ANALYTICAL HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF PENTOSAN AS FURFURAL IN THE PRESENCE OF HYDROXYMETHYLFURFURAL

by

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B.S., Chulalongkorn University
Bangkok, Thailand, 1979

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1982

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INTRODUCTION

The determination of pentosan has been the subject of numerous experiments for applied scientists having research interests in animal nutrition, brewing, cereals, fruits and vegetables, paper manufacturing, starch production, furfural production and the corn syrup industry. It is generally acknowledged that the crude fiber value in the analysis of feed produces complex information, and its use is questionable in the determination of nutritive value. A determination that reflects individual nutritive carbohydrate components (e.g., cellulose, hemicellulose and starch) is more applicable. However, a convenient direct analysis of hemicellulose is sought as an alternative to indirect gravimetric methods (Detergent system of fiber analysis: Hemicellulose = Neutral Detergent Fiber-Acid Detergent Fiber) which are now in use and are generally too labor intensive to be routinely applied.

Furfural has been used to quantitate pentosan. However, past methods have serious shortcomings such as destruction of furfural in the distillation step, instability of color product of colorimetric determination, difficulty in end point determination of titrimetric method, and non-stoichiometric reaction and solubility of condensation product of long and tedious gravimetric method. To circumvent those problems the present work was undertaken.

In the analysis of feed, the crude fiber is the only value for cell wall material found in common feed composition tables, and it is a legal standard for food and feed labeling. The determination of crude fiber assumes that the residue remaining after extraction of a food with a solvent, followed by digestion with dilute aqueous acid and dilute alkali represents the indigestible fraction. The percentage of nitrogen-free extract (NFE) is found by difference; the percentages of water, ash, protein, fiber and fat are merely added

together and the sum subtracted from 100 percent. In the cereals nearly all the NFE is starch. The forages are much lower in NFE, the majority of it consists of hemicellulose, pentosan and some portions of the cellulose. Both the crude fiber and NFE values do not represent the true carbohydrate value.

The major carbohydrate values that should be considered for ruminants are starch, which is easily digested, and the less digestible group, cellulose and hemicellulose (pentosan); the latter two are 70-80% digestible by ruminants. For monogastrics, starch is the primary nutritive carbohydrate. Hemicellulose, however, is another potentially nutritive carbohydrate component.

For cereal and food chemists, water-soluble pentosan of wheat flour contributes significantly to dough consistency, because of its properties in water binding capacity and gel formation. The role of pentosan in dough and bread properties and its relation to bread staling have been studied. Pentosan determination has been used as a basis for determining the degree of milling of flours. It also plays an important role during commercial separation of starch and gluten. Besides bread making, pentosan is also functional in soft and durum wheat products, baking quality of rye flour (4) and cooking properties of rice (all reviewed in 1, 2, 3). In the canning industry, furfural determination is needed to evaluate deterioration of citrus juice (9, 10).

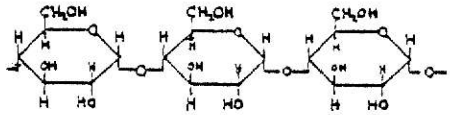
The purpose of this work is to provide a direct, convenient and accurate method which can be routinely applied for the determination of pentosan in cereal products. High performance liquid chromatography has gained popularity as a quality control instrument in cereal and related industries for the past five years. Using HPLC, the furfural and hydroxymethylfurfural (HMF), from acid hydrolysis of pentosan and starch respectively, can be separated and quantitated at the same time. Thus there is a potential to simultaneously determine starch and pentosan of cereal products.

LITERATURE REVIEW

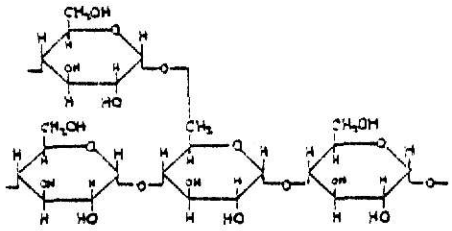
OCCURENCE, STRUCTURE, AND COMPOSITION OF CEREAL POLYSACCHARIDES

The main carbohydrate constituents of cereal grains are starch, cellulose, hemicellulose, cereal gum and free sugars (5, 6, 7, 9).

Starch constitutes a major portion of all cereal grains, Starch granules are organized structures of two polysaccharides. Both polysaccharides are polymer of D-glucose, one linear (amylose) and the other branched in a bush-like structure (amylopectin). The composition of the mixture, generally, is about 75% amylopectin and 25% amylose.



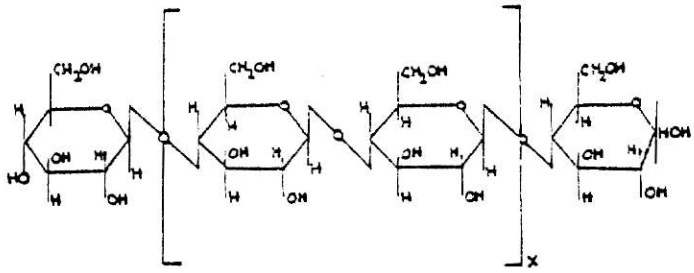
Amylose is a completely linear polymer containing only $\alpha(1\rightarrow4)$ glucosidic bonds.



Amylopectin is a highly branched polymer which contains primarily $\alpha(1\rightarrow4)$ bonds, the branching is introduced by $\alpha(1\rightarrow6)$ bonds. There is a branch, on the average, for every 20 to 25 units of the molecule.

Cellulose, the chief structural polysaccharides in plants, was found in the husk, straw or bran portion. It is believed to be identical in

chemical constitution whatever the source.



Cellulose is a linear polymer of D-glucose linked by β(1→4) glucosidic bonds.

According to Aspinall and Greenwood (11), to describe pentose-containing polysaccharides, the term hemicellulose shall be used to refer to the water-insoluble, nonstarchy polysaccharides present in cereal grain or to those plant cell wall polysaccharides, usually from lignified tissues, which may be extracted with dilute alkali but not with water. The term "cereal gum" has been used to describe the non-starchy water soluble polysaccharides from cereal grain.

Hemicellulose is composed largely of anhydro-D-xylose units. Hemicelluloses are extractable from cereal grains (after removal of starch and cereal gums) by means of 4% aqueous sodium hydroxide solution appear to be of two types (12). Husk-type hemicelluloses are found abundantly in the husk, integuments and perisperm (and also in leaf and straw hemicellulose), but are dispersed in smaller proportions throughout the grain. This type of hemicellulose was found to contain a small amount of uronic acid residues. The endospermic hemicelluloses are free from uronic acid residues and contain moderate (wheat) to large proportions (barley) of glucan.

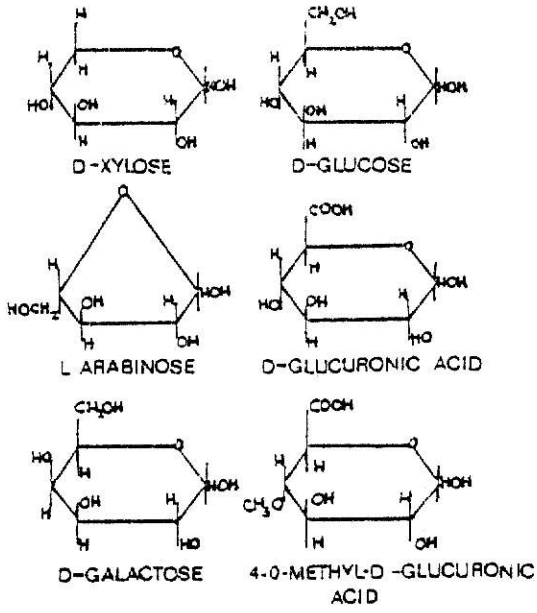
Cereal gum or water soluble non-starch polysaccharides are mostly found in endosperm. The chief characteristic is a mixture of polysaccharides which

contain protein moieties. They give viscous solutions in water and may well play an important role in the physical characteristics of the doughs of cereal flours (2). The major polysaccharides of cereal gums are composed of glucans and pentosans.

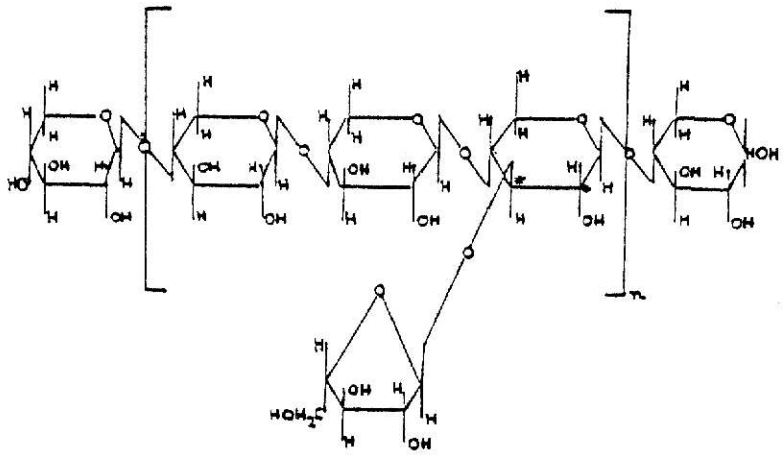
The non-cellulosic β-D-glucans are found to occur in large proportion in barley gum and oat gum (lichenin). The β-glucans are unbranched polymers containing both (1→3) and (1→4)-linked D-glucose occurring together in the same molecule.

The pentosans are mainly arabinoxylans containing glucose, galactose and uronic acids. Pentosan of cereal grains differ in molecular weight, degree and extent of branching and in the amount and kind of associated protein. Rye was found to be the best source of pentosan. The galactose-rich polysaccharides (arabinogalactans) could be separated from arabinoxylans by fractional precipitation with ammonium sulphate (13) or by chromatography on DEAE cellulose (14). Fincher et al (15, 16) reported the results of structural studies and chemical and physical properties of an arabinogalactin peptide isolated from water extracts of wheat endosperm.

It is generally accepted that the basic structure of the hemicelluloses and pentosans of cereal grains and grasses are characterized by the presence of L-arabinofuranose residues linked as single-units side chains to a backbone of D-xylopyranose residues linked β(1→4). Usually the side chains are attached to position 2 or 3 of D-xylose, but in some cases D-glucoronic acid or 4-O-methyl-D-glucuronic acid residues may be attached to position 2 or 3.

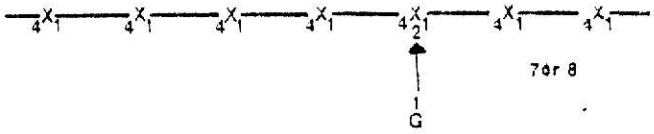


Pentose and Hexose Sugars Frequently Found in Cereal Pentosans



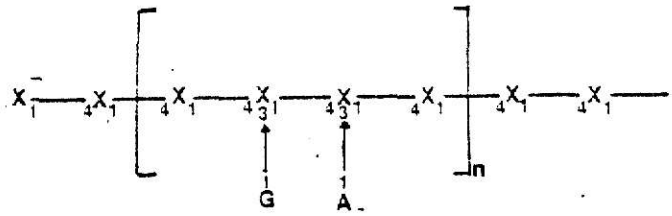
Typical Structure of a Water-Soluble Wheat Endosperm Pentosan

The hemicelluloses of straw, leaf and bran differ from the hemicelluloses derived from endosperm in that they contained uronic acid residues.



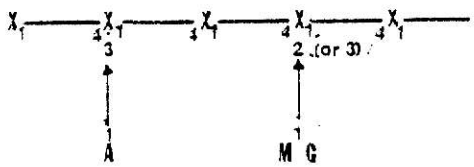
A Structure of Wheat Bran Hemicellulose

Adams (17) reported the composition of bran hemicellulose (alkali extraction): L-arabinose (59%), D-xylose (38.5%), and D-glucuronic acid (9.0%). The structure was proposed (18) to be comprised of the backbone structure of D-xylopyranose residue linked β(1→4), attached by a (1→2) glycosidic bond.



Structure of Wheat Leaf Hemicellulose

The hemicellulose of wheat leaf hemicellulose is a polyuronide containing D-xylose (88.5%), L-arabinose (6.9%), and uronic acid anhydride (5.3%) (19). A β-xylan chain of approximately 30 units with 3-L-arabinofuranose and 1-D-glucuronic acid side chains is connected through glucosidic linkages.

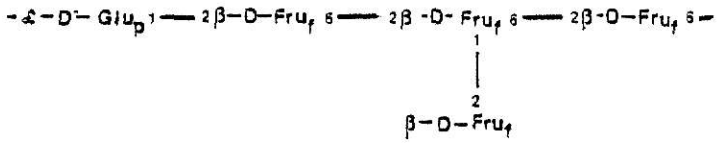


Structure of Wheat Straw Hemicellulose

The composition of the wheat-straw xylan which contains uronic acids is L-arabinose (12%), D-xylose (84%), and uronic acid (4%) (20). The structure of wheat-straw hemicellulose is a normal xylan backbone with arabinose side chain at position 3 of xylose and 4-O-methyl-D-glucoronic acid attached to position 2 or 3 of xylose (21).

The free sugars are extracted from grains with 80% ethanol. The monosaccharides are: fructose, mannose, glucose, galactose; the disaccharides: sucrose and maltose; and the trisaccharide, raffinose, occur as free sugars in cereal grains.

In wheat flour, there exists a series of oliogosaccharides composed of D-fructose and D-glucose called "glucofructans." The glucofructans are highly water soluble, non-reducing and have a molecular weight of around 2000. Although they are often referred to as oligosaccharides, the higher M.W. glucofructans might be referred to as polysaccharides.



The proposed structure for the major glucofructan fraction from durum wheat flour (22).

* Note that the term pentosan in this work refers to all of the types of polysaccharides in the gramineae, other than celluloses, starches and fructans.

CHEMISTRY OF THE FURFURAL FORMATION REACTION

The pentosans in cereal grains are chiefly those yielding xylose and arabinose upon hydrolysis. The monosaccharides formed can easily decompose, making it difficult to quantitatively determine them directly. Because of the difficulty in controlling the hydrolysis condition, the determination of pentosans is always based on their conversion through acid hydrolysis into pentose sugar. In the most commonly used methods, the pentosans are hydrolysed to pentoses, which immediately convert into furfural. The amount of furfural is determined, and used to calculate pentose sugar.

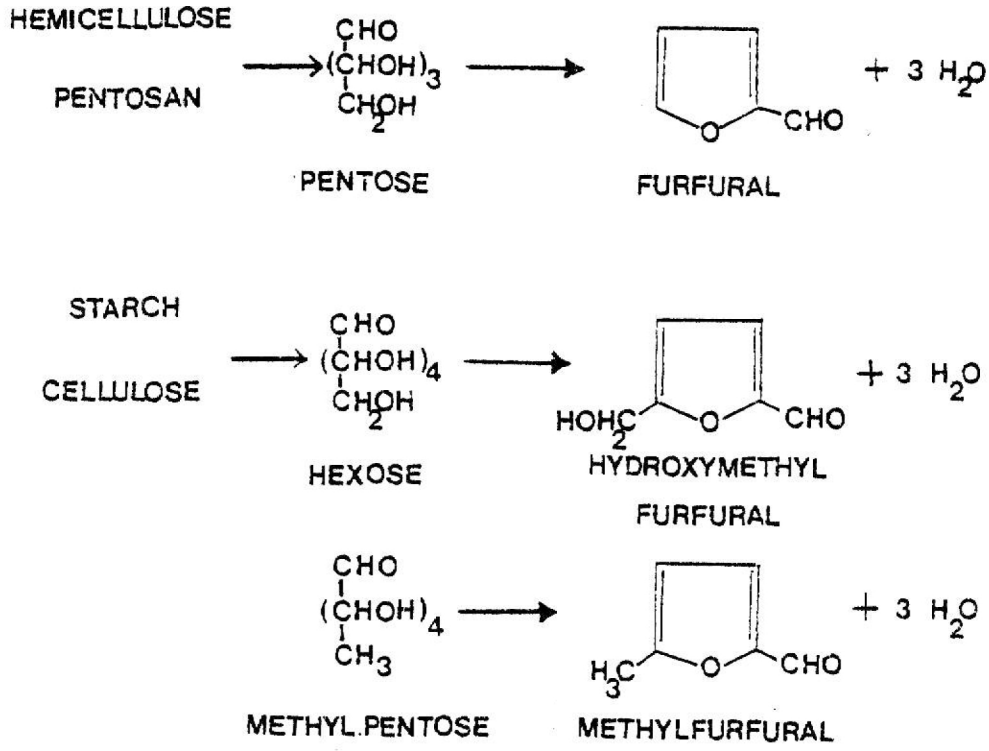
$$(C_5H_8O_4)_n \xrightarrow[\text{Hydrolysis}]{+H_2O} n\ C_5H_{10}O_5 \xrightarrow[\text{Dehydration}]{-3H_2O} C_5H_4O_2$$

Pentosan	Pentose	Furfural
M.W. 132.1	M.W. 150.1	M.W. 96.1

The hydrolysis of polysaccharides has been investigated (review in 23, 24, 25); hydrolysis of polysaccharides is probably affected by configuration, the ring size and conformation of the monosaccharide residues, the anomeric configuration of the glycosidic linkages (such as (1→2), (1→3), (1→4), and (1→6)), the presence of functional groups in the molecule, and the intensity of inter-molecular and intramolecular interactions. The studies of theory and kinetic of the hydrolysis were reviewed and the kinetic parameters of different polysaccharides were tabulated (24).

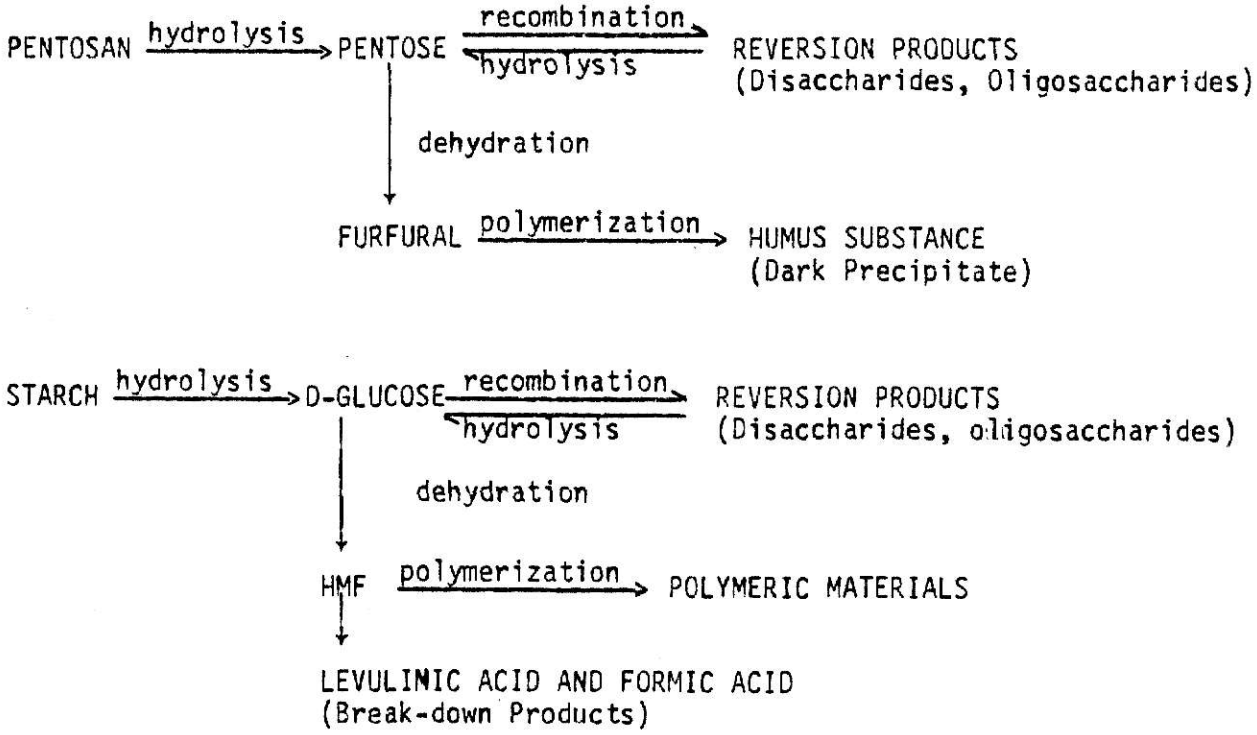
In dilute acid solution, furfural (2-furaldehyde) is formed in high yield from all the pentoses. It is obtained at almost theoretical quantitative yield from xylose, if it is removed from the solution while it is being formed. In relatively concentrated acid solution, other minor products are formed; reductive acid, formaldehyde, crotonaldehyde and acetaldehyde (26).

The hydroxymethyl furfural (5-hydroxymethyl-2-furaldehyde) formed from hexose is unstable; levulinic acid and polymeric materials were also found as major products. In addition, many minor dehydration products and products not formed solely by dehydration mechanisms (acetone, formaldehyde, acetaldehyde, propionaldehyde and 5-methyl-2-furaldehyde) were found in low yield (26).



Major Decomposition Products of Acid Hydrolysis

"Products Obtained by Heating Sugar with Mineral Acids"



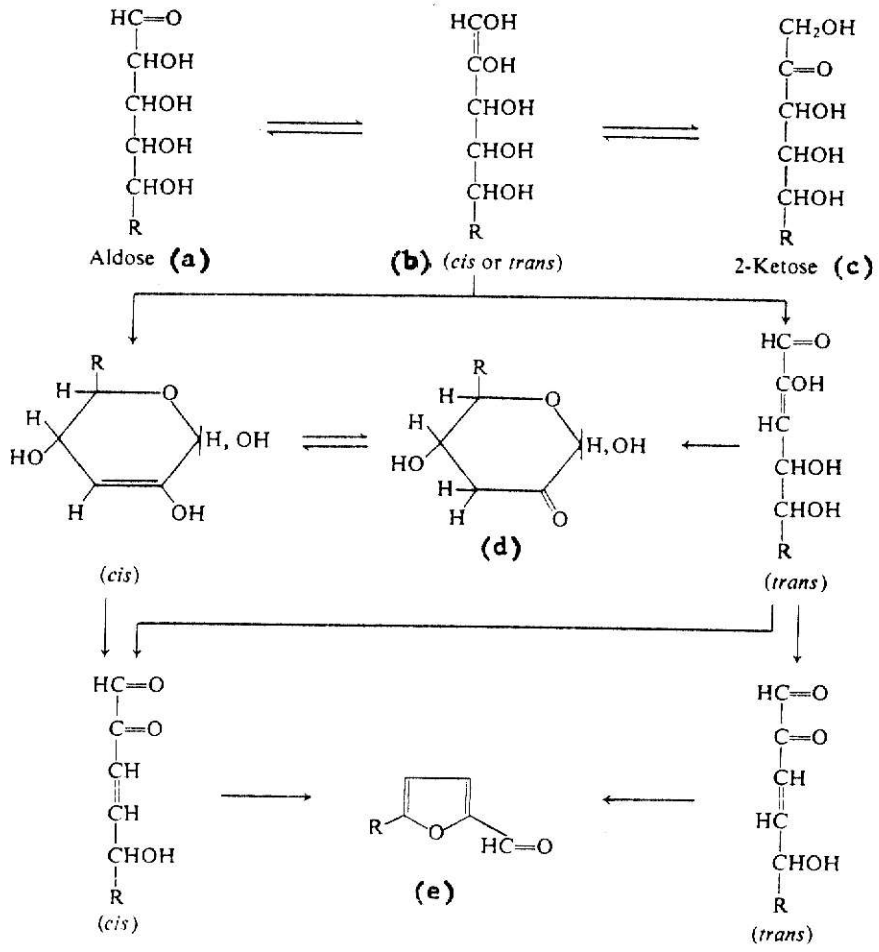
In a hot concentrated acid medium, the simple sugars formed upon the hydrolysis of pentosan and starch subsequently decompose to furfural and hydroxymethylfurfural respectively. The reversion products of monosaccharides will form under the conditions of hydrolysis and the recombination occurs in equilibrium. The "humus substances" (non-carbohydrate substance) have relatively high carbon content which darken the sugar solution. The hydroxymethylfurfural is less stable than furfural and can break down to levulinic acid and formic acid.

The mechanism for the formation of 2-furaldehyde was studied by many investigators. Several different mechanisms have been proposed (review in 27). Under vigorous hydrolysis conditions, the enolizations (which is a general reaction of acid-base catalyzed of carbon compounds having an alpha-hydrogen

atom) that occur lead to beta elimination (elimination of a hydroxyl or alkoxyl group in a beta-position relative to a carbonyl group) and the formation of furan compounds (with hexoses the degradation proceeds further to yield levulinic acid and formic acid).

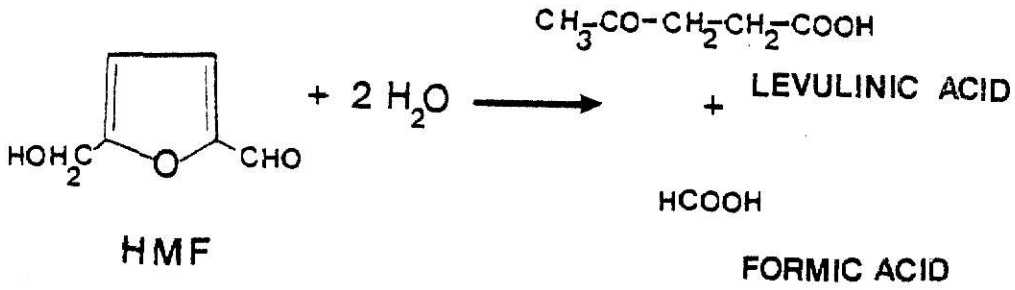
The mechanisms of the formation of 2-furaldehyde is analogous to the formation of 5-(hydroxymethyl)-2-furaldehyde. The evidence for the beta-elimination mechanism was obtained by Anet (28, 29).

Anet's scheme (described in (26) and (27)) for formation of 2-furaldehyde, shown below, was based on experimental evidence available that included the isolation, characterization, and further study of intermediate compounds.



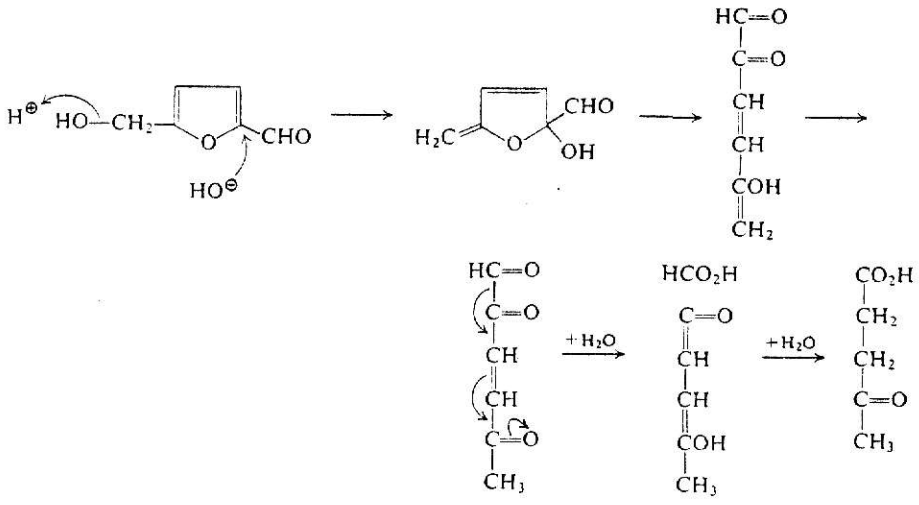
Since the rate-limiting step is the formation of the enediol (b), the rate of formation of the 2-furaldehyde depends on the ease of enolization of the sugar.

The preparation of 5-(hydroxymethyl)-2-furaldehyde from hexoses is more difficult because the product cannot be removed from the reaction mixture by distillation as it is formed, and also because it is less stable than 2-furaldehyde. The HMF is converted to levulinic acid and formic acid under the hydrolysis conditions (30).



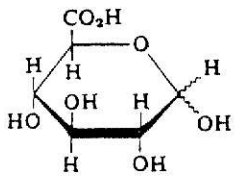
The hydroxyl group of 5-HMF is very reactive, being easily removed.

The mechanism for the reaction proposed by Isbell (31) is shown below.

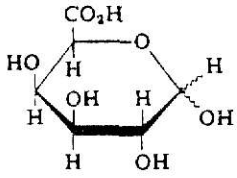


The determination of pentosans by conversion to furfural is complicated by (A) the simultaneous formation of furfural from materials other than pentosan.

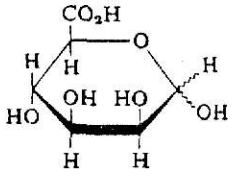
- Uronic Acids and Polyuronides. The glycuronic acids which commonly are found to occur in nature as D-glucoronic (A), D-galacturonic (B), and D-mannuronic acid (C). When combined in natural products, the uronic acids always seem to occur as glycopyranosiduronates.



A



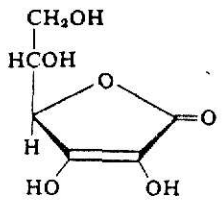
B



C

Glycuronic acids yield furfural and carbon dioxide upon the treatment of aqueous mineral acid (discussed in 32, 33). The yield of CO_2 is quantitative but the amount of furfural obtained is only in the vicinity of 30% of the theoretical yield and is not produced quantitatively. A more accurate figure for pentosans may be obtained by deducting from the total furfural yield the amount of furfural corresponding to the amount of CO_2 found in the uronic acid analysis.

- L-Ascorbic Acid and Hexulosonic Acid also liberate 2-furaldehyde and CO_2 upon treatment with acid (discussed in (26), (27)).



L-ascorbic acids are illustrated by the formula of the most important member of the group, L-threo-hex-2-enono-1,4-lactone, also known as vitamin C.

- Oxycellulose has also been claimed to produce furfural upon the distillation with hydrochloric acid (34).

(B) The formation of other substances which may be determined as furfural. In the distillate, in addition to furfural, HMF is produced from hexoses and hexosans, methylfurfural from methylpentoses and methylpentosans and small quantities of formaldehyde, acetone, and levulinic acid.

(C) The presence of large quantities of lignin and tannin may cause low recovery of furfural because they may react with furfural before furfural is removed by distillation.

ANALYTICAL METHODS FOR PENTOSAN

The analytical methods for pentosan determination generally consist of 2 steps, the reaction of the sample with acid to produce furfural and the determination of the furfural distilled from the reaction mixture during the hydrolysis reaction.

Acid Hydrolysis and Distillation

Many studies have been done on distillation procedures to improve the production and minimize the destruction of furfural (Review in 34, 35, 36); different kinds of acids (e.g., phosphoric acid, oxalic acid, trichloroacetic acid, nitric acid, hydrobromic acid, hydrochloric acid and sulfuric acid) were tried, addition of salts (e.g., sodium chloride, ammonium chloride) to maintain acid concentration, varying the concentration of acids and salts, optimization of temperature and duration of distillation, modification of

distillation apparatus and using steam distillation in order to remove furfural in a distillate to protect it from further destruction by the acid.

The hydrochloric acid of 12% concentration has had widest use. The sample is heated with 12% acid, the distillation is carried out at such a rate that some of the distillate are collected in 10 minutes, 30 ml more of 12% acid are then added and the process is repeated until no more furfural is produced or until a total of 360 ml of distillate is collected.

The theoretical yield from xylose was claimed (34) by distillation with steam from 12% HCl and salt, and later obtained complete recovery of furfural from arabinose and of methylfurfural from rhamnose by the same method (38). The bromide-bromate at $0^{o}C$ (described on page 17) was used for the quantitation of furfural.

Reeves and Munro (39) have found that the furfural is not destroyed upon refluxing with 12% HCl in the presence of a suitable high-boiling, immiscible solvent (e.g., xylene, n-butyl ether). The furfural, as it is formed, is rapidly extracted by the solvent to protect from further decomposition by acid. Xylose was converted to the theoretical quantity of furfural on distillation with 12% acid + xylene for 3 hours and with 15% acid and xylene for one hour. The furfural was determined by a Duboscq colorimeter with aniline acetate as a reagent. A correction factor was required to compensate for the partition coefficient of furfural between acid and the xylene (AACC method 52-11).

Determination of Furfural

The furfural produced from acid hydrolysis of pentosan may be determined by gravimetric, titrimetric, polarographic, colorimetric, ultraviolet spectrophotometric, gas chromatographic and liquid chromatographic methods.

Gravimetric Method

Different reagents have been used for precipitating the furfural (35, 36, 40), among the best known reagents are phloroglucinol, barbituric acid, thiobarbituric acid and dinitrophenylhydrazine. The use of the latter reagent was abandoned after the introduction of phloroglucinol by Councler (1894).

The phloroglucinol method (described in 35), which is the well known Kröber modification of the Tollens method (1901) is used as a standard AOAC method (41). A solubility correction is applied to the weight of the precipitate (furfural-phloroglucinol condensation product) and the corresponding weight of pentose or pentosan is calculated using empirical factors suggested by Kröber. These disadvantages, precaution and limitation of the method were discussed by many investigators (35, 36, 40).

Titrimetric Method

Furfural has been determined titrimetrically with a number of reagents; hydroxylamine, potassium bisulfite, phenylhydrazine and bromide-bromate reagent. The amount of bromine taken up by the furfural has been estimated by potentiometric titration (4) and more successfully by various modifications of the excess bromine method (37, 38). The potentiometric titration was reported difficult in obtaining a good end point, and failure to check the method against other methods (42, 43, 44, 45).

In the AACC method 52-10 (46) developed by Hughes and Acree (37), the furfural produced from the distillation with 12% HCl was allowed to react

with excess bromine (supplied by reaction of a bromide-bromate mixture with an acid) at $0^{o}C$ for 4 minutes (at $0^{o}C$ a mole of furfural combines with 2-gram atoms of bromine). The excess bromine is determined by addition of potassium iodide and titration of the liberated iodine with standard thiosulfate solution using starch solution as an indicator.

Since methylfurfural reacts more rapidly with bromine than does furfural under the same condition ($0^{o}C$), the method has also been applied to mixtures of the two aldehydes. By determining bromine consumption at two or three time intervals, it is possible to calculate the amounts of furfural and methylfurfural in mixtures of the two (38).

The titrimetric method has been found simpler in application, and more reliable than the gravimetric procedures. It can be used over a wide range of furfural concentrations which is especially suitable for dilute solutions, where gravimetric methods fail because of solubility effect. However, results are time and temperature dependent, therefore time and temperature must be carefully controlled.

Polarographic Method

The polarographic method for furfural determination can be done by both direct (49) and indirect method (47, 48). The indirect method is based on the reaction with hydroxylamine hydrochloride to form oxime which is easily reduced at the dropping mercury electrode. The indirect method was claimed to have doubly sensitivity as compared with the direct polarographic method (48) and the oxime formed is reduced at a more positive potential.

Colorimetric Method

Furfural forms highly colored compounds with aniline, xylidine, benzidine, orcinol, resorcinol, phloroglucinol and many other reagents. Schmieder (50)

critically reviewed the color reactions for pentosan determination from 12 different compounds (compounds containing amino group and phenolic group). Absorption spectra of chromophores produced by the reaction of furfural and hydroxymethylfurfural with various color producing reagents were given. Aniline acetate method was considered to be the method of choice with more selective and sensitive reaction.

The color differentiating reactions for hexose and pentose sugars are rare. They are not specific and give similar reaction with other aldehydes (e.g., HMF and methylfurfural). The interferences from hexose and other sugars are a very real source of error. Although the colorimetric method appeared to be less affected by methylpentoses and hexoses than the gravimetric and titrimetric methods, the colorimetric estimation of pentosans in the presence of hexose/hexosan material was explored to improve the reliability of the procedure.

Cracknell and Moye (51) adapted the method of Dische and Borenfreund (52) for the analysis of flour, starch and other cereal products. The Dische procedure is based upon a modification of the original phloroglucinol reaction of Tollens (1889). The interference from hexoses and other sugars can be eliminated by measuring the absorbance at two different wavelengths (552 nm and 510 nm). The absorbance of interfering sugars differs very little at these two wavelengths but is considerable for the pentosan. The absorption value at 552 nm minus that at 510 nm is directly proportional to the pentose content of the hydrolyzate. The modified method of Cracknell and Moye is considered to be a rapid method for determination of pentosans in cereal products (which eliminates the distillation step): 10 ml of a reagent mixture consisting of acetic acid, hydrochloric acid, phyloroglucinol/ethanol and glucose is added

to a flour sample (2-5 mg) in a test tube. The tube is then placed in a vigorously boiling water bath for 16 minutes and the absorbance of the resulting solution measured at 552 nm and 510 nm. Pentosan content is calculated by subtracting the 510 nm reading from the 552 nm reading and comparing the results to a standard curve based on a pure xylose.

Douglas, S.G., (53) investigated the modified method of Cracknell and Moye and found that by adding 2 ml of distilled water to the samples, the effect of the red/orange color of the reagent blank which imparted a negative value to the pentosan was negated. Twenty-five minutes was found to be the optimum reaction time and the expensive grades of acetic acid are not needed in the reagent to obtain an acceptable blank. The limitation of the method is the problem of sampling, larger samples will produce a solution which is too highly absorbed. The range of pentosan concentrations determined is not very wide (ca. 1.0% - 3.0% as D-xylose) and as with many colorimetric methods of analysis, the "color fading" is a major problem.

Cerning and Guilbot (54) proposed a specific method for the determination of Pentosans in cereal and cereal products. The distillation apparatus developed by Duffau (1946) was used, the pentosans are converted into furfural by hydrochloric acid and separated by steam distillation. The apparatus was designed to make the distillation take place without direct heating of the reaction medium and the total acid volume and its concentration are maintained constant during the whole operation. The difficulty presented by the instability of the aniline furfural color has been resolved by the use of buffer solution (anhydrous ammonium acetate, stannous chloride and 4.2 g stannic chloride and glacial acetic acid) which leads to a stabilization of the color reaction for at least 45 minutes.

Dinsmore and Nagy (9) studied a colorimetric furfural measurement as an

index of deterioration in stored citrus juices. They reported the improved colorimetric method for furfural (10) which is based on the aniline acetate reaction by addition of $SnCl_2$ and HCl to yield higher intensity and improved color stability. The reagent giving the best result contained 1% $SnCl_2$, 0.6 NHCl and 10% aniline in glacial acetic acid.

Ultraviolet Spectrophotometric Method

Earlier work by Tweeten and Wetzel (55) (Kansas State University) sought to eliminate the distillation step and quantitate the equilibrium concentration of furfural in the head-space of the sealed reaction vessel. Thus, without actual distillation the vapor phase was sampled to provide enrichment of furfural in relation to hydroxymethylfurfural. Quantitation was by absorption at 292 nm of the semicarbazone derivative.

Gas Chromatography

A method for the determination of pentosans by Gas Chromatographic analysis of furfural produced by acid treatment was proposed by Folkes, D.J. (56). The samples were reacted with 3.85 M hydrochloric acid under reflux and the furfural produced partitioned into dibutyl ether. (The dibutyl ether was reported to give a more favorable partition of furfural than the xylene used by Reeves and Munro (39)). The 4 hours reflux time was selected to obtain the least difference of the yields of furfural from xylose and arabinose, this minimized the error caused by the use of xylose only for calibration. Furfural was measured by GC with acetophenone as internal standard. Xylose was used as a calibration curve. The method was applied to starch and flour samples, the method showed good precision with a coefficient of variation of 2 - 4% depending upon sample type.

EXPERIMENTAL

A. HYDROLYSIS AND CHROMATOGRAPHIC DETERMINATION OF PENTOSAN

CEREAL GRAINS, MILLING PRODUCTS AND FEED INGREDIENTS

Current commercial samples of oats, sorghum, millet, corn, wheat and rice were examined. All except rice were grown in Kansas during the 1980-1981 crop year. Rough rice was obtained from the 1980 crops in Arkansas. The milling products of bran, shorts, germ, flour and rice hulls were taken from the Kansas State University flour mill. The feed ingredient available was dehydrated alfalfa meal.

Whole grain samples and rice hulls were ground through a Wiley Mill equipped with a 1 mm screen, mixed thoroughly, and stored in a refrigerator prior to analysis. Wheat milling fractions of bran, shorts and germ were used without grinding.

REFERENCE MATERIALS

The xylan purchased from Sigma Chemical Company, was dried in a vacuum oven (85^{o}C) for 4 hours before use as a standard. The commercial xylan was checked to determine percentage of xylose sugar. Sugar composition was determined by HPLC using a BIO-RAD ion-exchange resin column.

HPLC Quantitation of Xylose Sugar in Commercial Xylan: An aluminum heating block 12.5 cm square and 3.75 cm thick was fabricated. Eight holes were drilled in a square pattern to hold 20 mm O.D. x 58 mm, 5 ml micro reaction vessels with screw cap seals (Reliance Glass Works, Inc.). A 9th hole was drilled at the center of the block to hold a Weston Thermometer,

0-250^{o}C. The heat source was a TekPro^{TM} Heat Stir 36 stirring hot plate (Scientific Products).

The chromatographic system consists of the following components: Beckman Accu Flo pump (reciprocating single-piston pump), Hewlett Packard Model 1031 refractive index detector, Haake Model FK 2 circulator and Hewlett Packard Model 3390A reporting integrator.

The 2M Trifluoracetic acid was prepared by diluting 2.28 g concentrated acid to 10 ml with distilled water.

The liquid chromatograph mobile phase was prepared by deionizing redistilled water. The deionized water was filtered through a 47 mm millipore filter (EHWP 047 00; Millipore Corp., Bedford, MA) with a pore size of 0.5 µm. Before use, the water was degassed using a megason ultrasonic generator for 15 minutes.

Stock solution of xylose (Sigma Chemical Company, St. Louis, Mo.) was prepared by dissolving 0.18 g of xylose in 100 ml deionized water. The standard solutions were prepared by diluting appropriate amounts of the stock solution with water.

Trifluoroacetic acid hydrolysis of cell wall polysaccharides is described by Nevins et al., (57). Twenty mg of dried xylan was weighed out and placed in a 5 ml micro reaction vessel. Exactly 2 ml of 2 N trifluoroacetic acid was added to mix with the xylan and a magnetic triangular stirring bar was placed in each of the reaction vessels, the tubes were sealed with screw caps equipped with tetrafluoroethylene liners, the tubes were then placed in the aluminum heating block at 121^{o}C and allowed to hydrolyze for 1 hour. Following hydrolysis, the samples were removed from the block, allowed to cool 10 minutes, and evaporated to dryness at 50^{o}C with a jet of filtered air directed onto the liquid surface. The residue sugar was

redissolved in water and transferred to a 10 ml volumetric flask, and made up to volume. 1/4 teaspoon of mixed resin was added and shaken for 15 minutes. The hydrolyzates were filtered through 0.47 µm millipore filters (HAWP 01300) using a swinny adaptor. The samples were saved and stored in screw cap vials, covered with aluminum foil, prior to analysis.

Twenty µl of aliquot of the sample were injected into the liquid chromatograph using the following conditions: mobile phase as described; flow rate 0.6 ml/min.; refractive index attenuation 4; column Aminex HPX-87 (heavy metal form) 300 x 7.8 mm (Bio-Rad Laboratories); column temperature 85°C. Integrator parameters were: chart speed 0.6; zero offset 0.10; attenuation 2; peak width 0.16; threshold 1; area reject 50000.

INSTRUMENTATION AND CHEMICALS

Six aluminum heating blocks 7.5 cm in diameter and 5 cm thick were turned to produce a cavity 4 cm deep which would hold a 44 mm (O.D.) x 89 mm glass bottle (WHEATON). The heat source was a Lab-Line Pyro-Multi-Magnestir (Lab-Line Instruments, Melrose Park, Ill.). The Dial thermometer (Taylor Instrument, Arden, N.C.), 0-110°C, was used to measure the temperature of the aluminum block.

Sulfuric acid (15 N) was prepared by dilution of the concentrated acid (36 N) and the acid strength was checked by titration before being used.

The 2 N sodium acetate solution was prepared by dissolving 27.2 g of sodium acetate trihydrate with distilled water to the volume of 100 ml.

Column Packing Techniques and Apparatus: The analytical column, 4.1 mm I.D. x 25 cm, was .316 stainless steel (Handy and Harmon Tube Co., Norristown, PA). The exit end of the column was milled to contain a two micron stainless steel frit (0.1875 inch diameter x 0.125 inch thick, Mott. Metallurgical

corporation, Farmington, CT). The column ends were fitted with 1/4 inch to 1/16 inch Swagelok low dead volume reducing unions (Crawford Valve and Fitting Co., Cleveland, OH).

The packing system consisted of a Haskel pump (DSTV-122/CP4) pneumatic amplifier pump (Haskel, Inc., Burbank, CA), equipped with a two-way valve for packing solvent flow direction switching, a manual override valve, a ball valve and slurry packing chamber.

The column was packed by the procedure of Gere (58) described below:

1) Weigh out 2.7 grams of packing material. Place in clean, dry screw-cap bottles (50 ml) with plastic inner cap liner.

2) Add 10 ml of packing solvent consisting of 3 parts tetrachloroethane to 1 part dioxane. Pass packing solvent through an open silica column prior to use. Shake bottle 10 times, and place in ultrasonic bath for approximately 1 minute.

3) Remove from ultrasonic bath and wipe off water adhering to bottle.

4) Pour suspension into packing apparatus, with 4.1 mm x 25 cm stainless steel empty column attached. End terminator must be installed.

5) Top-off suspension in reservoir with 10 ml of excess packing solvent.

6) Turn on high pressure pump loaded with degassed hexane. Allow hexane to flow until a minimum of 10 column volumes have passed. Remove column and test. The column was packed at 8,000 psi.

7) Carefully remove excess packing material at top of column and insert upper terminator (a porous TFE disc, ca. 100 μm thick).

The column, after it was flushed with hexane, was then washed with chloroform, methanol and 60/40 methanol: water respectively, for a minimum of 20 minutes each at a flow rate of 1 ml/minute. The quality of the column was then checked by injecting a mixture of phenol and 2,6-xylenol and cal-

culating the number of plates per meter of the latter peak.

Liquid Chromatographic Mobile Phase: Water for use in the mobile phase was redistilled in an all-glass apparatus from alkaline permanganate solution. Methanol was prepared by refluxing using the Grignard reaction described by Vogel (59). The 80/20 water: methanol solution was used as a mobile phase; the mobile phase was sucked through a 0.45 µm microporous filter (EHWP 047 00, Millipore Corp., Bedford, MA), using a Millipore filter holder.

Liquid Chromatography: The chromatographic system consists of the following components: Altex Model 110A pump, stop-flow injector, Water Model 202 Differential UV detector (modified to monitor at 280 nm wavelength absorbance; Waters Associates, Milford, MA) and Hewlett Packard Model 3385A reporting integrator (Hewlett Packard Corp., Avondale, PA).

METHODS OF ANALYSIS

Acid Hydrolysis of Samples: Weigh sample (~0.1 g) containing 1 - 20 mg pentosan into a 60 ml glass bottle. 25 ml of 15 N H_2SO_4 was added to the sample with a magnetic stirring bar. The bottles were sealed and placed in aluminum heating blocks which were then heated on a hot plate with a 6 position stirrer at 95°C for 30 minutes. Allow to cool. The reaction mixture was pressure filtered through Whatman filter paper with the aid of a Swinney adaptor fitted to a 5 ml syringe. 1 ml of the filtrate was pipetted into a 10 ml volumetric flask and diluted to volume with 2 M sodium acetate solution to bring the pH up to around 3-4.

Chromatography of Reaction Mixtures: A 20 µl aliquot of the sample was then injected into the liquid chromatograph using the following set of conditions: Mobile phase as described; flow rate 1.0 ml per minute; column

4.1 mm ID x 25 cm μ Bondapak C-18 (10 μm mean particle size, Waters Associates, Milford, MA). A use of guard column prior to the analytical column is recommended. The system was operated at ambient temperature. The UV detector sensitivity was set at 0.32 absorbance units full scale.

A Hewlett Packard 3385A reporting integrator was used to quantitate the data. The integrator was programmed with the following set of conditions: Chart speed 1.00 cm/min.; attenuation 4-6; slope sensitivity 0.1; and area reject 1000.

RESULTS AND DISCUSSION

The method developed was simple, rapid, reliable and specific for pentosan determination. The distillation step was eliminated, and acid hydrolysis of a sample was performed in a sealed tube system for 30 minutes. High performance liquid chromatography was used for analysis of the liquid phase reaction mixture to determine furfural resulting from acid hydrolysis of pentosan without interferences from hydroxymethylfurfural and methylfurfural. In this way, by maintaining the same condition of reaction mixture, time, temperature and acid concentration, the relative pentosan of different samples can be determined by furfural yield in the sealed tube reaction. Commercial xylan, with its purity determined as an absolute amount of xylose, is used to make a calibration curve.

OPTIMIZATION OF HYDROLYSIS CONDITION

Although the rate constant of the hydrolysis of pentosans by hydrochloric acid is larger than for sulfuric acid (60), sulfuric acid is preferred for working in a sealed system. Hydrochloric acid with its high volatility can cause problems with laboratory electronic systems. Its high volatility also makes it difficult to maintain constant pH. For a given acid, the important reaction variables are the concentration, the temperature and the duration of heating.

To keep the heating time within 30 minutes, the concentration of acid should be in the range of 10-18 N. From Figure 1, as the concentration of acid increases, the maximum yield is reached in less time. For concentration of more than 15 N, the curve drops drastically after reaching the maxi-

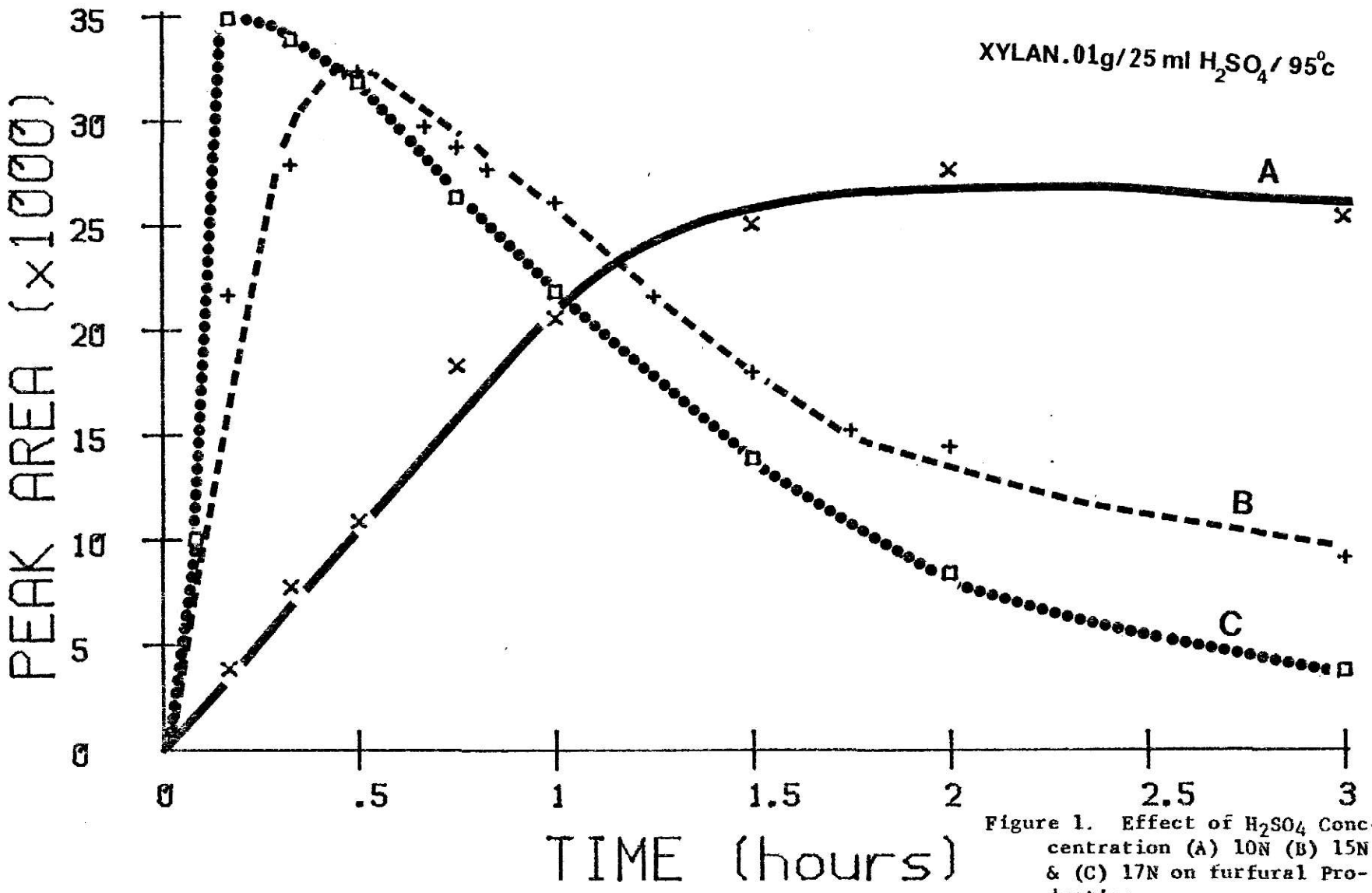


Figure 1. Effect of H_2SO_4 Concentration (A) 10N (B) 15N & (C) 17N on furfural Production.

mum point; therefore, the 15 N acid was chosen. The maximum furfural production of 15 N acid is between 30-40 minutes when the temperature is maintained at 95°C.

The temperature was varied (Figure 2); the maximum yield is reached in less time as the temperature is increased. When operated at 120°C, a very drastic change occurred after the maximum point. At 95°C, 20 percent change of furfural yield is noticed in the 30 to 60 minutes time period. (When operated at 120°C, 65 percent drop of furfural yield is found in the same time period.)

The optimum condition for hydrolysis of pentosan conversion to furfural must be a compromise between the relative rate of furfural production and the rate at which the furfural formed is destroyed. From Figure 3, when varying the sample size, straight lines were obtained from 0 to 30 minutes. The lines begin to curve and fall off after 30 minutes. The more furfural produced, the greater the destruction (25, 20 and 13 percent drop of furfural yield from .02, .01 and .005 g of sample respectively, during the 30-60 minute time period). The furfural yield (peak area) seems to be proportional to the amount of sample at 30 minutes hydrolysis time. The furfural yield from different samples vs. time was also observed (Figure 4). The results confirmed the suitability of a 30 minute reaction time.

CHROMATOGRAPHY OF FURFURAL

In previous work hydroxymethylfurfural (HMF) in tomato paste was determined by high performance liquid chromatography using a reverse phase Lichrosorb C_8 column, an ultraviolet detector operated at 284 nm, and isocratic elution with water (61). The HMF appeared on the chromatogram with the retention time between 6-8 minutes.

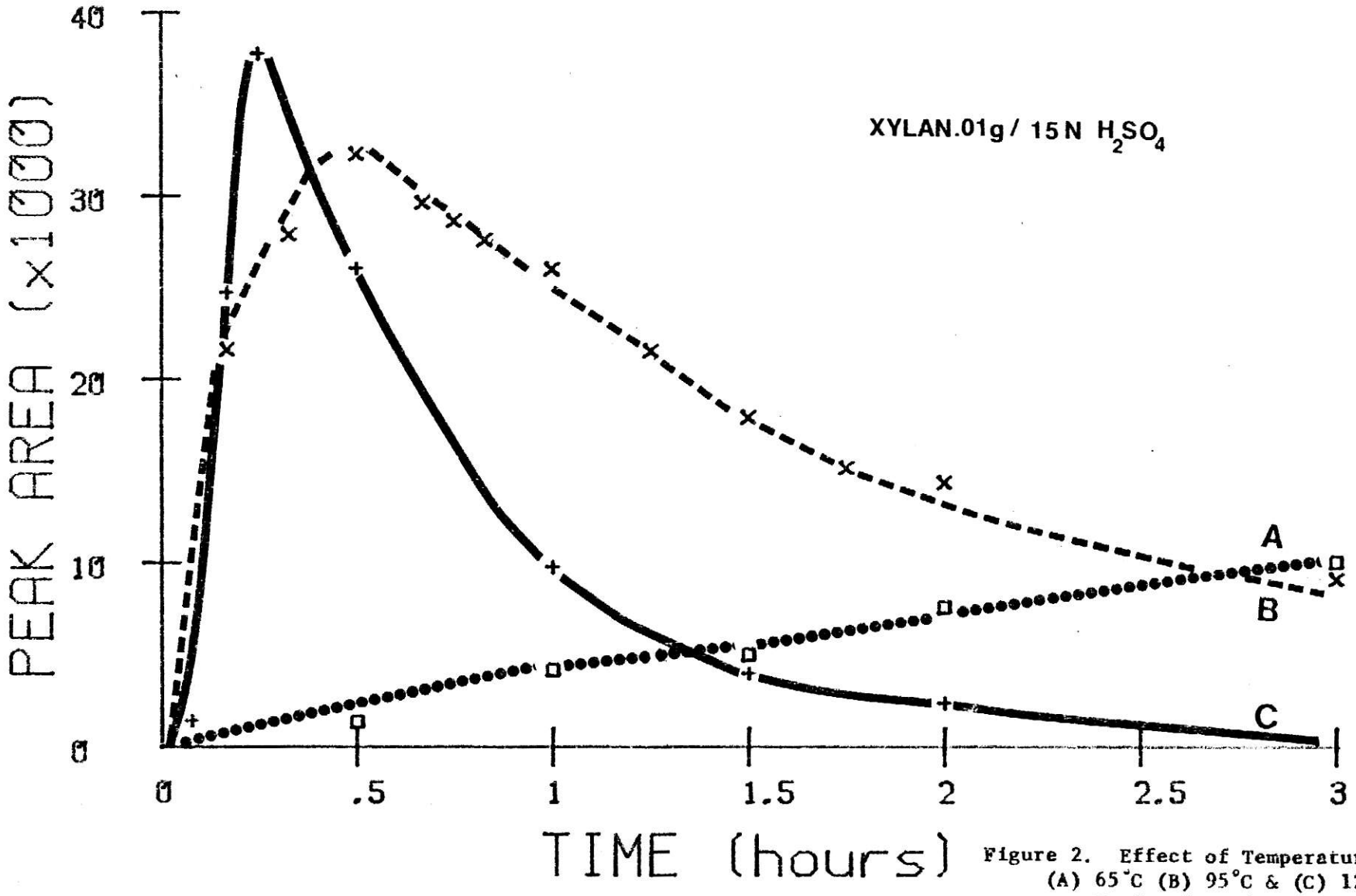


Figure 2. Effect of Temperature (A) 65°C (B) 95°C & (C) 120°C on Furfural Production.

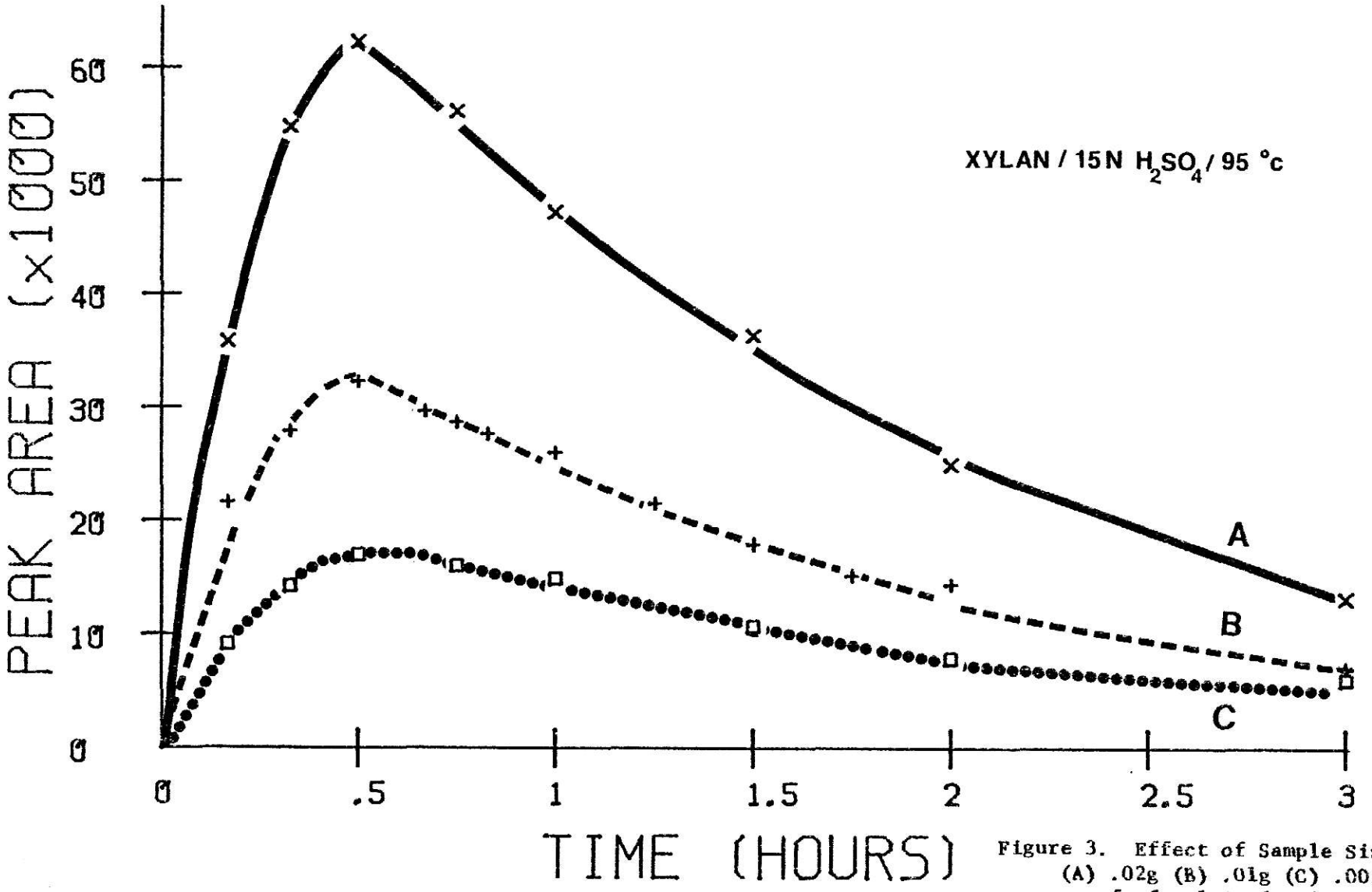


Figure 3. Effect of Sample Size (A) .02g (B) .01g (C) .005g on furfural Production.

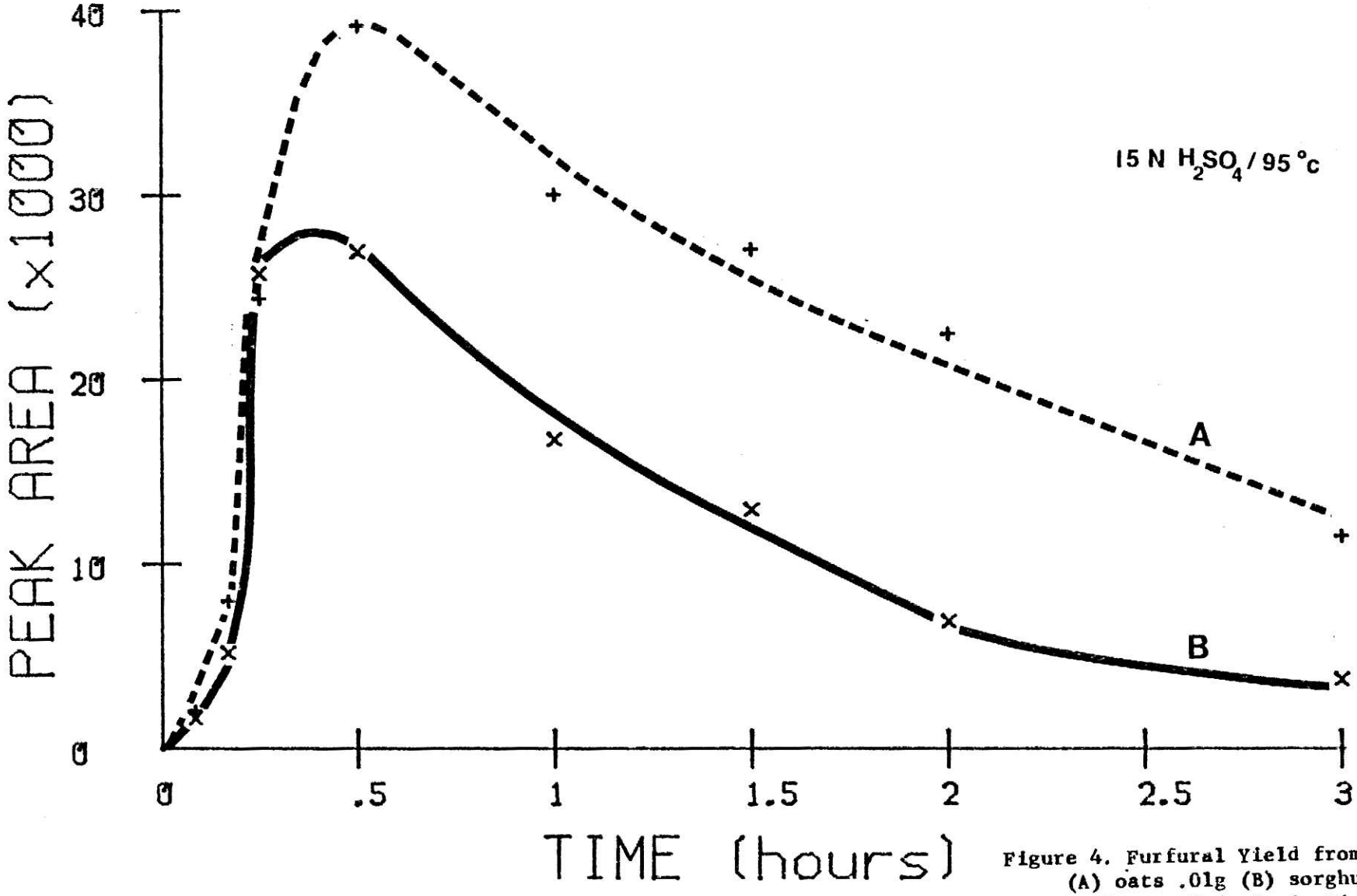


Figure 4. Furfural Yield from (A) oats .01g (B) sorghum .05g vs. Hydrolysis Time.

In this work, a reverse phase μ-Bondapak C_{18} column was used with a 25:75 methanol/water as a mobile phase to get a separation of furfural and HMF within 7 minutes. The k' value (observed capacity factor) of HMF is 1.7, of furfural is 2.5, and of methyl furfural peak is 4.8 (Figure 5).

UV absorption was selected as a method of detection. The UV absorption spectrum of furfural and HMF (Atlas of spectral data and physical constants, Vol. III) showed maximum absorption at 271 nm and 279 nm (ε = 14800) in methanol respectively, The Waters Model 202 UV detector was modified for measurement at 280 nm to allow measurements to be taken closer to the λ max of both compounds. The molar absorptivity (ε) of HMF in water = 18197 and of furfural in sulfuric acid = 1175 (Handbook of Chemistry and Physics).

The chromatograms of reaction products from various sources are shown in Figure 6. The simple chromatograms with no interference from other UV absorbing materials were obtained from all samples examined. The decomposition products of hydroxymethylfurfural were seen (Figure 6) having the k' value of 1.5 and 1.8. By using a spiking technique, the peak with k' of 1.5 appeared to be levulinic acid (A). The furfural (D) peak with k' value of 2.5 also appeared to be a break down product of hydroxymethylfurfural (B) (Figure 7). The third, unidentified decomposition product (C) has a k' value of 1.8.

CALIBRATION CURVE AND LINEARITY

Since pure pentosan standards are not available, absolute amounts of xylose in a relatively pure commercial xylan was determined by exhaustive hydrolysis to xylose followed by chromatographic determination of the xylose sugar by ion moderated HPLC to establish a standard.

The results indicate that only xylose sugar appeared in the chromato-

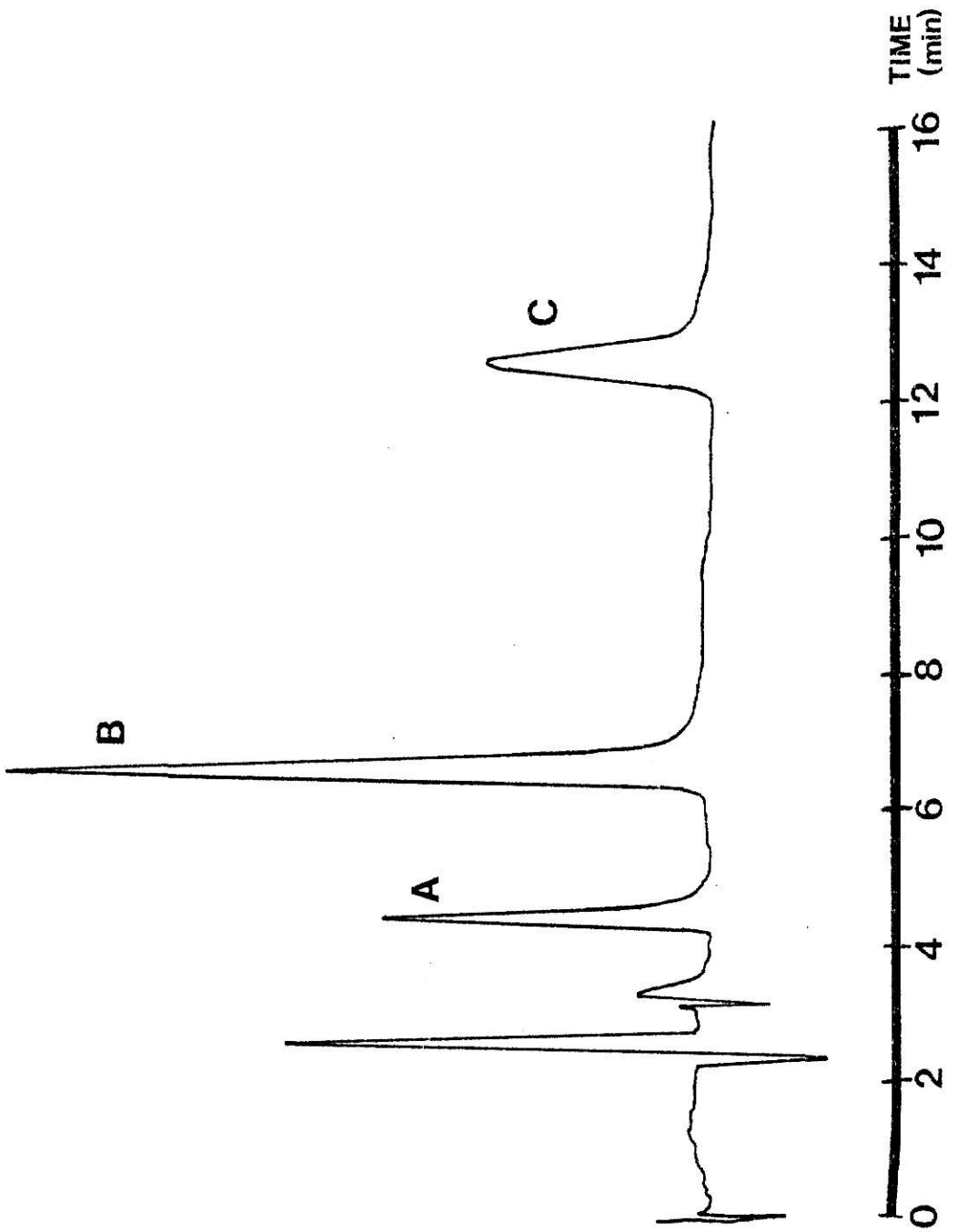


Figure 5. Chromatographic Separation of (A) Hydroxymethylfurfural (B) Furfural and (C) Methylfurfural.

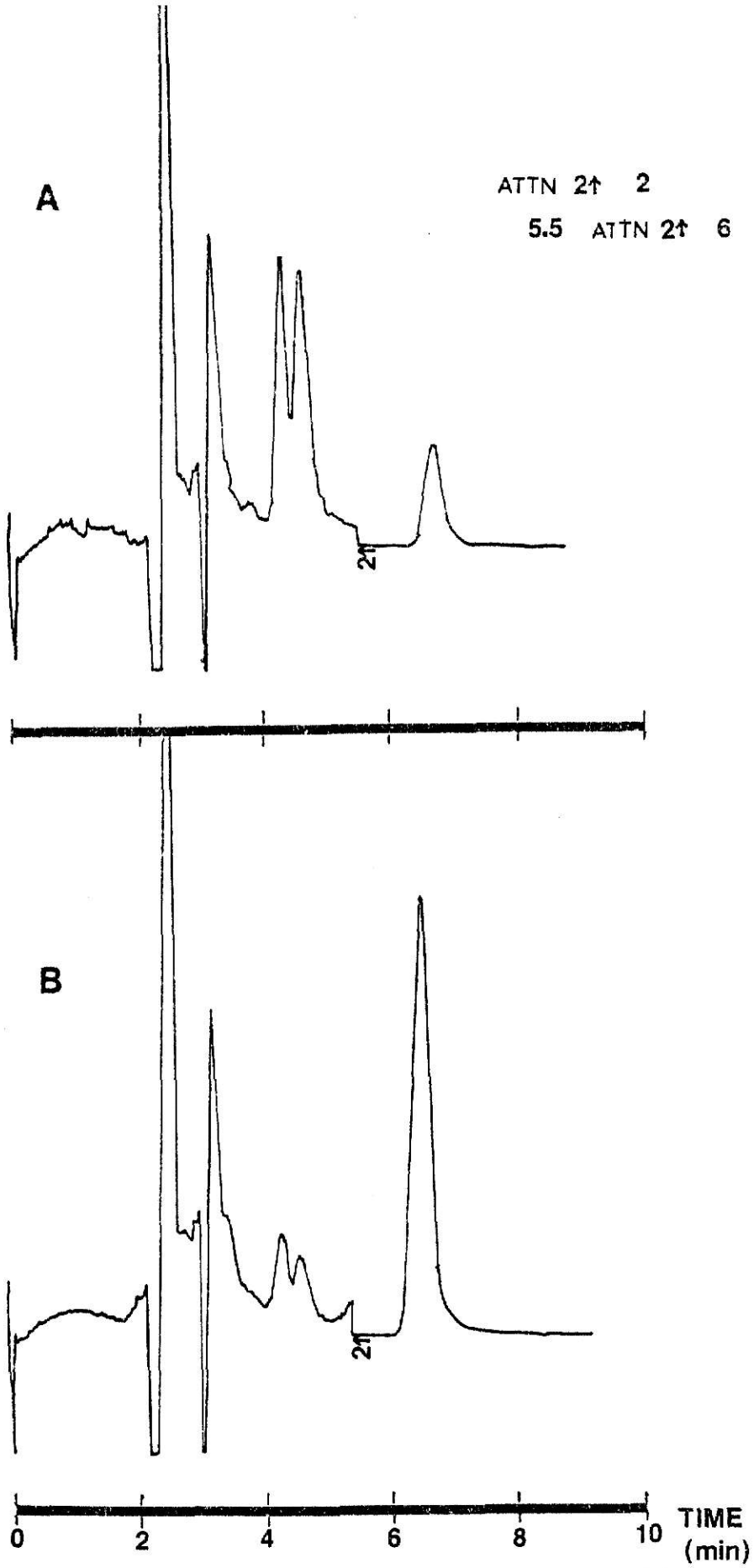
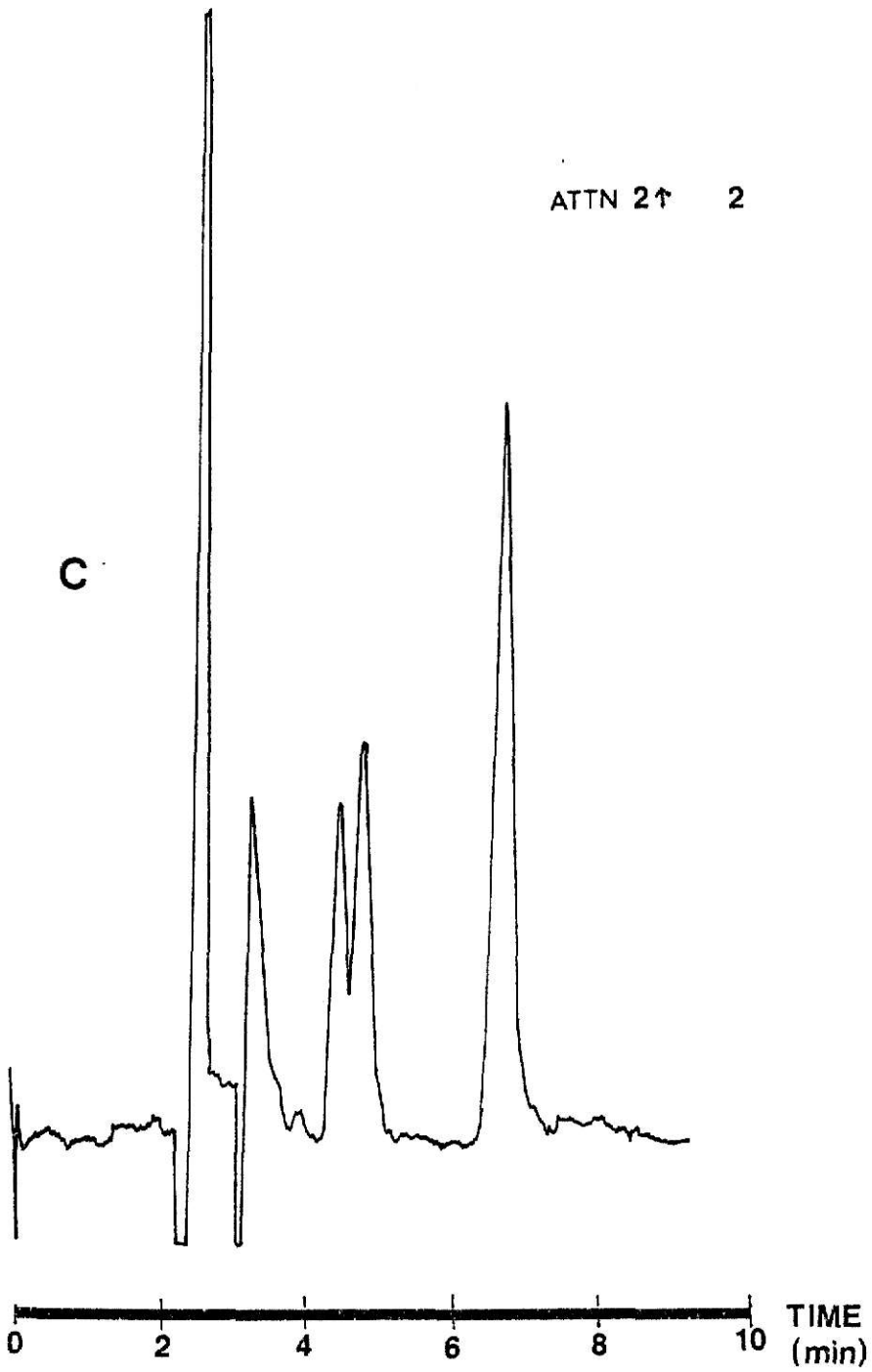


Figure 6. Chromatograms of Reaction Products from (A) Wheat (B) Bran & (C) Flour.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TIME
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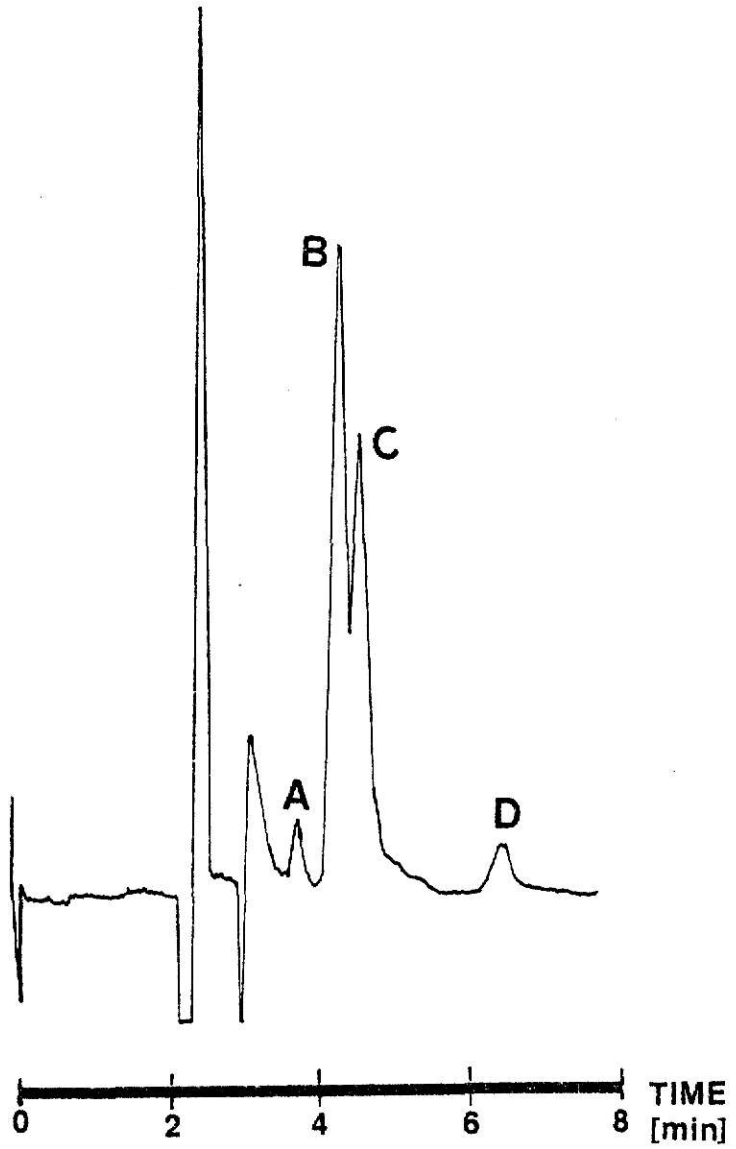


Figure 7. Chromatogram shows Destruction of Hydroxymethylfurfural in 15N H_2SO_4 (95°C/10 min.). (A) Levulinic acid (B) Hydroxymethylfurfural (C) Unidentified decomposition product & (D) Furfural.

gram of the commercial xylan (Sigma Chemical Company) TFA hydrolysate. Using xylose sugar as a primary standard for a calibration curve (Figure 8), the xylan was found to contain 65% xylose. The hydrolysates were also injected into the HPLC-UV system to check the % destruction of xylose. Less than 0.01% of xylose was found converted to furfural during the TFA hydrolysis process. To determine reproducibility of the chromatography and integration of xylose sugar seven injections of a given hydrolyzate were made. Reproducibility was calculated using peak area data. The seven injections yielded a mean peak area of 717,853, a standard deviation of 29029 and a relative standard deviation of 4.04% (Table 1).

Reproducibility of the overall analytical procedure was checked by running seven replicate samples at the same time under indentical conditions. The values used to calculate the mean, standard deviation and relative standard deviation were based on at least duplicate injection of every sample (Table 2). A relative standard deviation of 6.8% was obtained.

The relationship of the furfural production and hydrolysis time of xylose and xylan with equivalent amount of xylose was studied (Figure 9). Under the conditions described, the amount of furfural produced from xylose and equivalent xylan was found to be the same. The results indicated that the conversion to pentoses is rapid and the rate determining step is the dehydration to furfural. The use of xylan as a standard is preferred since it has a polymer structure.

The calibration curve was made by varying the amount of crude xylan to give the corresponding amounts of pure xylan in the range of 0.001 g to 0.24 g (Figure 10). Good linearity was obtained over a wide range Figure 11 shows good linearity up to 0.21 g of corresponding pure xylan (which corresponds

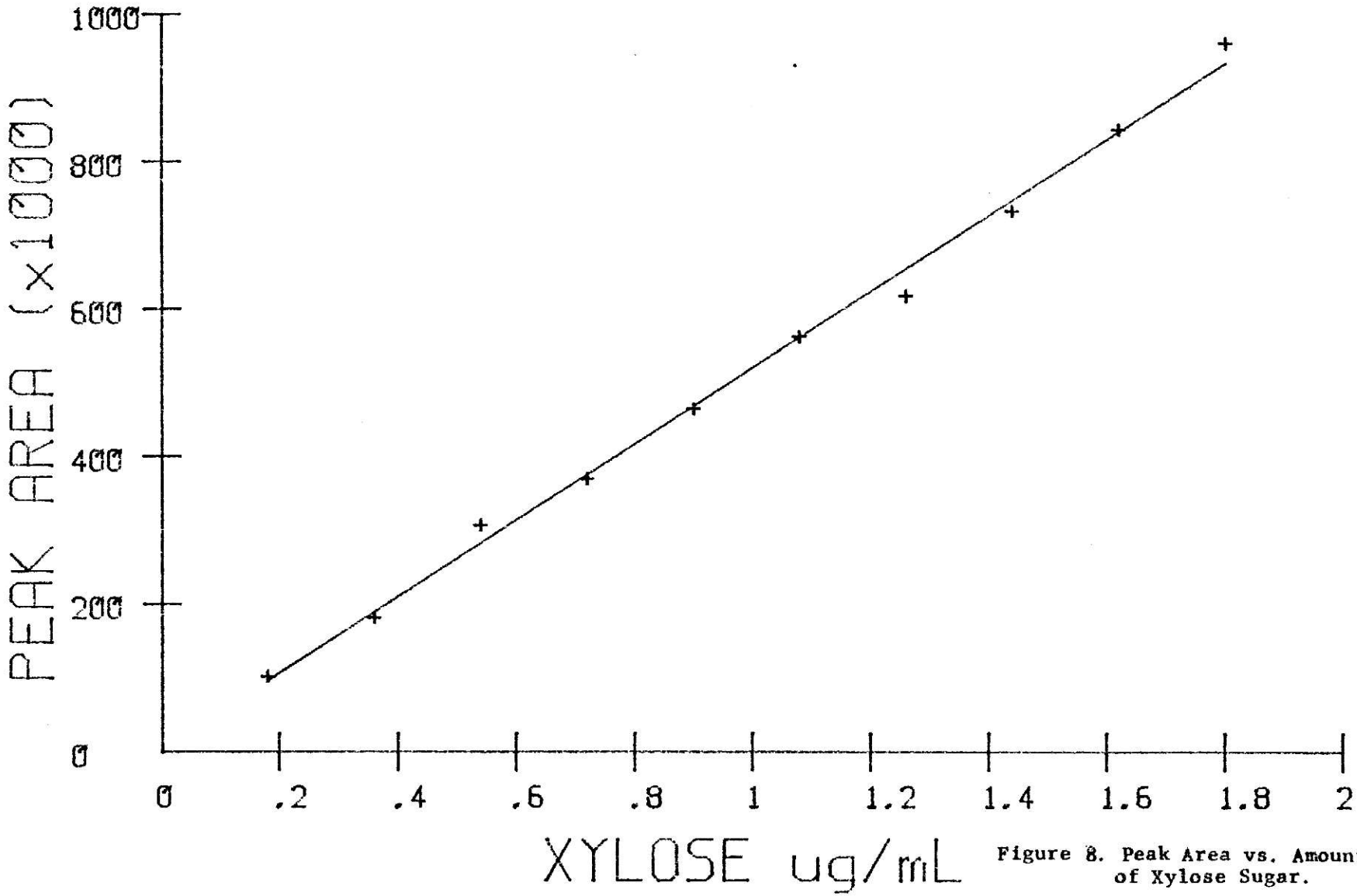


Figure 8. Peak Area vs. Amount of Xylose Sugar.

TABLE 1

REPRODUCIBILITY OF CHROMATOGRAPHY & INTEGRATION

(TFA HYDROLYSIS)

Injection No.	Peak Area
1	721770
2	731150
3	749370
4	731120
5	726310
6	706510
7	658740
Mean Peak Area:	717853
Standard Deviation:	29029
% Relative Standard Deviation:	4.04%

TABLE 2

REPRODUCIBILITY OF ANALYTICAL PROCEDURES

(TFA HYDROLYSIS)

Sample No.	Xylose (µg/ml)
1	1.28
2	1.30
3	1.27
4	1.30
5	1.29
6	1.35
7	1.08
Mean:	1.27
Standard Deviation:	0.09
% Relative Standard Deviation:	6.80%

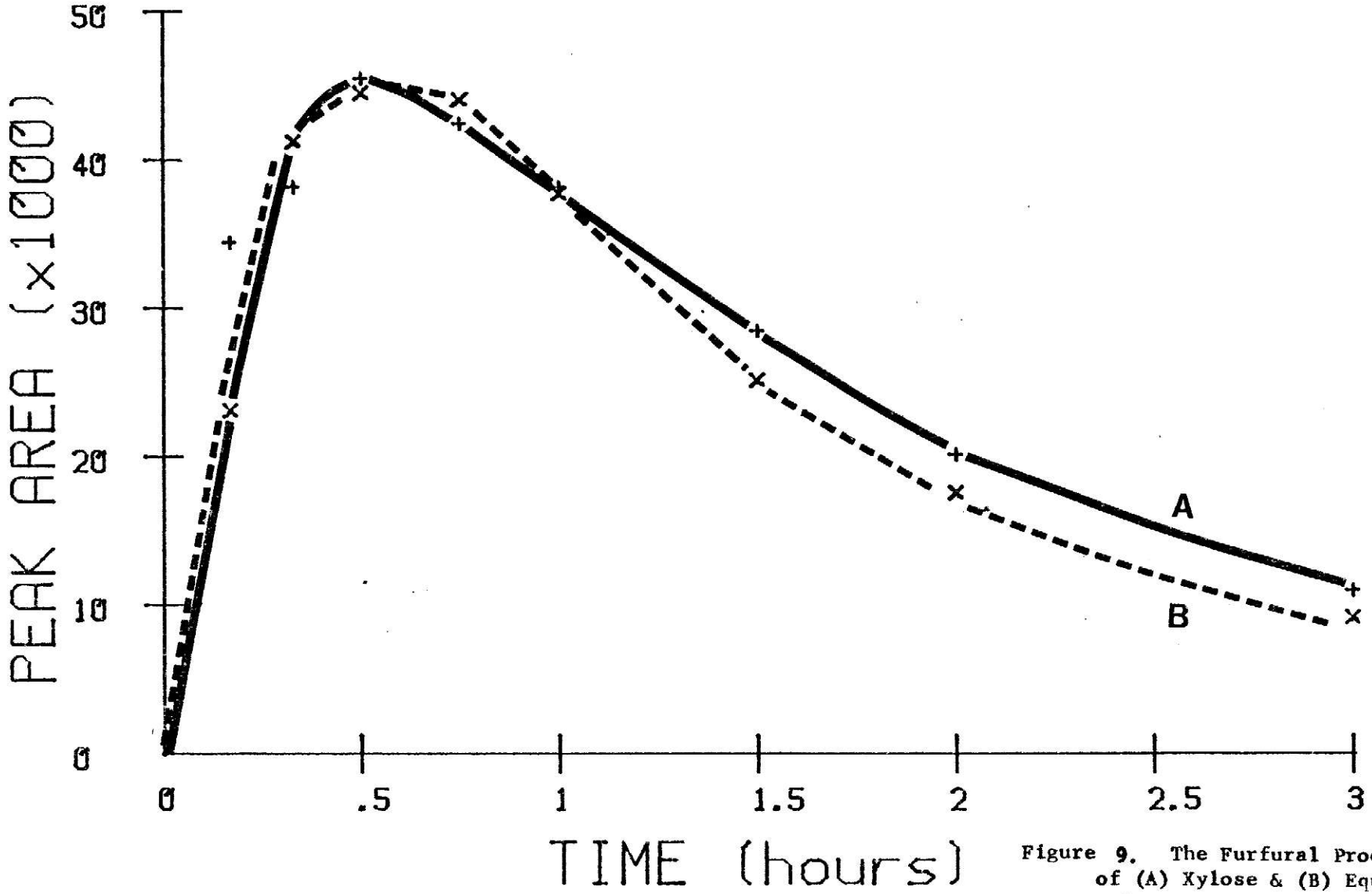


Figure 9. The Furfural Production of (A) Xylose & (B) Equivalent Xylan.

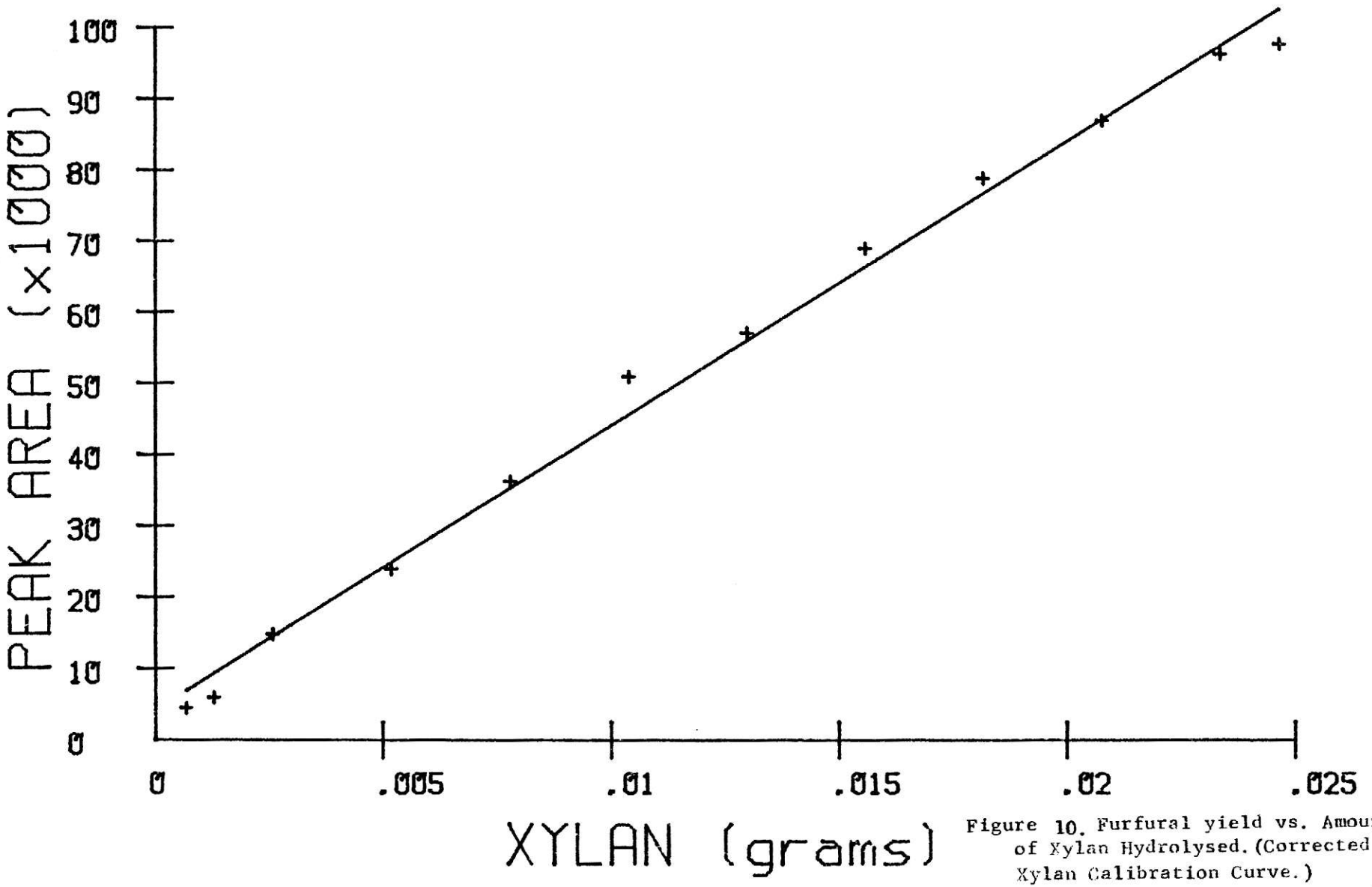


Figure 10. Furfural yield vs. Amount of Xylan Hydrolysed. (Corrected - Xylan Calibration Curve.)

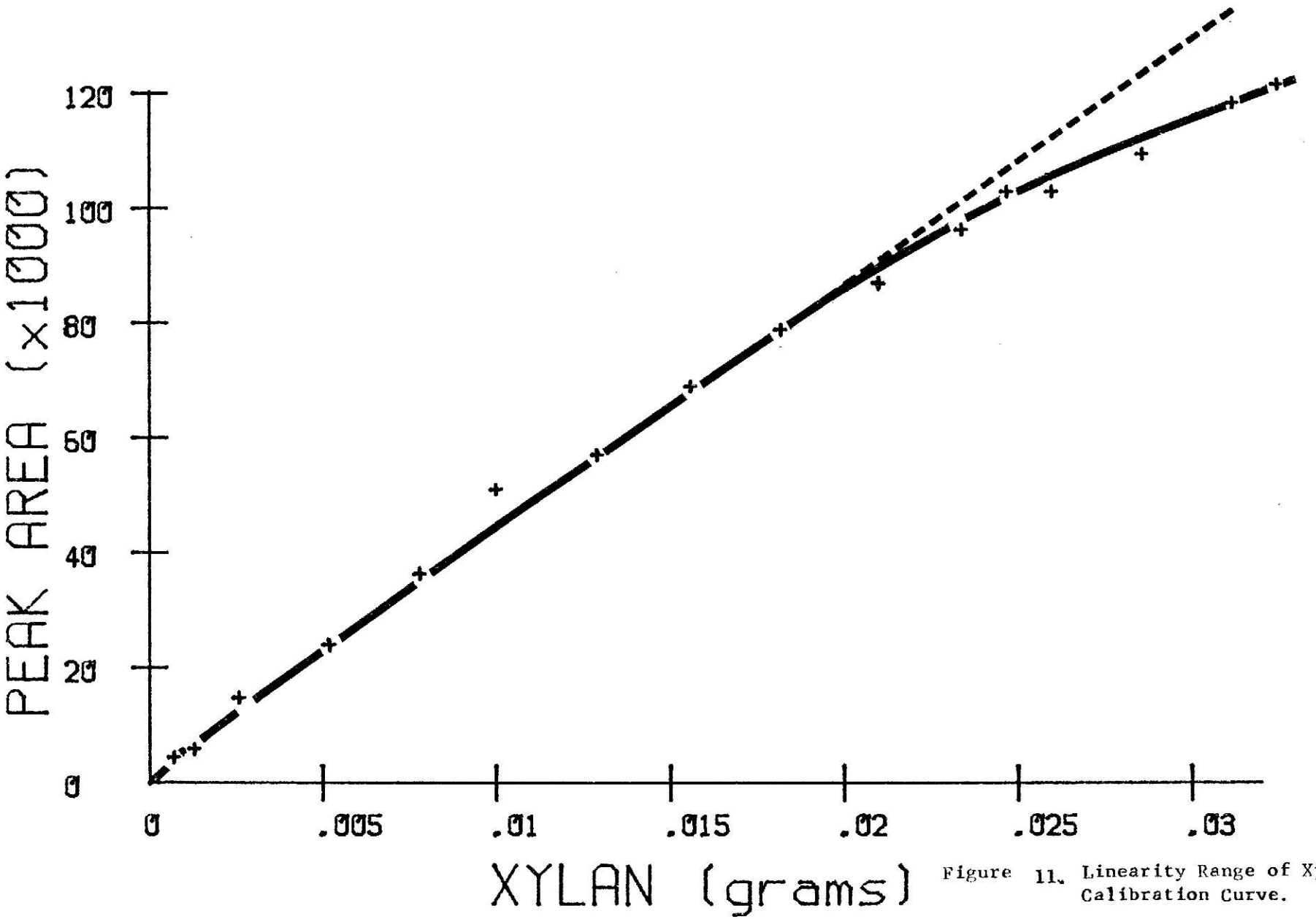


Figure 11. Linearity Range of Xylan Calibration Curve.

TABLE 3

% CONVERSION OF XYLAN TO FURFURAL

Xylan		Furfural	% Conversion
g	M x 10^{-4}	M x 10^{-5}	
.005	15.1	21.0	13.9
.008	24.2	33.0	13.6
.013	39.4	54.0	13.7
.016	48.5	65.5	13.5
.018	54.5	75.0	13.8
.021	63.9	83.0	13.0
.023	69.6	92.0	13.2
		Mean	13.5

to 0-21% pentosan of sample). The percent conversion of xylan to furfural under the conditions used was calculated to be 13.5% (Table 3).

$$\% \text{ conversion} = \frac{\text{concentration of furfural produced} \times 100}{\text{concentration of xylan}}$$

The furfural (99% Aldrich Chemical Company) was distilled under reduced pressure, with temperature kept below 130°C (119-121°C). The colorless freshly distilled furfural was used to make a calibration curve for determination of furfural produced.

Under the hydrolysis condition described, hydroxymethylfurfural can be converted to furfural, even though in small amounts (Fig. 7). The addition of large quantities of starch had an effect on the recovery of furfural from xylan. The result that the recovery of furfural was depressed by the presence of starch was unexpected. Figure 12a showed the depression of furfural production when 0.07 g starch was added to varied amounts of xylan. Figure 12b illustrates the addition of .07, .06 and 0.5 g starch affects the yield of furfural to the same extent. The addition of .01 g has no effect, the suppression of furfural yield can be seen when .03 g starch was added. Figure 12c shows that addition of varied amounts of starch to give a xylan/starch ratio of 3:7 had a negative effect when more than .015 g xylan was present. Suppression of furfural production was greater when larger amounts of xylan were present. The presence of hydroxymethylfurfural produced from hexoses in the reaction mixture could reduce the yield of furfural by causing co-polymerization to occur. Figure 12d shows a decrease in % response in the presence of higher amounts of starch.

A second set of xylan standards (Figure 12b) was prepared by adding

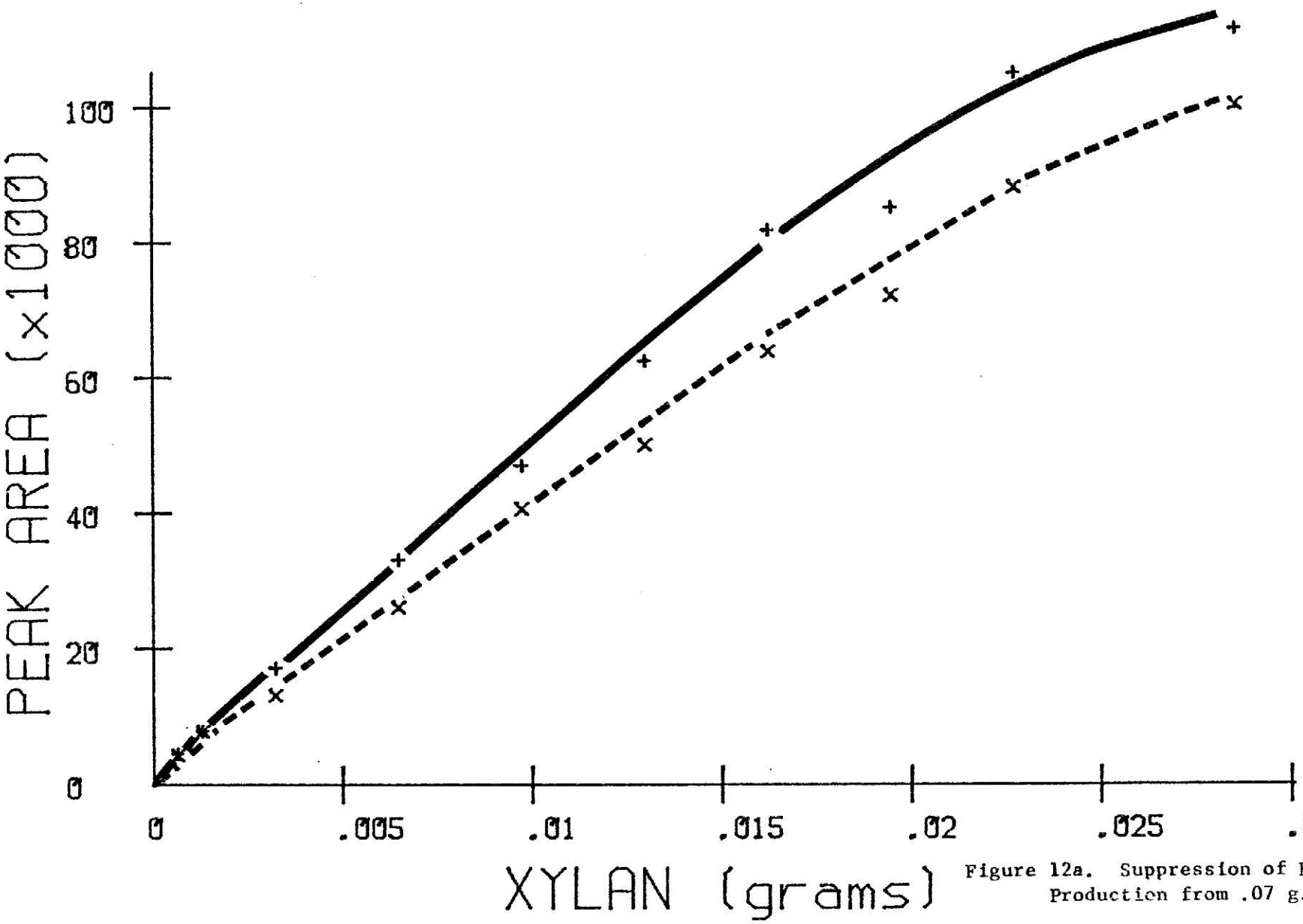


Figure 12a. Suppression of Furfural Production from .07 g. Starch.

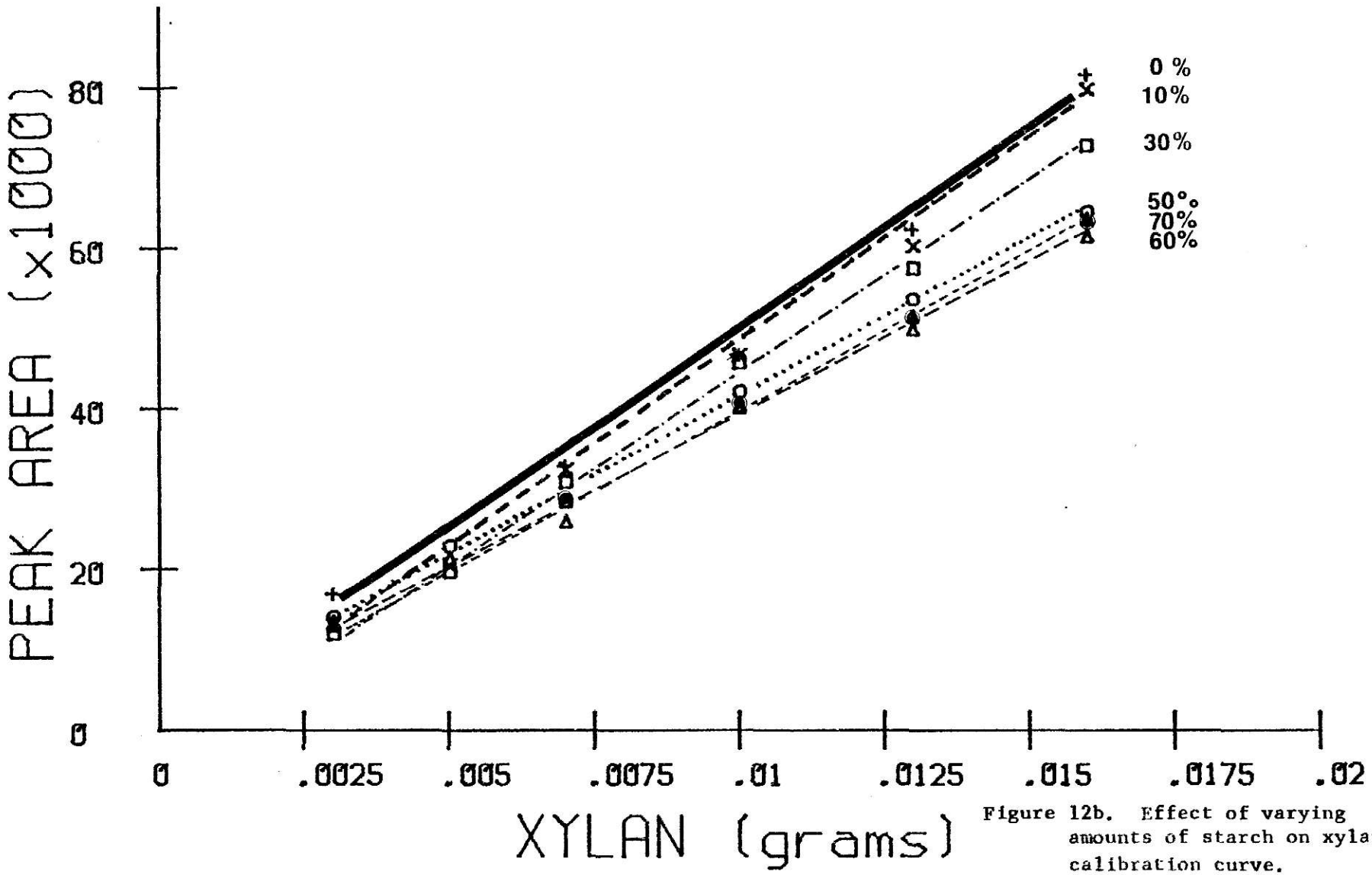


Figure 12b. Effect of varying amounts of starch on xylan calibration curve.

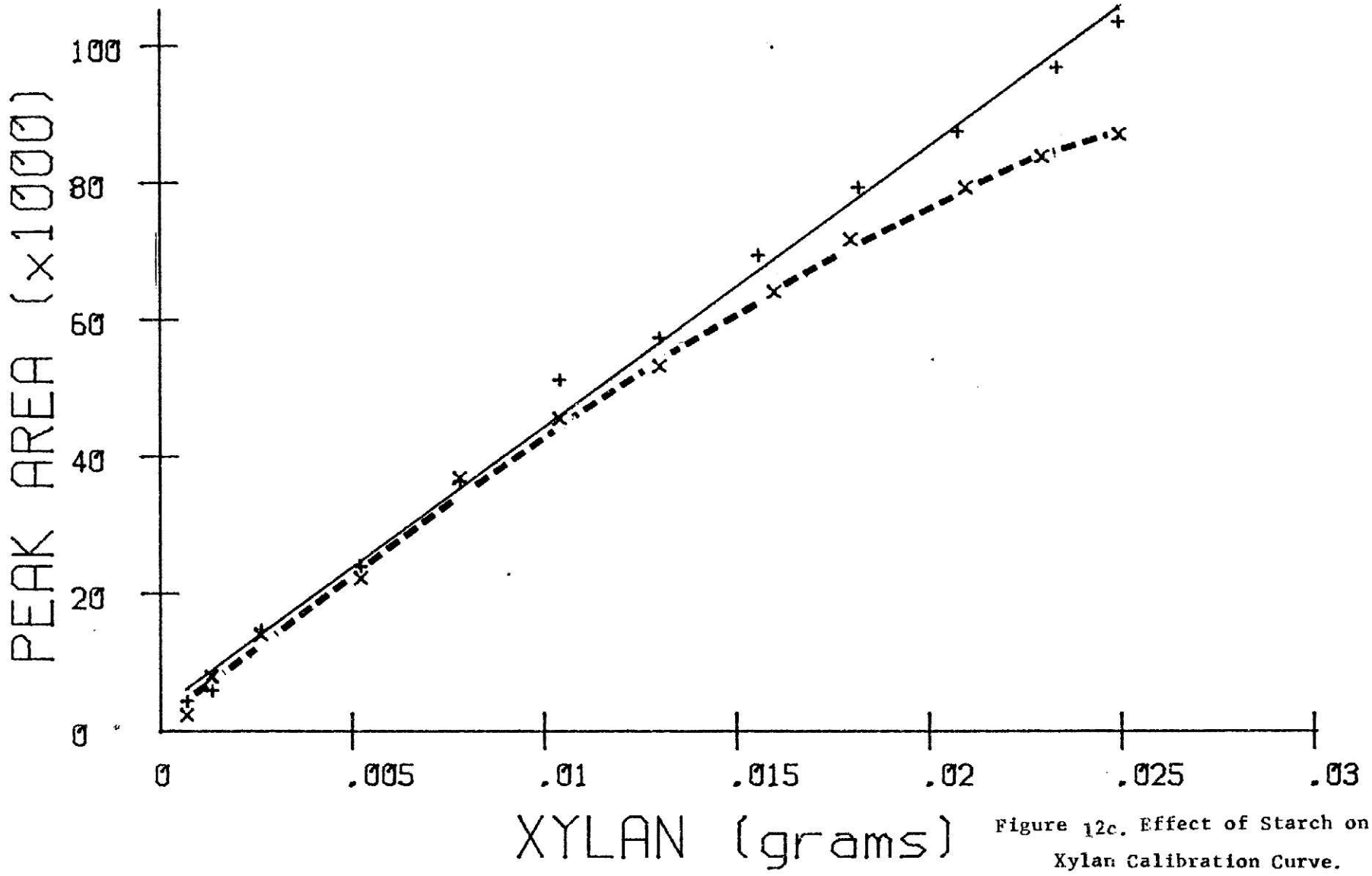


Figure 12c. Effect of Starch on Xylan Calibration Curve. Xylan/Starch ratio = 3:7.

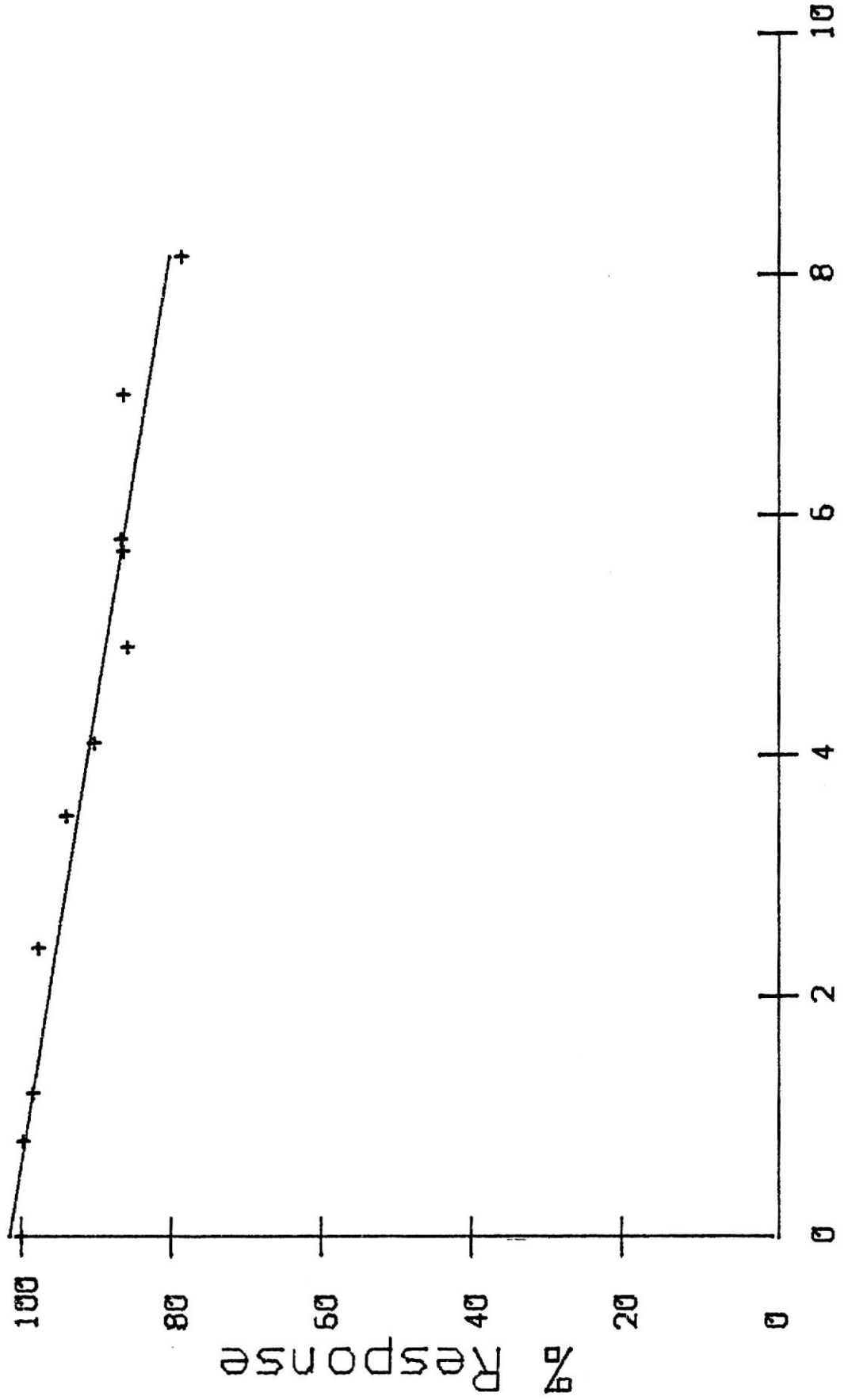


Figure 12d. Mole Ratio of Interference to Analyte vs. % Response of Furfural.

.07, .06, .05 g os starch (from potato, improved Lintner method; Sigma Chemical Company). These being the starch content of a typical flour and other different cereal grains (corn and sorghum (70%), wheat (60%), millet (50%)). The addition of varied amounts of starch lowered the recovery of furfural to about the same extent. Table 4 shows a comparison of results using the 0% starch calibration curve and a 70% starch added curve.

Figure 13 illustrates the difference in furfural yield from xylose and arabinose. Lower furfural yield is obtained from arabinose. Since it is recognized that different sources of pentosan have different xylose/arabinose ratios, using pure xylan as a calibration curve may involve a negative error in the calculation.

Since the composition of the polysaccharides is dependent upon the method employed in extraction (isolation) and subsequent purification, our knowledge of the exact proportions of the different pentose units in the cereal as a whole remain too fragmentary to permit making up a control mixture of pentose sugar in standard pentosan.

The xylose/arabinose ratio of pentosans from different samples can be determined by the aid of cation exchange column and HPLC technique. This will make it possible to establish the calibration curve with a mixture of xylan and araban, which comes close to that existing in the pentosan fraction of the cereal to be analyzed. (See appendix A.)

SAMPLE GROUPING

Samples were divided into 3 different groups based on the structure of the hemicellulose and pentosan of cereal grains and forages.

GROUP A: represents the pentosan of non-endospermic tissue, which is an acid arabinoxylan containing xylose, arabinose, D-glucuronic acid, and

TABLE 4

COMPARISON OF THE RESULTS FROM XYLAN CALIBRATION CURVE (A) & STARCH (.07g)-XYLAN CALIBRATION CURVE (B)

SAMPLE	% PENTOSAN (A)	(B)
FLOUR	1.46	1.85
SORGHUM	1.83	2.32
CORN	2.43	3.07
WHEAT	4.18	5.28
MILLET	2.85	3.61
OATS	7.24	9.16

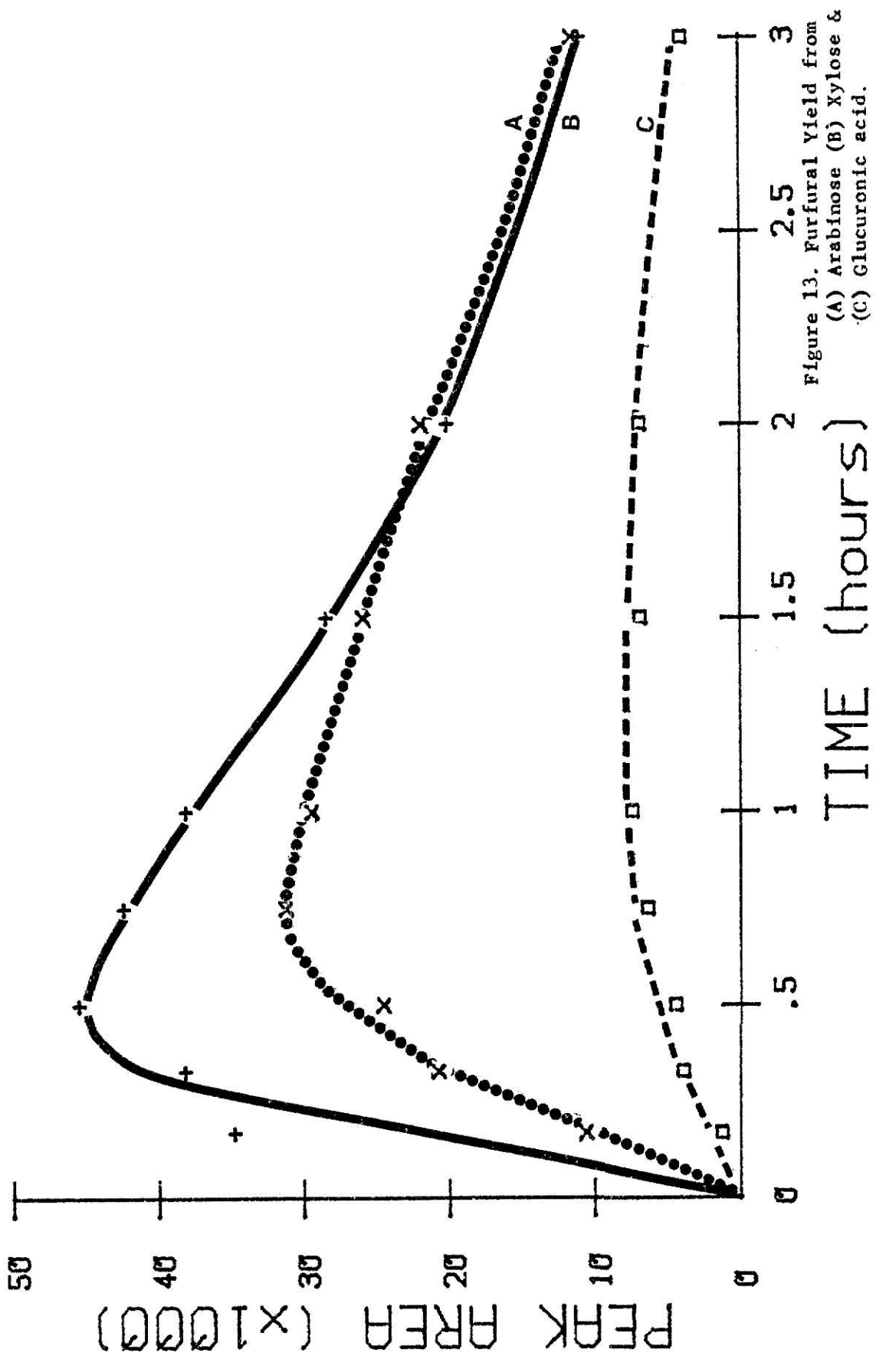


Figure 13. Furfural Yield from (A) Arabinose (B) Xylose & (C) Glucuronic acid.

4-O-methyl-D-glucuronic acid (alfalfa meal, bran, shorts and rice hulls).

GROUP B: represents the endospermic hemicellulose (pentosan) which is free of uronic acid (cereal starches or flours).

GROUP C: represents the pentosans of the whole grain samples (oats, rice, millet, corn, wheat and sorghum).

COMPARISON OF THE PENTOSAN DETERMINATION METHODS

Comparison of the results were made with the literature value (64, 65), a specific method for the determination of pentosans in cereals and cereal products proposed by Cerning & Guilbot (page 20), and a modified method of Disch & Borenfreund (page 19) (Tables 5, 6, 7).

The results for samples of group B and C agree to some extent with the literature value (Table 5). For group A samples, rice hulls which have the highest pentosan content give a lower result than expected. The results for rice hulls from the Cerning and Guilbot's method (Table 6) gives a pentosan content of 10% higher than the HPLC method. This may be due to interferences in the colorimetric method or the limitation of hydrolysis in a sealed tube system. The values obtained for oats, corn and sorghum are in satisfactory agreement with HPLC. For flour sample, the HPLC method gives a little lower value.

The modified method of Disch & Borenfreund was done by the research lab of Industrial Grain Products, Ltd., Montreal, Quebec. The method involves solubilization of the sample, reaction with the phloroglucionol reagent and subsequent boiling for a fixed amount of time. The absorbance of the solution is then read at 552 nm and the results are compared to a calibration curve prepared by use of pure xylose or arabinose. The method is used to determine the pentosan content of starches and flours in most cases. For

TABLE 5

COMPARISON OF THE HPLC METHOD WITH THE LITERATURE VALUE (REPORTED AS % PENTOSAN)

SAMPLE	HPLC	LITERATURE VALUE (1) mean min.	LITERATURE VALUE (1) no. max.
Group A			
Bran	18.3*	18.1[2]	
Shorts	16.4*	13.8[2]	
Germ	5.4*	3.7[2]	
Rice Hulls	15.2	21.4	5
		18.9	26.0
Dehydrated	7.9	13.1	5
Alfalfa Meal		12.9	14.1
Group B			
Flour	1.9*	2.0[2]	
Group C			
Sorghum	2.6	2.5	37
		1.8	4.9
Oats	10.0	7.5	4
		4.5	13.6
Yellow Corn	3.4	6.2	12
		5.9	6.6
Millet	3.8	7.2	1
Paddy Rice	3.7	2.3	84
		1.6	6.3
HRW Wheat	5.9	7.4	3
		6.3	8.3

(1) Composition of cereal grains and forages, Donald F. Miller. Publication 585, National Academy of Sciences, National Research Council, Washington, D.C. 1958.

(2) Compiled for USDA Mimeographed Publication ACE-189 (1942) (based on 13.5% m.b.).

(*) 13.5% m.b.

TABLE 6

COMPARISON OF THE HPLC METHOD (A) WITH CERNING & GUILBOT'S METHOD (B)

SAMPLE	% PENTOSAN (DRY BASIS) METHOD A	METHOD B
Group A		
Rice Hulls	15.7	25.3
Group B		
Flour	1.2	1.6
Group C		
Oats	13.0	13.5
Corn	4.3	4.4
Sorghum	2.3	2.8

TABLE 7

COMPARISON OF THE HPLC METHOD (A) WITH MODIFIED DISCH & BORENFREUND'S METHOD (C)

	% PENTOSAN (DRY BASIS)	
SAMPLE	METHOD A	METHOD C
Group A		
Bran	18.9	20.9
Shorts	17.2	19.0
Germ	5.8	7.6
Group B		
Flour	2.0	2.9

the analysis of bran, the material which is not water-soluble must be hydrolysed by the action of acid prior to dilution.

Table 7 shows that the two methods place the samples in the same order. For wheat derived substances, the HPLC method gives consistently lower results. The higher results of the colorimetric method could be due to the interference from other substances that can absorb light in the same region. The use of xylose as a standard curve when the rate of color formation with xylan is governed by the rate of hydrolysis of the xylan to xylose can cause a negative error to the result.

By reducing the sample size used, the furfural production was not improved. Under the condition used, the samples of high pentosan content (more than 20%) will give low results.

REPRODUCIBILITY OF THE PENTOSAN DETERMINATION

The reproducibility of the chromatography and integration was evaluated by making a series of seven replicate injections of a given reaction mixture. The relative standard deviation of peak areas was determined (Table 8). A relative standard deviation of 1.8% was obtained for these replicate injections.

To determine the reproducibility of the overall analytical procedure (conversion to furfural and chromatography), two sets of seven identical samples (oats: relatively high pentosan content, and sorghum: relatively low pentosan content) were run at the same time under identical conditions. Quantitation of each sample in % pentosan was obtained from the xylan calibration curve. The average value from duplicate injections of each sample was used to calculate the precision. % Relative standard deviations of 4.97 and 5.77% were obtained from oats and sorghum samples respectively(Table 9).

TABLE 8

REPRODUCIBILITY OF CHROMATOGRAPHY & INTEGRATION

(PENTOSAN DETERMINATION OF OATS SAMPLE)

Injection No.	Peak Area
1	39,860
2	39,690
3	41,390
4	38,990
5	39,720
6	39,630
7	40,090
Mean Peak Area:	39,910
Standard Deviation:	734.23
% Relative Standard Deviation:	1.8%

TABLE 9

REPRODUCIBILITY OF ANALYTICAL PROCEDURES

(PENTOSAN DETERMINATION)

SAMPLE NO.	% PENTOSAN	
	OATS	SORGHUM
1	12.75	2.80
2	12.40	2.70
3	12.70	2.50
4	13.30	2.40
5	14.10	2.70
6	13.80	2.70
7	12.60	2.50
Mean:	13.09	2.61
Standard Deviation:	0.65	0.15
% Relative Standard Deviation:	4.97%	5.77%

The reproducibility of the procedure was also checked on a day to day basis. Identical samples were also analyzed in duplicate for pentosan content on consecutive days and the mean values of both days' results are given in Table 10.

INTERFERENCE FROM GLYCURONIC ACID (HEXURONIC ACID)

The problem of interference from hexuronic acid was not completely solved. A more accurate figure for pentosans may be obtained by deducting from the total furfural yield the amount of furfural corresponding to the amount of carbon-dioxide found in the uronic acid analysis (discussed in 33, 35, 36).

Figure 3 shows the furfural yield from D-glucuronic acid is very low when compared to xylan of the same number of moles. When working with samples of low uronic acid content (e.g. whole grain samples), the interference is considered to be negligible. With samples of considerable uronic acid content (e.g., forages, bran, leaf and straw), the correction for the uronic acid should be done to get a more reliable value of pentosans.

TABLE 10

REPRODUCIBILITY ON "DAY TO DAY" BASIS

SAMPLE	% PENTOSAN (d.b.)	
	Day 1	Day 2
Group A		
Bran	18.86	18.97
Shorts	17.71	16.71
Germ	5.63	5.99
Rice Hulls	14.98	15.44
Deh. Alfalfa Meal	7.94	7.87
Group B		
Flour	2.04	1.93
Group C		
Sorghum	2.56	2.57
Oats	9.91	10.04
Yellow Corn	3.37	3.42
Millet	3.89	3.69
Paddy Rice	3.77	3.73
HRW Wheat	5.87	5.89

CONCLUSION

A high performance liquid chromatographic procedure for the determination of pentosan has been developed as a practical method for routine analysis. The procedure involves a 30 minute acid hydrolysis of samples in a batch system, followed by dilution with sodium acetate solution to raise the pH up to a safety range before injecting into the chromatographic system for the separation of furfural and hydroxymethylfurfural in less than seven minutes. A reverse phase ODS μ-Bondapak C_{18} column is used with a methanol/water mobile phase. Detection is obtained by measurement of UV absorption at 280 nm. Good linearity was obtained over a very wide range, which made it possible to determine the pentosan content in many different kinds of cereal products. This included flours, whole grains, other feed ingredients, and milling by-products. The limitation of this method is that samples of more than 20% pentosan will give low values. The method shows good precision with a relative error of approximately 5% depending upon sample type. The results also correlate with accepted methods.

This procedure is considered to be a simple and rapid one and can be modified to an automated system. In one eight hour work day, 30 samples can be analysed in duplicate with less labor needed.

The method has many advantages over the classical methods, beginning with elimination of the distillation step. Because of the high sensitivity of the detector used, the complete conversion of pentosan to furfural is not necessary. Using xylan as a standard under the same conditions, the results are not affected by destruction of furfural.

The detection of furfural itself in the HPLC method eliminates the

problems of the classical methods which require a secondary reaction to determine furfural in the distillate. For a colorimetric method, the formation of the colored-product is time-dependent and the instability of the colored product always causes problems. In the volumetric method of pentosan determination, the reaction of bromine and furfural is time and temperature dependent; the temperature must be controlled at 0-2^{o}C. The long and tedious gravimetric method has a problem of nonstoichiometry of condensation products and solubility of the precipitates.

The HPLC method is a specific method as the use of chromatographic separation makes it possible to simultaneously separate, determine and quantitate the compound of interest. The chromatogram shows clear separation of furfural, hydroxymethylfurfural and methylfurfural. By this method, the furfural is determined without any interferences from other UV absorption compounds.

By the aid of ion-moderated HPLC, the absolute amount of xylose in commercial xylan can be determined. This allows one to use xylan as a standard to make a calibration curve. The xylose to arabinose ratio in pentosan fraction of different samples was also determined. The correction factor obtained was applied to the % pentosan based on xylose only to give a more reliable pentosan value.

Suppression of the recovery of furfural was observed when sufficient amounts of starch were added. For samples of more than 30% starch, it was necessary to add a specific amount of starch to generate a calibration curve.

LITERATURE CITED

1. Patil, S.K. Ph.D. Dissertation, Kansas State University, Manhattan, Kansas, 1972.
2. D'Appolonia, B.L.; Kim, S.K. Bakers Digest. 1976, 50(3), 45-8, 53-4.
3. D'Appolonia, B.L. J. Texture Stud. 1980, 10(3), 201-16.
4. Weipert, D.; Zwingelberg, H. Chem. Abstr. 1980, 93, 112527d.
5. Montgomery, R.; Smith, F. J. Agri. Food Chem. 1956, 4(8), 716-720.
6. D'Appolonia, B.L.; Gilles, K.A.; Osman, E.M.; Pomeranz, Y.In. "Wheat: Chemistry and Technology"; 2nd ed.; Pomeranz, Y., Ed.; American Association of Cereal Chemists: St. Paul, Minnesota, 1971; Chapter 7.
7. D'Appolonia, B.L. In "Industrial Uses of Cereals"; Pomeranze Y., Ed.; American Association of Cereal Chemists: St. Paul , Minnesota, 1973; p. 138.
8. Wilkie, K.C.B. In "Advances in Carbohydrate Chemistry and Biochemistry"; Tipson, R.S.; Horton, D., Ed.; Academic Press: New York, 1979; Vol. 36, pp. 215-64.
9. Dinsmore, H.L.; Nagy, S. J. Food Sci. 1972, 37, 768-770.
10. Dinsmore, H.L.; Nagy, S. J. Assoc. Off. Anal. Chem. 1974, 57(2), 332-5.
11. Aspinall, G.O.; Greenwood, C.T. J. Inst. Brew., London, 1962, 68, 167-78.
12. Preece, I.A.; Hobkirk, R. J. Inst. Brew., London, 1954, 60, 490.
13. Medcalf, D.G.; D'Appolonia, B.L.; Gilles, K.A. Cereal Chem. 1968, 45, 539-49.
14. Preece, I.A.; Hobkirk, R. J. Inst. Brew., London, 1953, 59, 385-92.
15. Fincher, G.B.; Stone, B.A. Aust. J. Biol. Sci. 1974, 27, 117-132.
16. Fincher, G.B.; Sawyer, W.H.; Stone, B.A. Biochem. J. 1974, 139; 535-45.
17. Adams, G.A. Can. J. Res. 1955, 33, 56.
18. Schmorak, J.; Bishop, C.T.; Adams, G.A. Can. J. Chem. 1957, 35, 108.
19. Adams, G.A. Can. J. Chem. 1954, 32, 186.
20. Adams. G.A. Can. J. Chem. 1952, 30, 698.

21. Aspinall, G.O. In "Advances in Carbohydrate Chemistry and Biochemistry"; Wofrom, M.L., Ed.; Academic Press: New York, 1959; Vol. 14, p. 438.

22. Medcalf, D.G.; Cheung, P.W. Cereal Chem. 1971, 48, 1.

23. Dutton, G.G.S. In "Advances in Carbohydrate Chemistry and Biochemistry"; Tipson, R.S.; Horton, D., Eds.; Academic Press: New York, 1973; Vol. 28, pp. 14-23.

24. BeMiller, J.N. In "Advances in Carbohydrate Chemistry and Biochemistry"; Wolfrom, M.L., Ed.; Academic Press: New York, 1967; Vol. 22, pp. 85-106.

25. BeMiller, J.N. In "Starch: Chemistry and Technology"; Whistler, R.L.; Paschall, E.F., Ed.; Academic Press: New York, 1965; Bol. 1, Chapter 20.

26. Feather, M.S.; Harris, J.F. In "Advances in Carbohydrate Chemistry and Biochemistry"; Tipson, R.s., Horton, D., Eds.; Academic Press: New York, 1973; Vol. 28, pp. 174-193.

27. Pigman, W.; Anet, E.F.L.J. In "The Carbohydrage Chemistry/Biochemistry"; Pigman, W.; Horton, D., Eds.; Academic Press: New York, 1972; Vol. IA, pp. 175-186.

28. Anet, E.F.L.J. In "Advances in Carbohydrate Chemistry and Biochemistry"; Wolfrom, M.L., Ed.; Academic Press: New York, 1964, Vol. 19, p. 181.

29. Anet, E.F.L.J. Aust. J. Chem. 1965, 18, 240.

30. Ref. 27, p. 185.

31. Isbell, H.S. J. Res. Natl. Bur. Stand. 1944, 32, 45.

32. Ref. 26, p. 186.

33. Theander, O. In "The Carbohydrate Chemistry/Biochemistry"; 2nd ed; Pigman, W.; Horton, D., Eds.; Academic Press: New York, 1980; Vol. IB, p. 1013.

34. Pervier, N.C. and Gortner, R.A. Ind. Eng. Chem. 1923, 15(11), 1167.

35. Brown, C.A.; Zerban, F.W. "Physical and Chemical Methods of Sugar Analysis"; 3rd ed.; John Wiley & Sons: New York, 1941, pp. 703-8, 904-20.

36. Browning, B.L. In "Wood chemistry"; 2nd ed., Wise,L.E., Jahn, E.C., Eds; American Chem. Society Monograph series, Reinhold: New York, 1952; Vol. II, p. 1159.

37. Hughes, E.E.; Acree, S.F. Ind. Eng. Chem., Anal. Ed. 1934, 6, 123-4.

38. Hughes, E.E.; Acree, S.F. Ind. Eng. Chem., Anal. Ed. 1937, 9, 318-81.

39. Reeves, R.E.; Munro, J. Ind. Eng. Chem., Anal, Ed. 1940, 12, 551.

40. Pervier, N.C.; Gortner, R.A. Ind. Eng. Chem. 1923, 15(11), 1256.

41. Official Methods of Analysis of the AOAC. 10th ed.,; Horwitz, W., Ed.; AOAC: Washington, D.C., 197 ; Method 22.050, 22.051, p. 336.

42. Kline, G.M.; Acree, S.F. Bur. Standards J. Research, 1932, 8, 25.

43. Iddles, H.A.; Robbins, P.J. Ind. Eng. Chem., Anal. Ed. 1933, 5, 55-7.

44. Youngburg, G.E.; Pucher, G.W. J. Biol. Chem. 1924, 61, 741-5.

45. Magistad, O.C. Ind. Eng. Chem., Anal. Ed. 1933, 5, 253.

46. Approved Methods of the AACC. Schaefer, W.C., Ed.; AACC: St. Paul, Minnesota, 1969; Bol. II, Method 52-10.

47. Hampl, J.; Davidek, J.; et al. Getreide u. Mehl. 1963, 13, 73-4.

48. Davidek, J. Nature, 1963, 197, 378.

49. Hampl, J. Chem. Abstr. 1967, 66, 52844k.

50. Schmieder, W. Jena Rev. 1973, 19(2), 131-7.

51. Cracknell, R.L.; Moye, ClJ. 20th Annual Conference, Royal Australian chemical Institute. Canberra, australia, 17-20 August 1970.

52. Dische, Z.; Borenfreund, E. Biochem. Biophys. Acta. 1957, 23, 639-42.

53. Douglas, S.G. Food Chem. 1981, 7(2), 139-45.

54. Cerning, J.; Guilbot, A. Cereal Chem. 1972, 50, 176-84.

55. Tweeten, T.N.; Wetzel, D.L. Paper #97, AACC 66th Annual Meeting, Denver, Colorado, October, 1981.

56. Folkes, D.J. J. Sci Food Agric. 1980, 31, 1011-16.

57. Albersheim, P.; Nevins, D.J.; English, P.D.; Karr, A. Carbohyd. Res. 1967, 5, 340-45.

58. Gere, D. Private Communications.

59. Vogel, A.I. "A Textbook on Practical Organic Chemistry"; 3rd ed., Logmans, Green and Company, London, 1956, p. 169.

60. Hurd, C.D.; Isenhour, L.L. J. Am. Chem. Soc. 1932, 54, 317-30.

61. Allen, B.H.; Chin, H.B. J. Assoc. Off. Anal. Chem. 1980, 63(5), 1074.

62. Scobell, H.D.; Brobst, I.M.; Steele, E.M. Cereal Chem. 1977, 54, 905.

63. Goulding, R.W. J. Chromatogr. 1975, 103, 229.

64. Miller, D.F. "Composition of ceral grains and forages." Publication 585, National Academy of Sciences, National Research Council, Washington, D.C.; 1985.

65. Compiled for USDA Mimeographed Publication ACE-189(1942.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. David L. Wetzel, my major professor, for the advice and encouragement he provided. An acknowledgement is also made to Dr. Paul A. Seib and Dr. Larry M. Seitz for their assistance in serving on my thesis committee.

A special thanks is extended to Mr. Randy L. Wehling for his help and suggestions, and to Mr. Louis Morel, Industrial Grain Products, Ltd., Montreal, Quebec, for providing the useful information and data.

Finally, my fondest appreciation goes to my parents. Without their financial and moral support this goal could not have been achieved.

APPENDIX A

HPLC Determination of Xylose/Arabinose Ratio of Cereal Products

The xylose/arabinose ratio of cereal products was determined by the aid of the ion moderated HPLC technique. The samples were exhaustively hydrolysed to simple sugars with trifluoroacetic acid in a sealed tube reaction followed by chromatographic determination of the sugars. This way the extraction (separation) and purification of pentosan fraction is not necessary. Different sugars can be determined, separated and quantitated at the same time.

Trifluoroacetic acid (TFA) was used for hydrolyzing the polysaccharides because it could be readily removed by evaporation. Nevins et al (57) described the use of trifluoroacetic acid for hydrolysis of pentosan and claimed the yield to be at least equal to that obtained by hydrolysis with mineral acids. Dutton (23) discussed the hydrolysis of polysaccharides to their sugar components with different acids.

The HPLC separation of unsubstituted carbohydrates can be done by using porous ion moderated cation-exchange columns which require operation at an elevated temperature with water as an eluting solvent. The works by Scobell and co-workers (62) and Goulding (63) show the capability of the column in the separation of a series of mono- and oligosaccharides in a relatively short time.

Instrumentation & Chemicals: as described on pages 22, 23.

Experimental Procedures: 20-40 mg of ground samples (depending on the amounts of pentosan in the samples) were weighed out and placed in 5 ml micro reaction vessels. The samples were hydrolyzed with 2N trifluoro-

acetic acid as described on page 23. After evaporation to dryness, 4 ml of deionized water and 0.1 g of mixed resin were added and stirred for 5 minutes. The hydrolyzates were pressure filtered through .47 µm millipore filters (HAWP 01300) using a swinny adaptor.

A 100 µl aliquot of the hydrolyzate was injected into the liquid chromatograph using the following conditions: mobile phase as described (page 23); flow rate 0.6 ml/min.; refractive index attenuation 8; column Aminex HPX-87 (heavy metal form) 300 x 7.8 mm (Bio-Rad Laboratories); column temperature 85°C. The integrator was programmed as described on page 24. INTG ( ) Key function #4 was performed.

Results & Discussion: The chromatograms show the separation of 4 major sugars within 17 minutes. The order of retention observed is glucose < xylose < galactose < arabinose. Since the response factor of xylose and arabinose is equal, the ratio of peak area was used to calculate the xylose/arabinose ratio.

TABLE A-1

Samples	Xylose/Arabinose	% Pentosan (based on xylose only)	Corrected % Pentosan
Rice Hulls	6.2	15.2	16.2
Wheat Bran	1.7	18.3	22.0
Deh. Alfalfa Meal	2.4	7.9	9.1
Rice	4.7	3.7	4.0
Oats	5.0	10.0	10.5
Millet	1.0	3.8	4.9
Sorghum	1.2	3.6	3.3
Wheat Flour	1.4	1.9	2.3

From data of Figure 13, under the condition used for pentosan determination, arabinose produced only 55% of the furfural produced by an equivalent amount of xylose. By knowing % arabinose in a pentosan, a corrected % pentosan can be calculated by using a correction factor.

$$\text{correction factor} = 1 - 0.45\ (\%\ \text{arabinose}/100)$$

$$\text{corrected \% pentosan} = \frac{\text{uncorrected \% pentosan}}{\text{correction factor}}$$

Conclusion

Samples with high pentosan content and low xylose/arabinose ratio will give a poor result for the pentosan content when using xylose as a calibration curve. For millet, sorghum and wheat flour with a xylose/arabinose ratio of approximately 1:1, the error caused by using xylose as a calibration curve is in the range of 20%. Wheat bran which has relatively high pentosan content with a xylose/arabinose ratio approximately 2:1 also gives a much lower result than the corrected value.

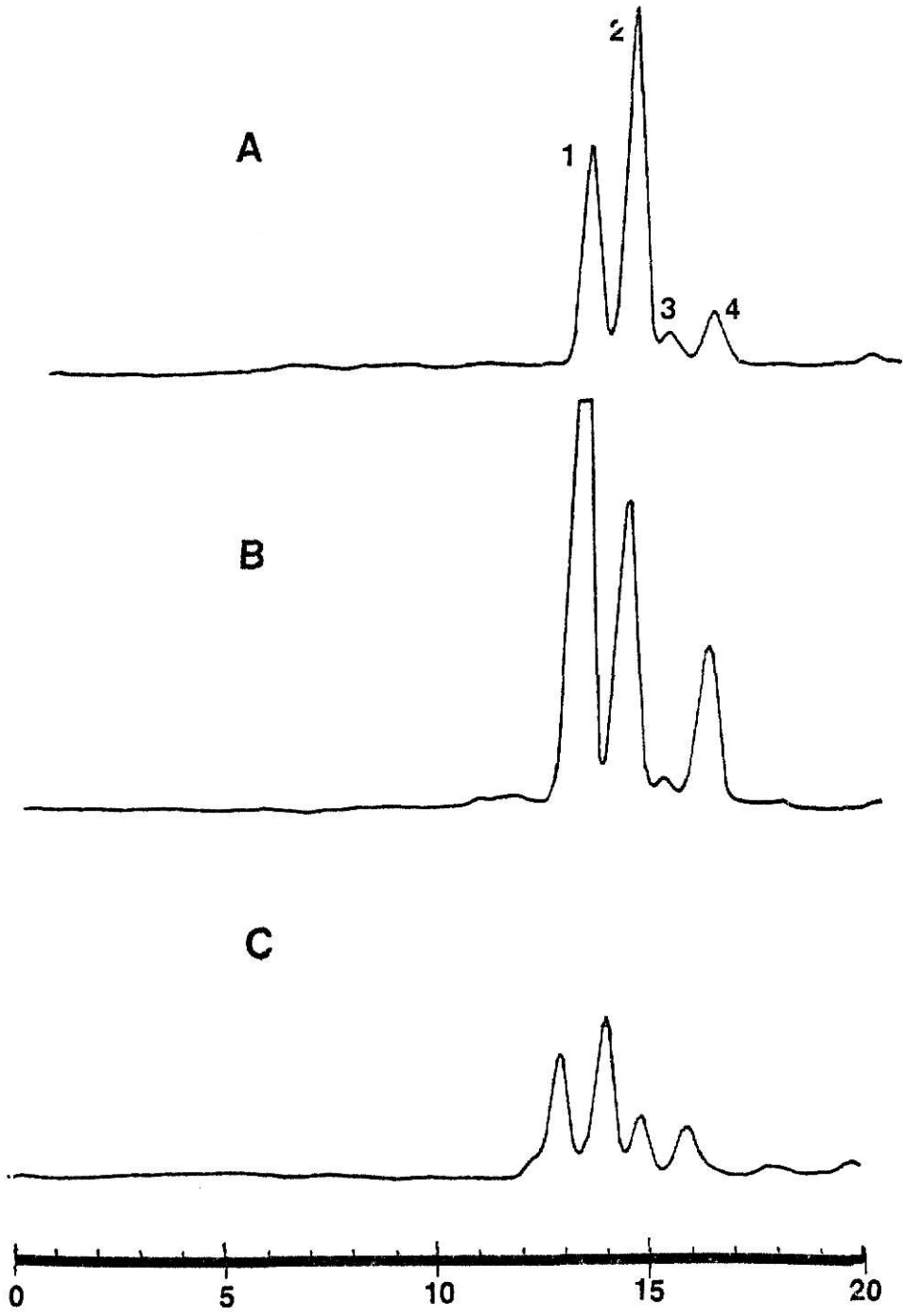
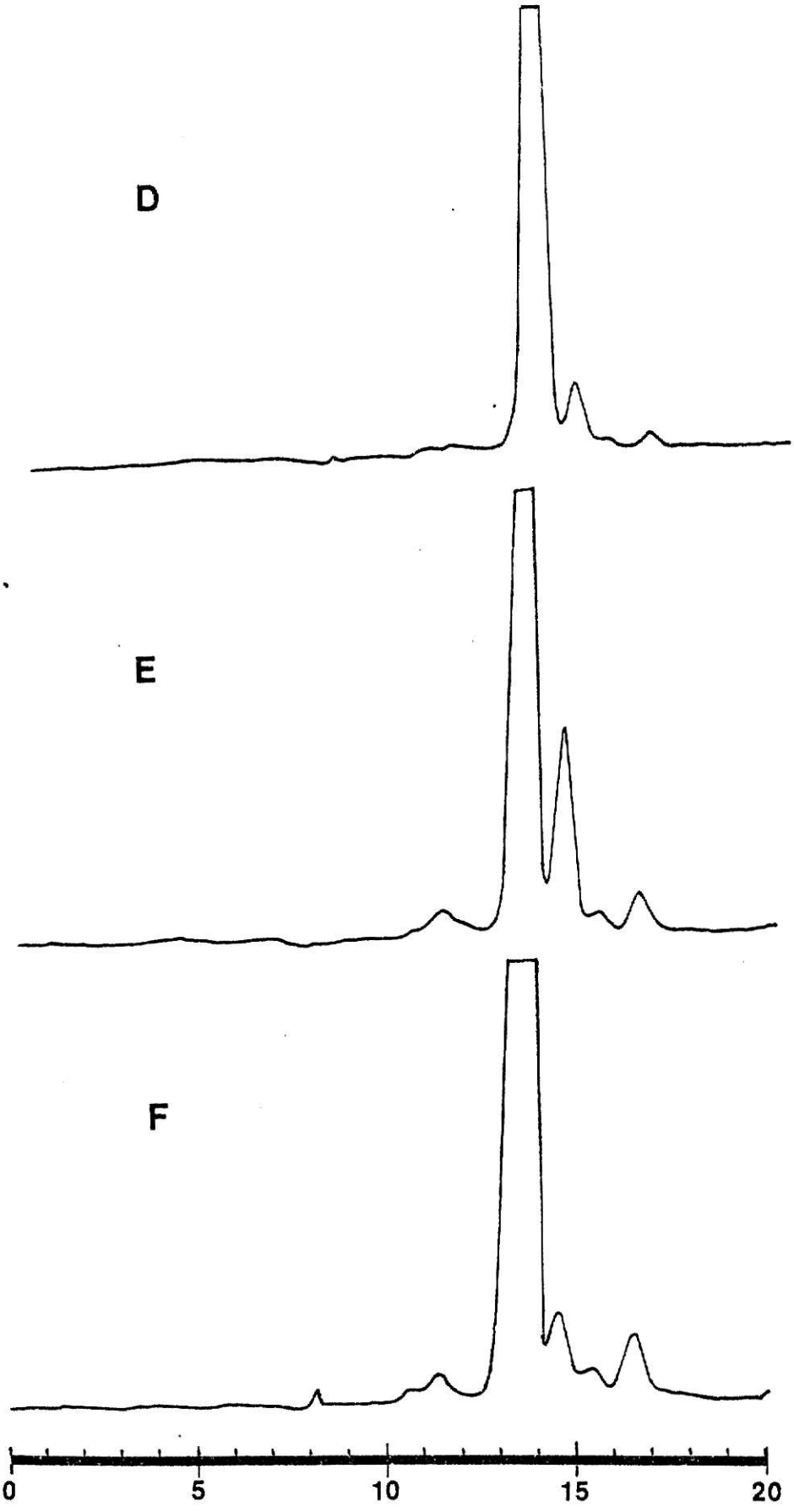


Figure A-1. Chromatograms show separation of 1. glucose 2. xylose 3. galactose & 4. arabinose from trifluoroacetic acid hydrolysates of (A) rice hulls (B) bran (C) dehydrated alfalfa meal (D) rice (E) oats .02g & (F) millet .04g.


D
E
F
0
5
10
15
20

APPENDIX B

Potential of Using HPLC for Simultaneous Determination of Starch and Pentosan

With the aid of high performance liquid chromatography, the furfural and HMF (from acid hydrolysis of pentosan and starch respectively) can be separated and quantitated at the same time. This makes it possible to simultaneously determine starch and pentosan content of cereal products. Note that under the conditions used, cellulose cannot be hydrolyzed to glucose and HMF. As shown in the chromatograms of various samples (Figure 6), the unstable HMF can be converted to several decomposition products. The unidentified decomposition product, which has an observed capacity factor ($k' = 1.8$) close to HMF ($k' = 1.7$) and cannot be completely resolved from the HMF parent peak, is a major problem. There are 3 different ways in which the determination of starch can be done.

I) Use a mild condition: by reducing the acid concentration to prevent decomposition of HMF and increasing hydrolysis time to obtain furfural production in a steady state segment of the curve (furfural production vs. time). 7N H_2SO_4 was used with a hydrolysis time of 3.25 hours. Under this condition, the conversion of HMF to the unidentified peak with $k' = 1.8$ was not observed on the chromatogram.

II) Use the optimum condition for pentosan determination (15N H_2SO_4/ hydrolysing time = 30 min.): the quantitation was done by using a response factor of HMF (determined at zero time) and a response factor of the unidentified decomposition product (X) in terms of the weight of parent HMF converted to product X (denoted as area) under the condition used.

Response factor of HMF:

$$R_{HMF} = \frac{\text{HMF (g)}}{\text{Peak Area}}$$

Under the chromatographic condition used

$$R_{HMF} = 7.7 \times 10^{-12} \text{ g/unit area}$$

Relative response factor of the unidentified decomposition product (X):

HMF was weighed to give an amount of HMF equivalent to that produced from a sample containing 50-70% starch. HMF was hydrolysed by 15N H_2SO_4 as described on page 26. Under this condition, all HMF was converted to X.

Relative response factor of X in terms of HMF = $\frac{\text{HMF (g)}}{\text{Peak Area (x)}}$

$$R_x = 1.4 \times 10^{15} \text{ g/unit area}$$

Total HMF (from starch) = $R_{HMF}(\text{Area}_{HMF}) + R_x(\text{Area}_x)$

III) Use a drastic condition to completely convert HMF to the unknown product (X). The preliminary investigation was done on the destruction of HMF (in 15N H_2SO_4) from a corn sample. The result showed that by increasing the hydrolysis time until all HMF converted to X, the furfural production was on a declining portion of the curve (furfural production vs. time).

EXPERIMENTAL

Instrumentation and Chemicals: as described on page 27.

Acid hydrolysis of samples: as described on page 26. (7N H_2SO_4 was used for experiment I with hydrolyzing time of 3.25 hours)

Chromatography of Reaction Mixture: as described on page 26. The integra-

tor was programmed to change slope sensitivity from 0.1 to 0.25 and attenuation from 2 to 4 at 5.5 minutes. A use of guard column prior to the analytical column is recommended to protect the analytical column.

RESULTS AND DISCUSSION

Experiment I: 7N H_2SO_4/Hydrolysis time 3.25 hours/95^{o}C. The calibration curve was made by varying amounts of starch (from Potato, Improved Lintner method; Sigma Chemical Company). Good Linearity was obtained from measurement of HMF peak area from varied amounts of starch in the range of 0.01 - 0.09 g (which corresponded to 10-90% starch in samples). (Figure B-1.)

Corn samples which had been determined for starch content by polarimetric method were analyzed and the results compared. Table B-1 shows that both methods put the sample in the same order; however, the HPLC method gives higher values for every sample.

TABLE B-1

COMPARISON OF HPLC METHOD (7N H_2SO_4)(A) WITH POLARIMETRIC METHOD*(B)

Corn Sample #	% STARCH	
	METHOD A	METHOD B
121	72.86	63.66
127	70.04	59.36
128	72.36	62.90
719	70.92	56.79
725	68.73	55.18

* The data was supplied by Mr. David Gottneid, American Maize-Products Co., Hammond, Indiana.

Experiment II: 15N H_2SO_4/Hydrolysis time 30 min./95^{o}C.

Calibration curve, Figure B-2, was made by varying amounts of starch. The peak areas of both peaks were multiplied by their response factors. Total HMF (from starch) was the sum of the two values.

$$\text{Total HMF (from starch)} = R_{HMF}(Area_{HMF}) + R_x(Area_x)$$

Since the relative response factor of X is greater in magnitude than the response factor of HMF itself. The value of $R_{HMF}(Area_{HMF})$ which corresponded to the amount of HMF left was negligible when compared to the HMF converted to X $[R_x(Area_x)]$. The relationship of $R_x(Area_x)$ and different amounts of starch shows linearity within the working range (corresponds to sample of 10-90% starch).

Conclusion:

Although the use of 7N H_2SO_4/3.25 hours gave reasonable results for the simultaneous determination of starch and pentosan, the long hydrolysis time (3.25 hours) made it less desirable for routine analysis. The attempt to quantitate both HMF and decomposition product peak under the condition of 15N/30 min. was done. The linearity of the calibration curve showed good potential for the simultaneous determination of starch along with pentosan from the 7 minute chromatogram.

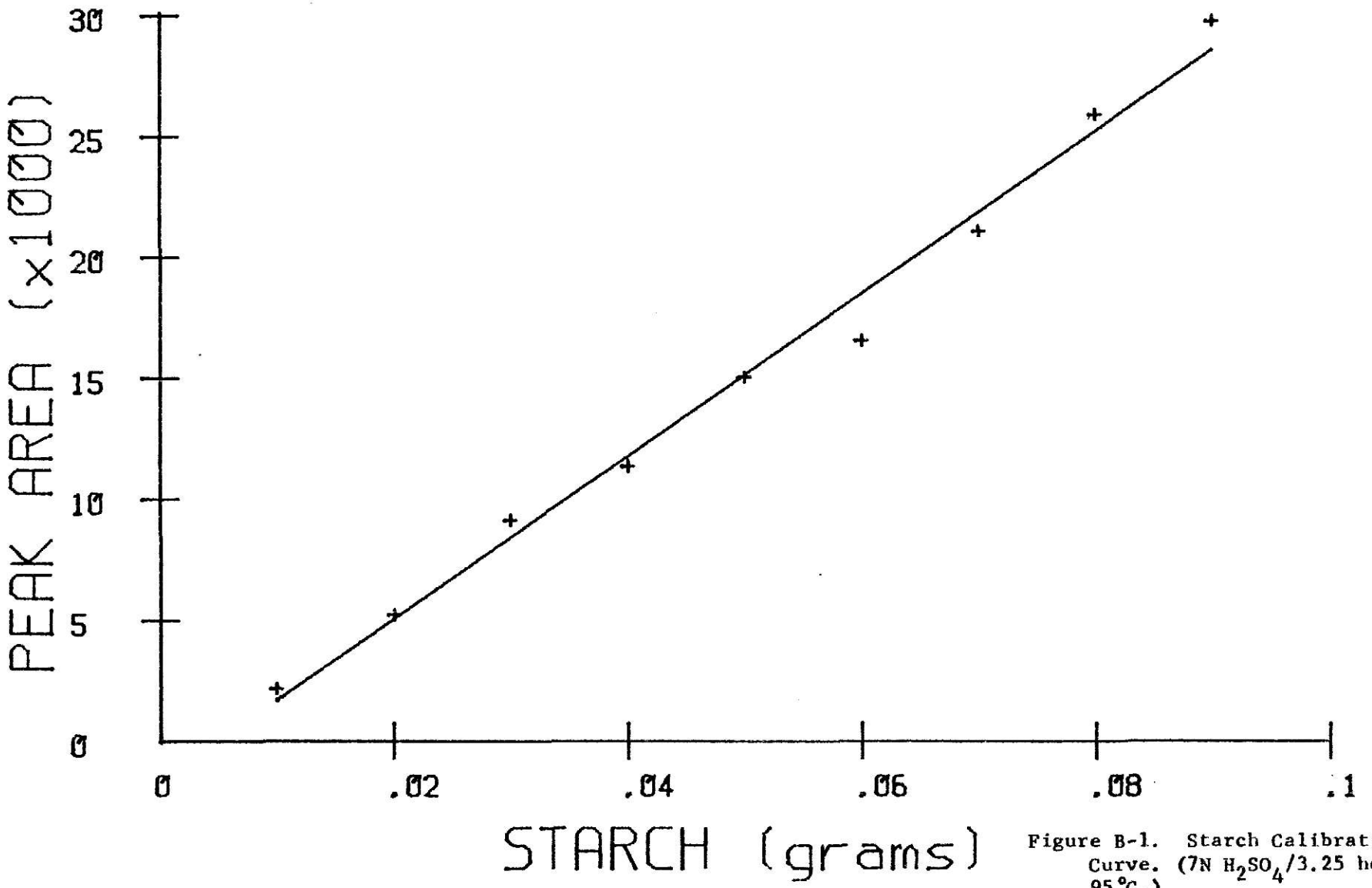


Figure B-1. Starch Calibration Curve. (7N H_2SO_4/3.25 hours/ 95°C.)

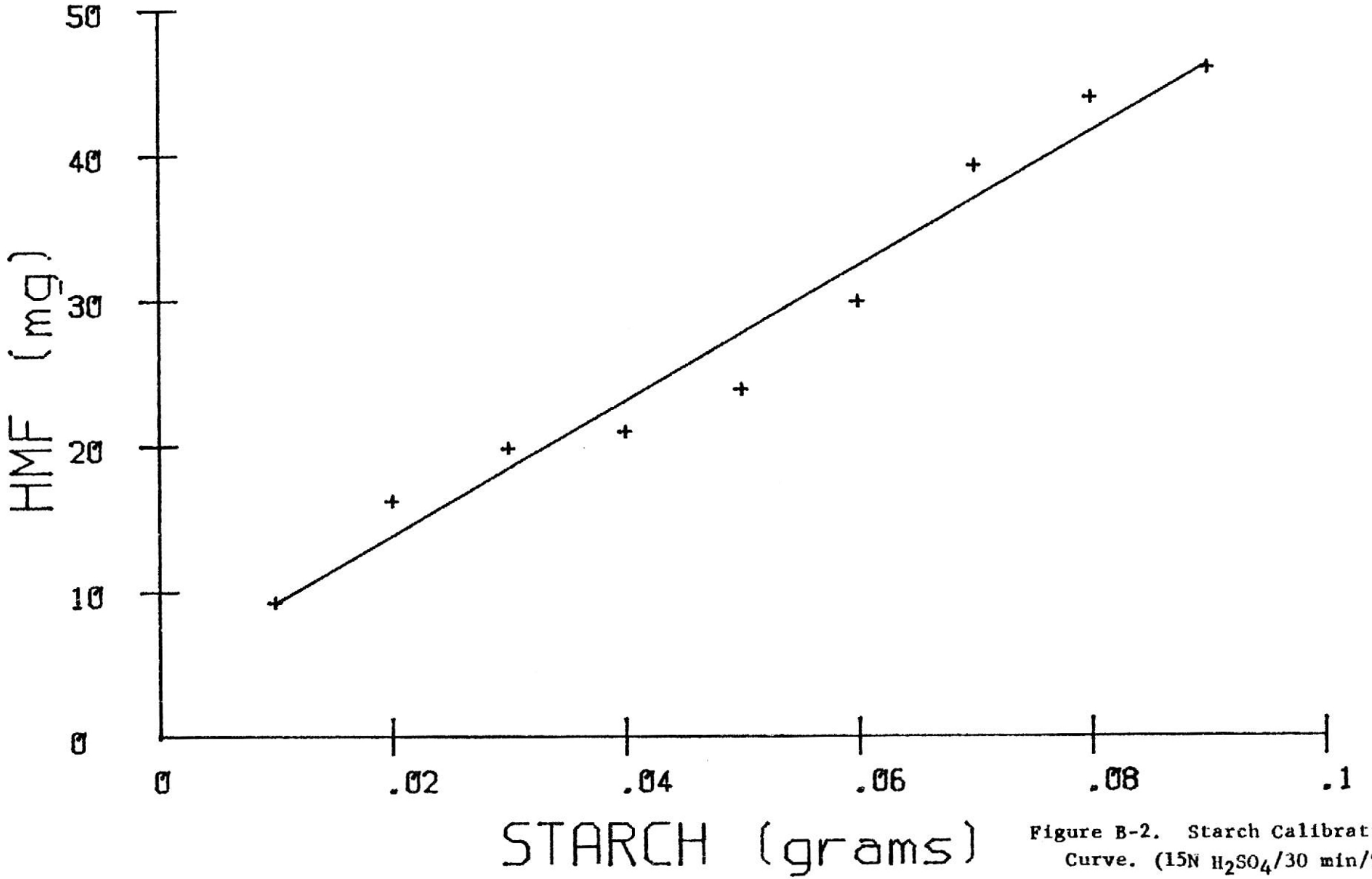


Figure B-2. Starch Calibration Curve. (15N H_2SO_4/30 min/95°C)

APPENDIX C

Recommended Procedure for Pentosan Determination

A. Group A samples (pentosan 10-20%, starch 0-20%)

a. Hydrolysis of samples

Procedure 1-1 Weigh 100 mg of sample into 60 ml sealed glass bottles.

1-2 Add 25 ml of 15N H_2SO_4 to the sample and a magnetic stirrer bar.

1-3 Place the bottles in aluminum heating blocks which have been heated on a hot plate with 6 position stirrer at 95°C for 30 min. (Ethylene glycol bath with an overhead stirrer can be used on top of the hot plate, this allows the heating of 10-15 bottles at the same time.)

1-4 Allow to cool for 45 minutes.

1-5 Pull out the reaction mixture with 5 ml syringe and pressure filter through Whatman No. 5 filter paper with the aid of a Swinny adaptor.

1-6 Pipette 1 ml of the filtrate into a 10 ml volumetric flask and dilute to volume with 2M sodium acetate solution.

1-7 Inject 20 µl of aliquot into the chromatographic system.

b. Preparation of the Calibration Curve

Procedure 2-1 Dry xylan (or xylose) in a vacuum oven at 85°C for 4-5 hours.

2-2 Weigh 0.002, 0.005, 0.01, 0.015, 0.02, 0.025, 0.03, 0.035 g of xylan (containing approx. 65% xylose) into 60 ml sealed glass bottles (multiply the weight by 0.65 and use the values to plot curve). (If pure xylose is used, weigh 0.001, 0.002, 0.005, 0.0075, 0.01, 0.015, 0.02, 0.025 g.)

2-3 Repeat steps 1-2 through 1-7.

B. Group B samples (pentosan 5-10%, starch 40-70%)

a. Hydrolysis of samples

Procedure Same as Group A samples (procedure 1).

b. Preparation of the Calibration Curve

Procedure 3-1 Repeat steps 2-1 and 2-2.

3-2 Add 0.07 g starch into each bottle.

3-3 Repeat steps 1-2 through 1-7.

C. Group C samples (pentosan 1-2%, starch 70%)

a. Hydrolysis of samples

Procedure Same as Group A samples (procedure 1) except 200 mg of sample is used.

b. Preparation of the Calibration Curve

Procedure Same as Group B samples (procedure 3).

ANALYTICAL HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF PENTOSAN AS FURFURAL IN THE PRESENCE OF HYDROXYMETHYLFURFURAL

by

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Bangkok, Thailand, 1979

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1982

Abstract

A high performance liquid chromatographic method has been developed for the determination of pentosan as the furfural reaction product in the presence of other carbohydrates. This simple, rapid, reliable and specific method for pentosan determination (typical precision of 5% relative depending upon sample type) is intended for routine application. The results obtained correlate with the accepted method. Furfural produced from pentosan during a 30 minute batch reaction is determined in the liquid phase reaction mixture. Separation of furfural from hydroxymethylfurfural, methylfurfural and other (280 nm) absorbing materials in the hydrolyzates is accomplished using a reverse phase separation mode with μ Bondapak C_{18} column and a methanol/water solvent system.

The calibration curve was linear over a wide range corresponding to samples of 1-20% pentosan content. The apparent limitation of the procedure is that samples reported to contain more than 20% pentosan give lower values by this method.

By the aid of ion-moderated HPLC, the absolute amount of xylose in trifluoroacetic acid hydrolyzate of commercial xylan was determined. It was shown that xylose or xylan gave identical conversion to furfural under the conditions of the new procedure, thus xylan or xylose can be used to make a calibration curve. The effect of starch on furfural production was measured and the ratios of xylose to arabinose in the pentosan fraction of different samples were determined to calculate the sample dependent variation resulting in the expression of pentosan as furfural.