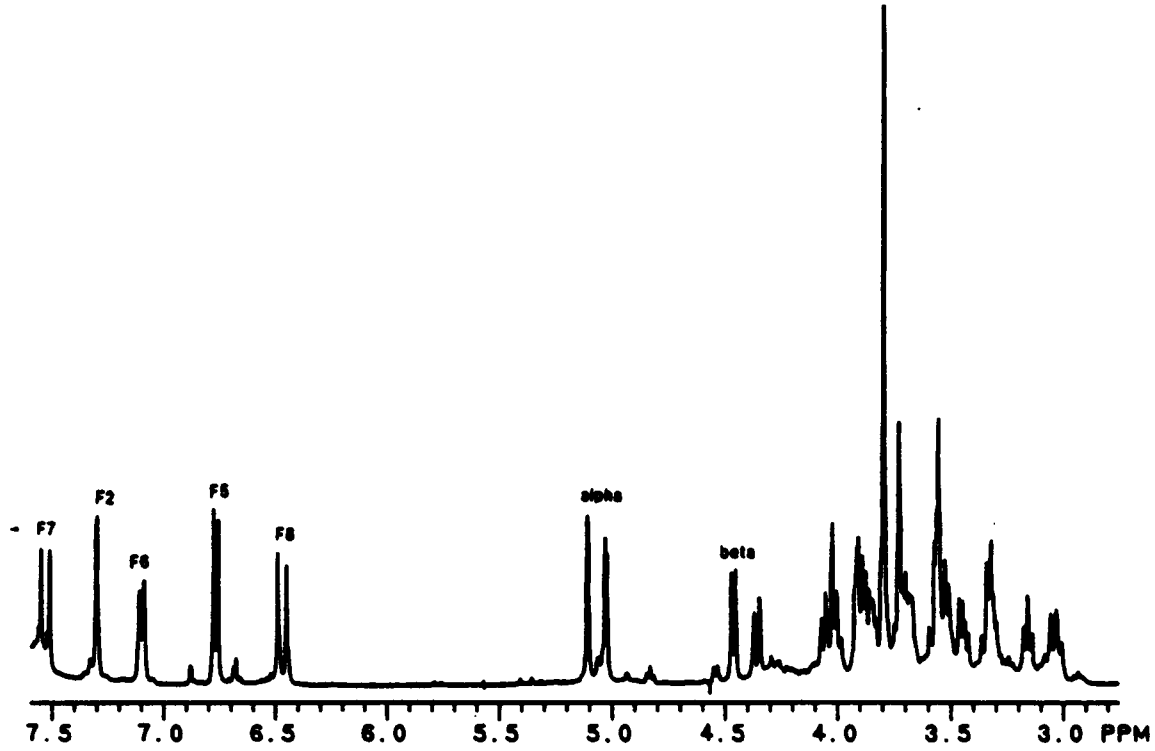


Figure 39. Proton (400 MHz) NMR spectrum of FGXA recorded in DMSO at room temperature (F = ferulate ester, A = arabinose, X = xylose, G = galactose).

obtained for FA, FXA, and literature values (Mueller-Harvey et al., 1986) for ferulic acid / carbohydrate esters. Again, coupling constants of 16 Hz for H-7 and H-8 (Table 9) indicated that the ferulate ester occurs as the *trans* isomer (Jackman and Sternhell, 1972).

Figure 40 shows the two-dimensional, H homonuclear, J-correlated spectrum (COSY), and Figure 41 shows the 2D-J proton NMR spectrum for the carbohydrate portion of FGXA. Three anomeric peaks were easily detected in the spectrum: a beta proton (4.4 ppm), an alpha proton (5.0 ppm), and the anomeric proton of the furanose ring (5.1 ppm). This is consistent with the C-13 data which suggested beta-xylose, alpha- galactose, and arabinofuranose. The lack of signals between 4.0 and 4.4 ppm suggested that ferulic acid was not attached at arabinose C-5 as compared to the proton spectrum obtained for FA and FXA. A comparison of chemical shifts and coupling constants (Table 9) for galactose in FGXA and mono-O-acetylated methyl alpha- and beta- galactopyranosides (Jansson et al., 1987) indicated that signals at 4.36 and 3.95 are indicative of an ester linkage at galactose C-6. Upon removal of ferulic acid (carboxylesterase) these signals are lost (Fig 42), supporting results which indicate galactose C-6 as the point of ferulic acid attachment.

Table 9. Proton NMR chemical shifts and coupling constants (Hz, in parentheses) for FGXA.[b]

Assignment	Ferulic acid	L-Ara*f*	β-D-Xyl*p*	α-D-Gal*p*
H-1		5.13 (1.6)	4.46 (7.2)	5.03 (2.8)
H-2	7.30 (2.0)	3.92 (4.0)	3.16 (8.0)	3.58 (10.2)
H-3		3.89 (6.4)	3.34 (8.4)	-[a] -[a]
H-4		3.84 (6.4)	3.53 (10.4)	3.72 (3.6)
H-5	6.78 (8.0)	3.43 (3.6)	3.78 (5.2)	4.04 (8.0)
H-5'		3.58 (10.4)	3.06 (10.4)	
H-6	7.12 (8.4)			4.00 (3.6)
H-6'				4.37 (10.4)
H-7	7.53 (16.0)			
H-8	6.46 (16.0)			

[a] Not obtained owing to complexity of the spectrum.
[b] Structure shown in Figure 43.

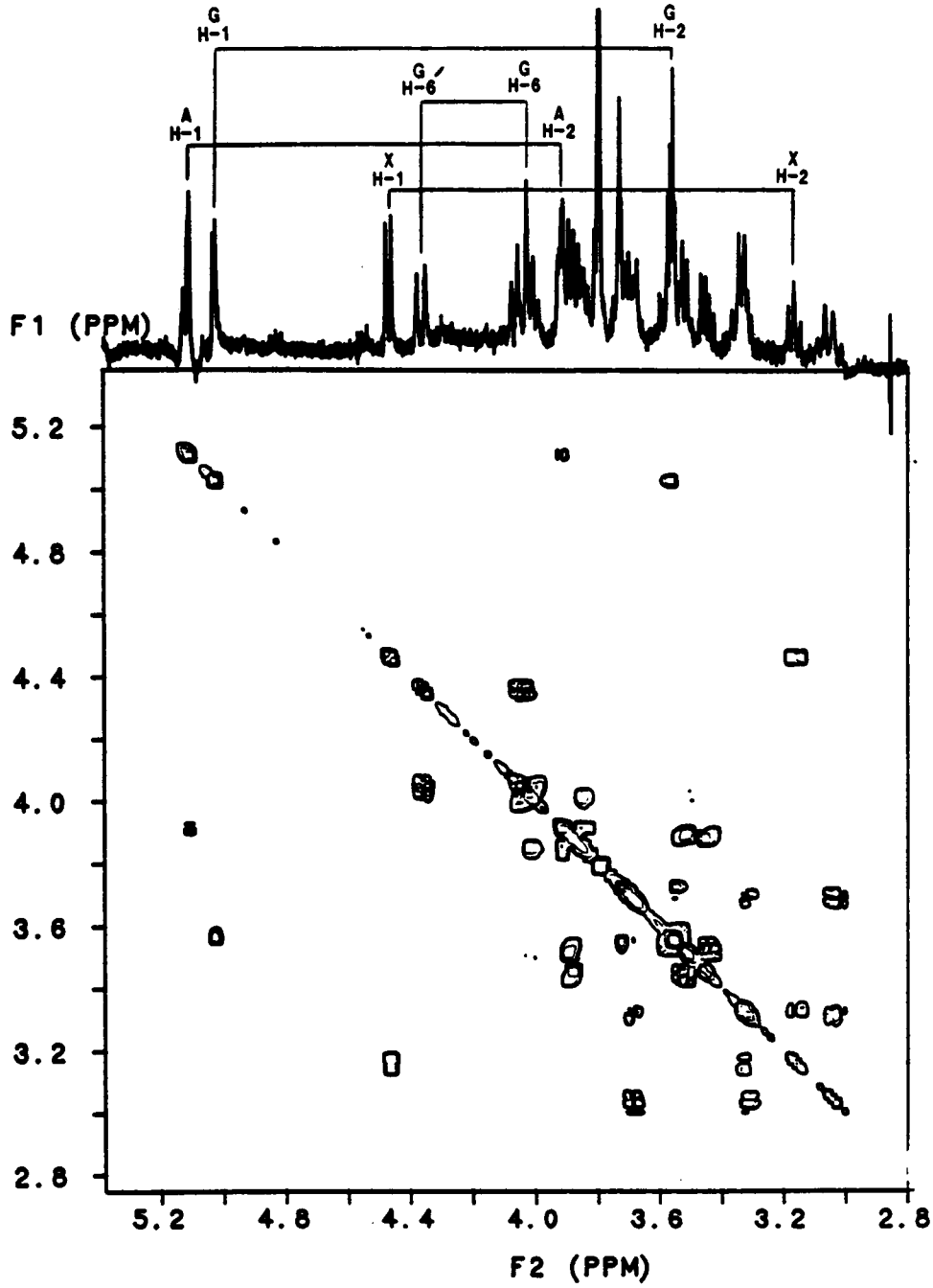


Figure 40. 2D-COSY proton (400MHz) NMR spectrum of FGXA focusing on the carbohydrate spectral region. Connective lines illustrate correlated proton signals (X = xylose, A = arabinose, G = galactose). See table 9 for signal assignments and coupling constants.

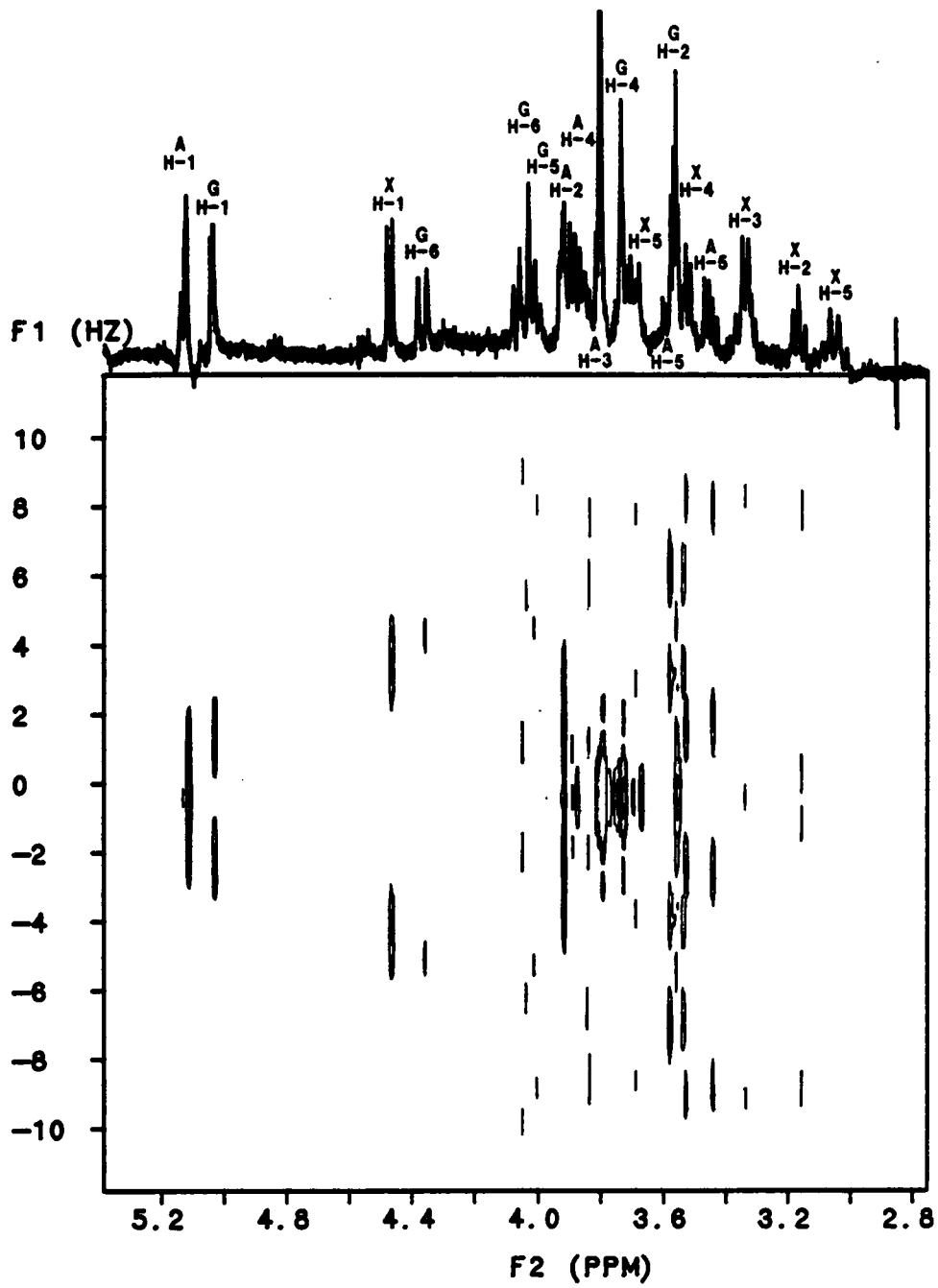


Figure 41. 2D-J proton (400 MHz) NMR spectrum of FGXA (X = xylose, A = arabinose, G = galactose). See table 9 for signal assignments and coupling constants.

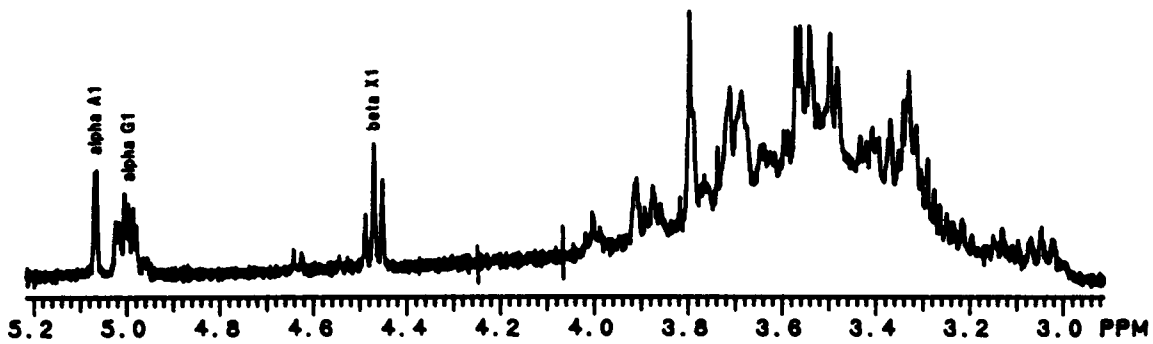


Figure 42. Proton (400 MHz) NMR spectrum of GXA recorded in DMSO at room temperature (F = ferulate ester, A = arabinose, X = xylose, G = galactose).

8. Proposed structure of FGXA

Results indicate FGXA is O-(6-O-*trans*-feruloyl-α-D-galactopyranosyl)-(1→4)-O-β-D-xylopyranosyl-(1→2)-L-arabinofuranose (FGXA). The proposed structure for FGXA is given in Figure 43.

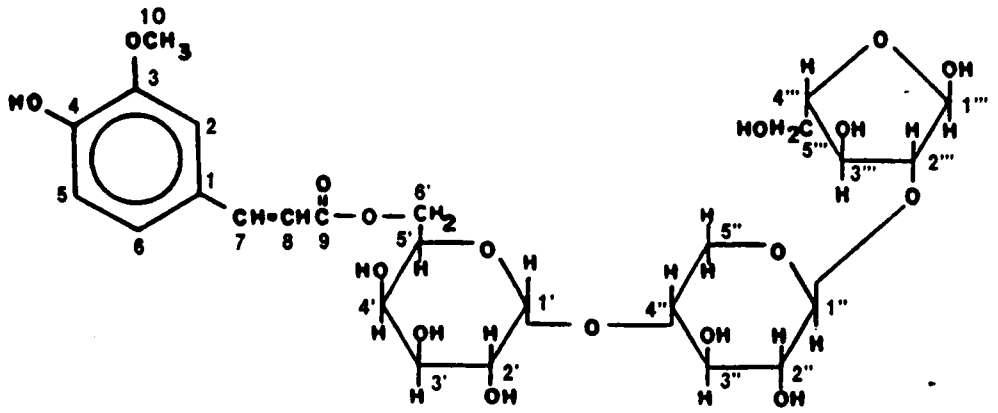


Figure 43. Proposed structure of FGXA: O-(6-O-*trans*-feruloyl-α-D-galactopyranosyl)-(1→4)-O-β-D-xylopyranosyl-(1→2)-L-arabinofuranose.

DISCUSSION

The widespread occurrence of ferulic acid in plant cell walls raises interest as to its location and function within the cell walls. Previous research on various mono- and dicotyledon cell walls has shown ferulic acid to be esterified to the cell wall carbohydrates (Fry, 1982; Morrison, 1973; Kato et al., 1987; Ahluwalia and Fry, 1986; Kato and Nevins, 1985; Smith and Hartley, 1983; Muellaer-Harvey et al., 1986). Since corn hulls contain a relatively high percentage (3%) of ferulic acid, corn hull cell wall carbohydrates may also contain ferulic acid esters. The research tested this hypothesis by using mild acid hydrolysis and chromatographic techniques to isolate feruloyl oligosaccharides from corn hulls. Structural elucidation of isolated feruloylated oligosaccharides showed a portion of the ferulate in corn hull cell walls occured in three highly specific side chains, characterized as 5-O-(*trans*)-feruloyl-L-arabinofuranose (FA), 2-O-β-xylopyranosyl-(5-O-*trans*-feruloyl arabinofuranose) (FXA), and O-(6-O-*trans*-feruloyl-α-D-galactopyranosyl-(1→4) -O-β-D-xylopyranosyl-(1→2)-L-arabinofuranose (FGXA).

Fractionation of corn hull hydrolyzate showed the highest concentration of ferulic acid in the 50% MeOH soluble fraction which consisted of small chain length cell wall fragments. Fractions high in ferulic acid also contained high levels of arabinose, xylose, and galactose which

are found in corn hull hemicellulose (CHH) side chains (Whistler and BeMiller, 1956; Montgomery et al., 1957). These results are similar to research which found ferulic acid esterified to arabinose side chains of arabinoxylans in spinach (Fry, 1982), ryegrass (Morrison, 1973), sugar-cane baggase (Kato et al., 1987), barley endosperm (Ahluwalia and Fry, 1986), Zea shoots (Kato and Nevins, 1985), wheat bran (Smith and Hartley, 1983), and barley straw (Muellaer-Harvey et al., 1986),

The carbohydrate structure of isolated feruloyl oligosaccharides in this study are similar to non-feruloylated side chains previously isolated from corn hull hemicellulose (Whistler and Corbett, 1955; Srivastava and Smith, 1956, Whistler and BeMiller, 1956; Montgomery et al., 1957). The trisaccharide component of FGXA was similar to the trisaccharide Gal-(1→4)-Xyl-(1→2)-Ara reported by Whistler and Corbett (1955). However, they did not determine anomeric configuration of the sugar components, or the ring structure of arabinose. In the current study, FGXA is shown to contain galactose in the alpha configuration, xylose in the beta configuration, and arabinose in the furan ring form. The use of NMR spectroscopy aided in the identification of both anomeric configuration and ring form.

Similarities were also seen between the disaccharide component of FXA and a disaccharide previously isolated from corn hull. Whistler and Corbett (1955), and Montgomery et al, (1957) reported the structure of an isolated disaccharide side chain from corn hull as 3-O-α-D- xylopyranosyl-arabinose. In the

present study, xylose in FXA was shown to be in the beta configuration linked (1→2) to arabinofuranose, as opposed to alpha (1→3) as previously reported. The differences may be due to the method of analysis. In the previous study anomeric configuration was determined by optical rotation and linkage positions by methylation analysis. The present study used C-13 and two dimensional proton NMR in combination with specific hydrolases. However, since the present study was limited to isolating only feruloyl oligosaccharides, it is possible that both forms of the disaccharide exist in corn hulls.

Earlier work on corn hulls characterized the structure of the hemicellulose fraction, but phenolic acid esters were not identified. The absence of ferulic acid esters during these studies was most likely due to the method of isolation. Alkaline treatments (lime water) were used to isolate the hemicellulose fraction, which would saponify ferulic acid esters. When the resultant hemicellulose structure was characterized, ferulic acid esters were already removed.

All three isolated feruloyl oligosaccharide side chains contained arabinofuranose as the reducing end sugar. This explained the selective removal of these side chains by mild acid (30 mM oxalic) hydrolysis. Furan ring glycosidic linkages were found to be less acid stable than pyran ring linkages (Perlin, 1951). Selective removal of arabinose side chains by mild acid hydrolysis was also reported by Ahluwalia and Fry (1986) for barley arabinoxylan, and by Fry (1982) for spinach

cell-walls. In addition, removal of arabinose side chains caused precipitation of the xylan backbone. Similar results were found for corn hull. Mild acid hydrolysis of the 50% MeOH insoluble fraction caused precipitation of material which was latter identified as xylan.

Specific glycosidases were used to identify the sequential position and anomeric configuration of galactose in the feruloyl-trisaccharide. Enzyme treatment was ineffective on the feruloyl trisaccharide, while the deferuloylated trisaccharide was readily hydrolyzed. Fry (1982) found similar results for the feruloyl di-galactose residue from spinach. Upon deferuloylation, Fry (1982) found glycosidases released galactopyranose from the non- reducing end. This suggested that ferulate ester attached to galactose inhibited enzyme attack, possibly due to steric hindrance. The location of ferulic acid at C-6 of the galactose residue of FGXA was confirmed by NMR comparing chemical shift of the feruloylated and de-feruloylated trisaccharide.

A large percentage of the cell wall ferulate in corn hull was associated with arabinose and galactose residues. A comparison of the feruloyl oligosaccharides isolated in this study showed ferulic acid attached to C-5 of arabinofuranose and C-6 of galactopyranose, indicating a specificity for primary hydroxyl groups. These results supported the theory that feruloylation of cell wall carbohydrates is not a random process. It is likely that feruloyl-transferases (i.e. feruloyl-coenzyme

A) with high specificity for the feruloyl acceptor may exist (Fry, 1982, Yomamoto and Towers, 1985; Hartley and Jones, 1976).

The present study used C-13, APT C-13, proton, and two dimensional proton NMR to characterize FA, FXA, and FGXA from corn hulls. Assigning NMR signals for oligosaccharides can be difficult when overlapping signals of two or more similar sugars exist (i.e. xylobiose, xylotriose, etc.). In this study, none of the isolated oligosaccharides contained two of the same sugars. In addition, both FGXA and FXA contained the same disaccharide sequence of β-xylosyl-(1→2)- arabinofuranose. The only difference between the NMR pattern for the two structures was additional galactose signals for FGXA, and the sugar to which ferulic acid was attached. This enabled complete signal assignments for the isolated feruloyl oligosaccharides, which are similar to signals reported for the structure of FAXX from *Zea* shoots (Kato et al., 1985), FAX from wheat bran (Smith and Hartley, 1983), and for FAXX characterized from barley straw (Mueller-Harvey et al. 1986).

The yield of feruloyl oligosaccharides from corn hulls in comparison to the total amount of corn hull xylose (moles of isolated feruloyl oligosaccharide moles of corn hull xylose) suggests that about 1 in every 166 xylose units contains FA, 1 in every 304 xylose residues contains FXA, and 1 in every 600 xylose residues contains FGXA side chains. In comparison, it was estimated that there is approximately 1 phenolic residue per 400 sugar in *Lolium multiflorum* leaf walls (Hartley and Jones, 1976),

1/240 in wheat coleoptiles (Whitmore, 1974), about 1/2400 in barley endosperm (Fincher, 1976), 1/150 in wheat bran cell walls (Smith and Hartley, 1983), and 1/121 sugar residues in barley straw cell walls (Mueller-Harvey et al., 1986). Painter and Neukom (1968) suggested that a substitution ratio of about 1 phenolic residue per 50 pentose residues was sufficient to enable oxidative coupling of cell wall polymers. Considering the total amount of ferulic acid in corn hulls is 3.0%, and hemicellulose constitutes about 60% of the the corn hull dry weight, it is estimated that 1 in every 14 sugar residues contains ferulic acid. The ferulic acid substitution ratio in corn hulls appears to be sufficient to allow for oxidative coupling reactions within the corn hull hemicellulose matrix. However, lack of information about the three dimensional structure of hemicellulose in corn hulls makes it difficult to predict whether the feruloyl oligosacchrides would be suitably oriented to allow interaction.

The results of this research showed that ferulic acid carbohydrate esters exist in corn hull. These results provide a better understanding of the location of ferulic acid within the corn hull structure. However, the functional role of these feruloyl oligosaccharides is still not understood. It is possible that ferulic acid may dimerize to form natural cross linkages in the corn hull cell wall structure as illustrated in other plant cell walls (Geissmann and Neukom, 1973; Hartley and Jones, 1976; Fry, 1979, 1982; Chen et al., 1982). Such dimerization/cross linking may explain the low solubility

(<1.0%) and low waterholding capacity (3.5 g water/ g corn hull) of corn hulls, even though hemicellulose is the major cell wall fraction. Alkaline treatment of corn hulls readily solubilizes the hemicellulose fraction which may be due to the disruption of feruloyl oligosaccharide /protein interactions in addition to hydrogen bonding. However, the existence of diferulic acid was not examined in the present study. Further research confirming the existence of diferulic acid and/or ferulic acid - protein complexes in corn hulls may help explain the functional role of these feruloyl oligosaccharides.

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