Studies on pentosans in Indian wheat varieties varying in *chapati* making quality

A Thesis

Submitted to the Department of Biochemistry

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In fulfillment of the requirement for the Degree of

Doctor of Philosophy In Biochemistry

By

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May 2009

Dedicated to

My beloved Parents and Teachers

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DECLARATION

I hereby declare that the thesis entitled "**Studies on pentosans in Indian wheat varieties varying in *chapati* making quality**" submitted to the University of Mysore for the award of the Degree of Doctor of Philosophy in Biochemistry is the result of research work carried out by me under the guidance of **Dr. P.V. Salimath,** Head, Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore during the period of November 2003 - November 2008.

I further declare that these results have not been submitted for any other degree or fellowship.

Place: Mysore **Revanappa**

Date: May 2009

Dr. P.V. Salimath,
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CERTIFICATE

This is to certify that the thesis entitled "**Studies on pentosans in Indian wheat varieties varying in *chapati* making quality**" submitted by Mr**. Revanappa** for the award of the Degree of Doctor of Philosophy in Biochemistry, to the University of Mysore is the result of research work carried out by him in the Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore under my guidance during the period of November 2003 – November 2008.

P.V. Salimath
Guide

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Revanappa

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

DF	Dietary fiber
%	Percent
°C	Degree Celsius
h	hours
k Da	Kilo Dalton
OD	Optical Density
RI	Refractive index
RT	Room Temperature
Ve	Elution volume/
Vo	Void volume
µg	Microgram (s)
mg	Milligram (s)
g	Gram (s)
mm	Millimeter (s)
cm	Centimeter (s
µL	Microliter (s)
mL	Milliliter (s)
L	Liter (s)
$[\alpha]_D$	Specific rotation
min	Minute (s)
TFA	Trifluroacetic acid
N	Normal
TCA	Trichloroacetic acid
M	Molar
HCl	Hydrochloric acid
mM	Millimolar
H_2SO_4	Sulphuric acid
eV	Electron volts
V	Volts
v/v	Volume/volume
w/v	Weight/volume
rpm	Revolutions per minute
PC	Paper chromatography
HPLC	High performance liquid chromatography
GLC	Gas liquid chromatography
NMR	Nuclear magnetic resonance spectroscopy
HPSEC	High performance size exclusion chromatography
MS	Mass spectroscopy
Rha	Rhamnose
Fuc	Fucose
Ara	Arabinose
Xyl	Xylose
Man	Mannose
Gal	Galactose
Glc	Glucose
AX	Arabinoxylans
Xyl_p	Xylopyranose
Ara_f	Arabinofuranose
BE	Barium hydroxide extract
Hem A	Hemicellulose A
Hem B	Hemicellulose B
D_2O	Deuterium oxide

Synopsis

Title: Studies on pentosans in Indian wheat varieties varying in *chapati* making quality

By

Mr. Revanappa

Cereals are staple foods for human nutrition and their use into a wide range of products is of great economic importance. Wheat is one of the major cereals across the world and is used mainly for the preparation of bread. Wheat in India is consumed mainly in the form of unleavened flat bread known as *chapati*. *Chapaties* constitute an important source of dietary proteins, calories, some of the vitamins and minerals for a large section of Indian population. Arabinoxylans are the pentose containing carbohydrate polymers and hence are often referred to as pentosans. Arabinoxylans (pentosans) were first identified in wheat flour among cereals and they have been of interest to cereal chemists and technologists because of their technological importance.

Pentosans (arabinoxylans) have been shown to significantly affect cereal-based processes such as milling, brewing and bread making. Furthermore, they offer nutritional benefits of soluble and insoluble dietary fiber, and because of the presence of phenolic moieties in their molecular structures, they also offer antioxidant properties. Arabinoxylans constitute a significant portion of human dietary fiber intake because they occur in wide variety of cereals and are consumed in large quantities.

Pentosans are the major non-starch polysaccharides in wheat and consist of xylan backbone with branches of arabinose residues in various linkages. The pentosans are known to play an important role in water balance of dough, rheological properties of dough, retrogradation of starch and bread making quality. These arabinoxylans compete with other constituents of dough

for water added to the flour. The ability of arabinoxylans to imbibe and hold water was found to increase with the cross linking density of the gel network. The chemical composition and structures of arabinoxylans vary significantly in different cereals and among wheat varieties and has been a subject of great academic interest. Variation in the amount, chemical composition and structures of pentosans brings about differences in their physico-chemical, solubility and functional properties. Pentosans in different varieties of wheat are known to vary and have relation to bread / *chapati* making quality.

To establish molecular basis of arabinoxylan functionality, it is necessary to gain more insight into the structural diversity of arabinoxylans from wheat varieties differing in *chapati* making quality. There have been few studies to relate the wheat *chapati* making quality with respect to structure-function relationship of pentosans. Hence, the present study is aimed to undertake a detailed investigation on the carbohydrate profile and structure–function relationships of pentosans in Indian wheat varieties varying in their *chapati* making quality.

The thesis is presented in 5 chapters

1. Introduction, 2. Materials and Methods, 3. Results and Discussion, 4. Summary and Conclusion, 5. Bibliography

1. First chapter consists of introduction on wheat and its chemical composition. Structural features of cereal pentosans reported in literature, physicochemical properties and nutritional significance of pentosans are presented. These aspects are cited with appropriate references. The first chapter ends with scope and objectives of the present investigation.

2. Second chapter deals with various materials used and methodologies employed during the course of the investigation. It deals with various analytical methods, isolation procedures and combination of methods employed in structural analysis of pentosans.

3. Third chapter deals with the results obtained and are discussed with contemporary results. This is divided into four sub-sections. Each sub-section has a brief Introduction, followed by Results and Discussion.

3.1. Carbohydrate composition of pentosans isolated from wheat varieties having differences in *chapati* making quality

Different varieties of wheat were screened for *chapati* making characteristics, of which DWR-162 and GW-322 showed good and MACS-2496 and HD-2189 revealed poor *chapati* making quality. They were chosen to study the carbohydrate profile and elucidate structural features of arabinoxylans. Various polysaccharide fractions such as water-soluble, barium hydroxide-soluble and alkali-soluble polysaccharides (viz., hemicelluloses A and B) were isolated from these wheat varieties and their carbohydrate compositions are studied.

Water-soluble fractions showed glucose as the major sugar along with small amounts of arabinose and xylose. Barium hydroxide-soluble polysaccharides were rich in arabinose and xylose with small amounts of glucose. Hemicellulose A fractions contained mainly xylose. Arabinose, xylose and glucose were the major sugars observed in hemicellulose B fractions. Alkali-insoluble residue was basically cellulosic in nature and was strongly associated with arabinoxylans. Differences were observed in arabinose/xylose (A/X) ratios in different fractions. Arabinose to xylose (A/X) ratio was more in varieties having good *chapati* making quality compared to poor *chapati* making varieties.

3.2. Phenolic acid profile and antioxidant properties of wheat varieties

Both free and bound phenolic acids contained ferulic and p-coumaric acids. Free phenolic acids in addition contained protocatechuic, vanillic and syringic acids. Ferulic acid was the predominant phenolic acid present in bound form. The contribution of bound phenolic acids to the total phenolic acid contents and antioxidant activities was higher than that of free phenolic acid in all the wheat varieties. Bound phenolic extracts of MACS-2496 showed highest antioxidant activity followed by HD-2189. In free phenolic acids, HD-2189 followed by MACS-2496 revealed maximum antioxidant activity.

Dietary fibre contents and their chemical composition differed in these varieties. Dietary fibre analysis revealed that whole-wheat flours from MACS-2496 and HD-2189 had higher amounts of soluble and insoluble dietary fibres. Arabinose, xylose and glucose were the major sugars present in both soluble and insoluble dietary fibres.

3.3. Structural studies on purified pentosans (arabinoxylans) from wheat varieties differing in *chapati* making quality

Pentosans (arabinoxylans) purified from barium hydroxide-soluble (BE) polysaccharides and hemicelluloses B (Hem B) were rich in arabinose and xylose with glucose as a minor contaminant. Hemicellulose A (Hem A) was rich in xylose along with small amounts of arabinose and glucose. The purification of pentosans was done by glucoamylase digestion and alcohol precipitation. Methylation analysis of pentosans from BE of wheat varieties indicated a xylan backbone in β-(1-4) linkages to which arabinose residues are substituted at O-3 or at both O-2 and O-3. The wheat varieties, DWR-162 and GW-322 known for good *chapati* making quality had branched arabinoxylans to a greater degree compared to MACS-2496 and HD-2189 having poor *chapati* making quality. Arabinoxylans from DWR-162 and GW-322 revealed high amounts of disubstituted xylose residues. Terminal arabinosyl residues were present in furanose form, whereas the xyloses were present in pyranose form.

^{13}C and ^{1}H NMR results substantiated the differences observed by methylation data. Signals due to anomeric carbon atoms of xylose residues were observed in the region between 101.01- 103.2 ppm and were characteristic features of β-linked xylopyranose residues. The signals due to anomeric carbon atoms of α-linked arabinofuranose were observed at 108.42-109.23 ppm. ^{1}H NMR analysis of arabinoxylans from wheat varieties indicated signals around 5.40, 5.30, and 5.20 corresponding to anomeric protons of α-L-arabinofuranose substituted at O-3 (monosubstituted) and at both O-3 and O-2 (disubstituted) of xylose residues, respectively.

Fourier transform infrared spectroscopy (FT-IR) of arabinoxylans showed the characteristic bands which could be assigned to the associated water, C-O, C-C and C-OH stretching and bending modes. Optical rotation measurements indicated that the xylan backbone of arabinoxylans was mainly made up of β-linkages. Periodate oxidation and Smith degradation analysis results indicated higher degree of substitution in varieties such as DWR-162 and GW-322. These results were further substantiated by the release of formic acid and products obtained as glycerol, arabinose and xylose, in Smith degradation samples.

Hemicellulose A mainly consisted of xylose along with small amounts of arabinose residues. High negative optical rotation value indicates preponderance of β-linkages in the xylan backbone.

Pentosans from hemicellulose B of wheat varieties showed the basic structure of xylan backbone in β-(1,4) linkages to which arabinose residues are attached at O-3 and /or O-2 and O-3 positions by α-linkages. Methylation analysis revealed that arabinoxylans (AX) from DWR-162 and GW-322 are more branched. Proton NMR analysis of hemicellulose B fractions from wheat varieties revealed signals for anomeric protons of terminal α-D-arabinofuranosyl residues around 5.2-5.4 ppm and of β-D-xylopyranosyl residues around 4.4-4.7 ppm. FT-IR spectra showed the bending and stretching vibration characteristics of arabinoxylans. Ferulic acid contents were more in good *chapati* making varieties compared to poor varieties.

3.4. Changes in textural quality and arabinoxylan characteristics of whole-wheat dough by the incorporation of peroxidase

Effect of peroxidase on textural parameters such as hardness, cohesiveness and adhesiveness and physico-chemical characteristics of wheat dough pentosans (arabinoxylans) were studied in the DWR-162 wheat variety. Significant increase in dough hardness was observed with the incorporation of peroxidase in the dough and it was maximum at 1mg peroxidase /100 g flour, with a hardness value of about 7.16 N. Adhesiveness decreased in dough with the incorporation of peroxidase. Pentosans isolated from dough incorporated with peroxidase (1 mg) had higher molecular weight compared to control. Peroxidase (1 mg) treated doughs also yielded pentosans with higher viscosities compared to control. Pentosans from peroxidase treated dough had higher arabinose to xylose ratio, ferulic acid and protein contents compared to native dough extracts. Thus, addition of peroxidase to dough may catalyze cross linking of arabinoxylans as well as formation of protein-arabinoxylan complexes that could be responsible for the alteration of physico-chemical properties of dough.

Fourth Chapter deals with Summary and Conclusions of the present study described in third chapter.

Fifth Chapter: The thesis concludes with a **Bibliographic** citation of all the chapters.

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Guide

(Revanappa)
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Chapter 1
Introduction

INTRODUCTION

Wheat (*Triticum aestivum*) is the most important and widely cultivated cereal crop in the world. It is grown in different climates around the world. It is obvious that the different varieties of wheat, grown under such different climate and other conditions, will greatly vary in appearance and in characteristics (Hoseney, 1994). Botanically, wheat belongs to the genus *Triticum* of family graminae. Although, as many as 18 species of wheat have been recognized in the world, only three species of wheat namely, *Triticum aestivum* (Bread wheat), *Triticum durum* (Macaroni wheat) and *Triticum dicoccum* (Emmer wheat) are being cultivated in India. Among these species bread wheat (aestivum) occupies 88% of the total area under cultivation in India (Sidhu, 1995).

When wheat flour is mixed with water, unique visco-elastic dough is formed. Due to this unique characteristic, wheat flour can be processed into a variety of food products such as bread, biscuit, *chapati* and pasta, among others (Pomeranz, 1989; Wrigley and Beitz, 1988). Commercial wheat cultivars have been classified into different classes according to technologically relevant properties such as kernel hardness, bran colour and protein content. The structure of the wheat kernel is shown in Fig. 1. The constituent tissues of the kernel are bran, endosperm and embryo (Hoseney, 1994).

The bran coat consists of several distinct layers as is evident from the graphic details in Fig. 1. The outer bran layers consist of the outermost epidermis cells, the hypodermis, tube cells and cross-cells. The inner bran layers are the testa or seed coat, nucellar epidermis, and aleurone cell layer. The embryo or germ lies at the base of the grain on its dorsal side and consists of the plumule or stem tip and the radical or root tip. The plumule and radical are connected by the cotyledon, which is surrounded by a layer of epithelial cells forming the so called scutellum. The largest part of the wheat kernel is endosperm. Wheat kernels contain 13-17% bran, 2-3% germ and 81-84%

endosperm (Hoseney, 1994; Meuser and Suckow, 1986). The bran layers are rich in protein, cellulose, hemicelluloses, and ash; pericarp and aleurone contain more pentosans (35% and 39%, respectively). The germ is rich in proteins, fat, sugars, and ash. The endosperm largely consists of starch (Pomeranz, 1989).

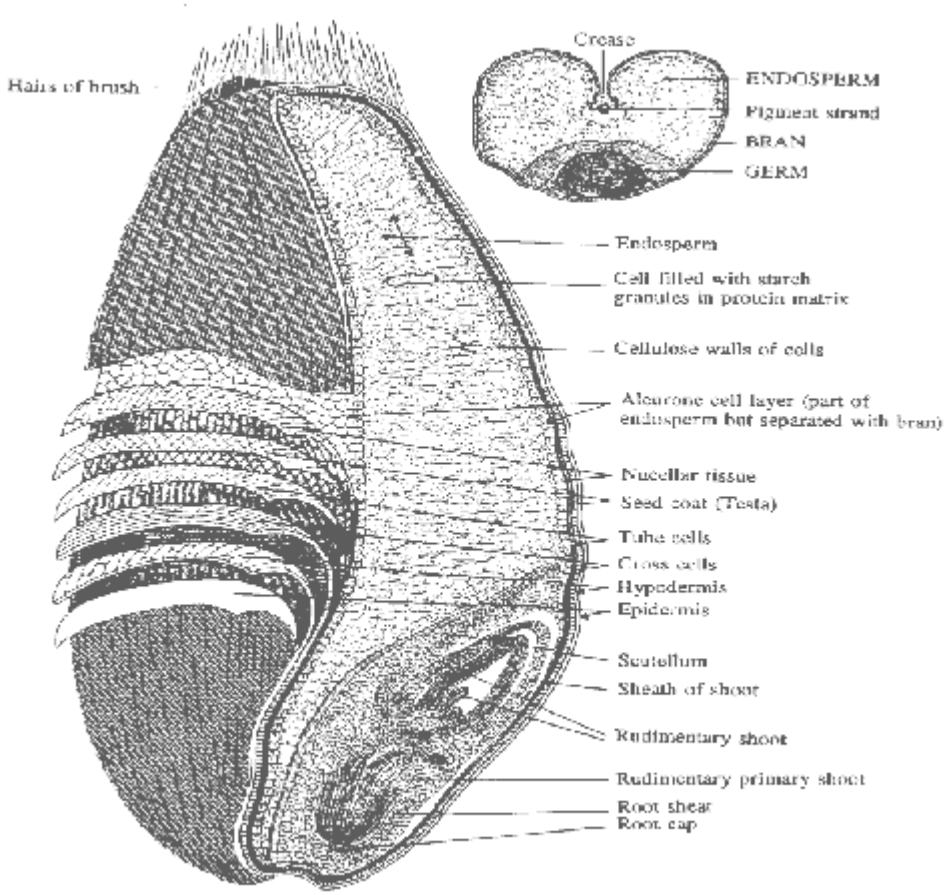


Fig. 1: The structure of the wheat kernel (Hoseney, 1994)

CHEMICAL COMPOSITION OF WHEAT FLOUR

Wheat flour, mainly consists of starchy endosperm of the kernel, contains carbohydrates (70 - 80%), proteins (8 -18%), lipids (1.5 - 2.5%) and non-starch polysaccharides (2 - 3%) all expressed as percentage on dry matter (MacRitchie, 1984; Meuser and Suckow, 1986).

Starch

Starch is the most abundant component of wheat flour. Starch granules consist of amylose and amylopectin. Starch plays an important role in baking properties. Starch dilutes the gluten to an appropriate consistency, furnishes maltose by amylase action for fermentation, provides flexibility for loaf expansion during partial gelatinization while baking and sets the loaf structure by providing a rigid network to prevent loaf collapse upon cooling (Shelton and D 'Appolonia, 1985; Sasaki et al., 2004).

Proteins

Proteins are the most important components of wheat flour with respect to bread-making characteristics. Albumins and globulins account for 20% of the total protein in flour; gliadins and glutenins represent about 80% of the total protein in flour (Wrigley and Bietz, 1988). The gluten proteins are the quantitatively most important fraction in dough. It has been suggested that the gliadins generally contribute to the viscosity of dough, whereas the glutenins contribute to their elasticity (Janssen et al., 1996).

Non-starch polysaccharides

Non-starch polysaccharides originating from the cell wall of the aleurone and endosperm of wheat kernel represent different polysaccharides, which are built up of mainly pentose sugars (named pentosans, or arabinoxylans) and small amounts of hexose sugars. Arabinoxylans together with cellulose, β-glucan, arabinogalactan-peptide and other minor constituents like glucomannan and xyloglucan referred to as non-starch polysaccharides and

are constituents of hemicellulosic polysaccharides (Amado and Neukom, 1985; Dupont and Selvendran, 1987; Whistler, 1993).

Glucomannans and galactomannans are the major cell wall components of gymnosperms. It consists of a backbone of glucose or galactose in β (1→ 4) linkage to which mannose residues are attached. Xyloglucans are found mainly in tamarind seeds. They consist of a linear polymer of glucose residues in β (1→ 4) linkage to which xylose residues are attached by α (1→ 6) linkages (O'Neill and Selvendran, 1985; Onweluzo et al., 2002).

Xylans include arabinoxylans and glucuronoxylans. In nature, pure xylans are rarely found. Xylans consist of a backbone of linear xylose residues in β (1→4) linkage. (Ebringerova and Heinze, 2000). Arabinose residues can be linked to xylose at 2 and/or 3 positions. Substitution of xylose residues at 2 and 3 positions occur in highly substituted arabinoxylans. Sometimes extended side chains are present in which arabinose residues carry additional substituents. Glucuronoxylans consists of 4-O-methyl-α-D-glucopyranosyl uronic acid residues attached by 1 → 2 linkages to D-xylose units in the backbone (Ebringerova et al., 2006; Courtin and Delcour, 1998).

ARABINOXYLANS FROM WHEAT

Arabinoxylans (AX) are the major non-starch cell wall polysaccharides of wheat grains and consist predominantly of arabinose and xylose residues and are often referred to as pentosans. AX have been identified in all major cereal grains, including wheat, barley, oats, rye, rice, sorghum, maize and millets, as well as in other plants, such as psyllium, panola grass, bamboo shoots and rye grass (Izydorczyk and Biliaderis, 1995; Roubroeks et al., 2000; Sun et al., 2004).

AX were first identified in wheat flour by Hoffman and Gortner (1927) and they have been of interest to cereal chemists and technologists ever since because of their technological importance. Arabinoxylans have been shown to significantly affect cereal-based processes such as milling, brewing and bread

making (Hoseney, 1984; Amado and Neukom, 1985). Furthermore, arabinoxylans offer nutritional benefits of soluble and insoluble fiber and because of the presence of phenolic moieties in their molecular structures, are likely to impart antioxidant properties (Katapodis et al., 2003; Lu et al., 2000; Renger and Steinhart, 2000). AX might constitute a significant portion of human dietary fiber intake because of their consumption in higher amounts and also that they occur in a wide variety of cereal crops (Carpita, 1996; Shyamprasad Rao and Muralikrishna, 2007).

AX from wheat flour have been studied extensively because of their role in bread making quality. The AX have been shown to affect water balance and rheological properties of dough and retrogradation of starch. AX from wheat have the basic structure of xylopyranose backbone to which arabinofuranosyl residues are substituted. The ratio of Ara/Xyl from wheat endosperm has been shown to vary from 0.50-0.71. A wide structural diversity was demonstrated in wheat flour extractable fractions (Vinkx and Delcour, 1996; Cleemput et al., 1995).

Pentosans, despite their low content (2-3%) in wheat flour, are very important in determining dough properties and bread quality (Amado and Neukom, 1985; Michniewicz et al., 1991; Virkki et al., 2008). These components may affect dough and gluten properties by interacting with gluten. Pentosans exhibit some unique physical properties that have been the subject of many studies. The ability to immobilize water and to form viscous solution or gels by covalent cross-linking are important attributes that also can have direct functional implications in gluten formation and properties (Hoseney and Faubion, 1981). The water holding capacity of pentosans probably affects the distribution of moisture among dough constituents and thereby affects the rheological properties of dough (Jeleca and Hlynka, 1971; Izydorczyk et al., 1990).

ARABINOXYLANS FROM OTHER CEREALS

Arabinoxylans (AX) from rice consists of highly branched xylan backbone. AX have been characterized from endosperm and bran. Apart from arabinose residues as constituents, it was shown to contain galactose, glucose and glucuronic acid (Yui et al., 1995; Sun et al., 2000).

Sorghum is one of the important cereal crops of India, and is consumed mainly in the form of *roti* (Indian flat bread). AX isolated from sorghum flour with alkali showed a highly branched xylan backbone and were found to contain other substituents like galactose and glucuronic acid in addition to arabinose residues (Nandini and Salimath, 2002; Verbruggen et al., 1995; Woolard et al., 1976).

Maize bran contains 40% (w/w) AX. Maize bran AX was found to be composed of a backbone of (1→4) linked β-D-xylose residues, highly substituted at O-2 and/or O-3 by single units of arabinose, xylose of glucuronic acid and by side chains of 2 or 3 unis of arabinose, xylose and galactose (Chanliaud et al., 1995; Xiao et al., 2001).

Pentosans of barley have gained importance because of their contributions to the brewing quality of barley and adjuncts (Sun and Sun, 2002; Vietor et al., 1994). Barley endosperm comprises 20% pentosans (w/w). Barley AX have higher A/X ratio than wheat AX. Total barley grain and barley endosperm contain 0.6% and 1.4% AX, respectively (Henry, 1987; Virkki et al., 2005).

Apart from cereals, AX have also been found in gums of sapote, brea, bromelia and watsonia gums (Izydorczyk and Biliaderis, 1995).

In cereal grains, arabinoxylans are localized mainly in the cell walls of starchy endosperm and the aleurone layer, in the bran tissues, and in the husk of some cereals. The cell walls of wheat and rye starchy endosperm and the aleurone layer are built up mainly of arabinoxylans (60-70%) (Aspinall, 1980; Meuser and Suckow, 1986). The molecular structure of arabinoxylans may also

vary depending on the specific tissue from which these polymers are derived. The outer layers of cereal grains (husk and bran) appear to contain acidic arabinoxylans (glucuronoarabinoxylans) containing glucuronic acid in addition to arabinose and xylose residues (Izydorczyk and Biliaderis, 1995). Cereal AX exhibit a high degree of endogenous microheterogeneity. It is therefore, not possible to assign a single structure to AX. Despite its high heterogeneity, all the studies point to a non-random distribution of arabinosyl residues along the xylan backbone (Cleemput et al., 1995; Nilsson et al., 2000).

The level of arabinoxylans in cereals depends on genetic and environmental factors. Among the cereal grains, rye has the highest content of arabinoxylans, followed by wheat and barley (Cyran and Saulnier, 2007). Significant genetic and environmental variations in arabinoxylan content have been reported for durum wheat and barley. Also, the exposure of plants to UV light increased the degree of cross-linking in arabinoxylans, but the effect on the total content of these polymers was not clearly established (Lempereur et al., 1997).

ISOLATION OF ARABINOXYLANS

The most common approach to isolating arabinoxylans from various plant materials involves aqueous or alkali extraction of these polymers either from whole grains or from specific plant tissues (Fang et al., 1999; Gruppen et al., 1992; Maes and Delcour, 2001).

Arabinoxylan chains can be covalently cross-linked to each other or to other cell wall constituents. As a consequence of these cross-links, a major portion of arabinoxylans cannot be extracted from the plant materials with water and requires treatments with alkali solutions to liberate them from the networks of covalent and non-covalent bonds, as well as physical entanglements (Maes and Delcour, 2001). Izydorczyk and Biliaderis, (1993) obtained nearly protein-free pentosan extracts from wheat flour after adsorption of contaminating proteins in the water extracts on various clays.

Gruppen et al., (1991) reported isolation of highly purified arabinoxylan-enriched cell wall material from wheat endosperm based on dough kneading in combination with wet sieving. Dupont and Selvendran (1987) developed a method for the isolation of arabinoxylans based on wet sieving of wheat flour in aqueous ethanol to remove starch granules, followed by sonication or removal of starch and intracellular proteins by organic solvents to improve the purity of the preparations.

STRUCTURAL FEATURES OF ARABINOXYLANS

Glycosidic linkages

Arabinoxylans consist of linear (1→ 4)-β-D-xylopyranosyl chains to which ά-L-arabinofuranosyl residues are attached as side branches. Arabinose residues can be attached to xylose units at O-2, O-3, or both O-2 and O-3 positions (Fig. 2), resulting in four structural elements in the molecular structure of arabinoxylans: monosubstituted Xyl_p at O-2 or O-3, disubstituted Xyl_p at O-2, 3, and unsubstituted Xyl_p (Cyran et al., 2003). The relative amount and the sequence of distribution of these structural elements vary depending on the source of arabinoxylans. Majority of arabinofuranosyl residues in arabinoxylans are present as monomeric substituents. However, a small proportion of oligomeric side chains consisting of two or more arabinosyl residues linked via 1→2, 1→3 and 1→5 linkages have also been reported (Ebringerova et al., 1990; Shibuya, et al., 1983).

The molecular structure of arabinoxylans from rice, sorghum, finger millet and maize bran is more complex than that from wheat, rye and barley. The side branches contain, in addition to arabinose residues, small amounts of xylopyranose, galactopyranose and α-D-glucuronic acid or 4-O-methyl-α-D-glucuronic residues. Glucuronopyranosyl residues constitute about 4% of the arabinoxylans from barley husk and are also present in arabinoxylans from wheat bran (Izydorczyk and Biliadersis, 1993).

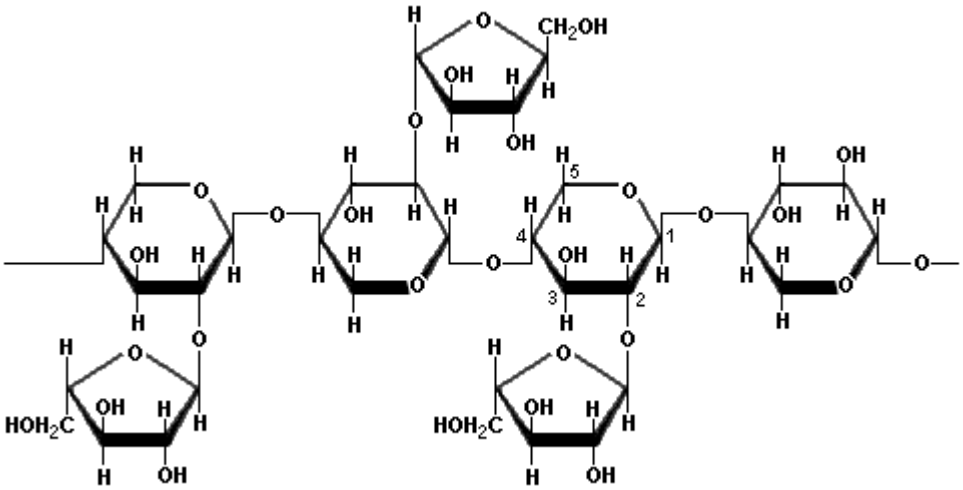


Fig. 2: General structure of arabinoxylans

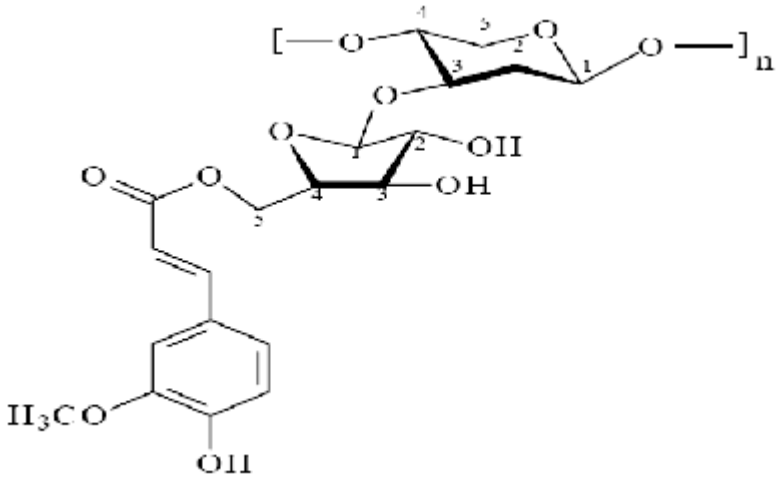


Fig. 3: Structure of ferulic acid bound to arabinoxylans (Neukom and Markwalder, 1978).

Ferulic acid residues and intermolecular cross-linking

An unusual feature of the structure of arabinoxylans is the presence of ferulic acid (4-hydroxy-3-methoxycinnamic acid) residues covalently linked via an ester linkage to O-5 of the arabinose residues of arabinoxylans (Fig. 3). Therefore, it is the natural component of pentosans (Neukom and Markwalder, 1978). Free, soluble-bound and insoluble-bound ferulic acid has been found in wheat flour (Sosulski et al., 1982). Rattan et al., (1994) reported that arabinoxylans from flours of several Canadian wheat varieties contained 0.63 to 1.37 mg of ferulic acid per gram of purified polymers. It was reported that the amounts ranged from 18 to 60 ferulic acid residues per 10,000 xylose residues and that purified arabinoxylans from wheat and barley contained more ferulic acid than those from rye and triticale (Dervilly et al., 2001).

Ferulic acid residues can act as cross-linking agents between polysaccharides or between polysaccharides and lignin. The cross-linking is effected by ferulate dimerization by either photochemical or more importantly, free radical coupling reactions of ferulate-polysaccharide esters (Geissmann and Neukom, 1973; Schooneveld-Bergmans et al., 1999). Ferulate esters dimerize via phenoxy radicals to form dehydrodiferulate esters. Cross-linking of cell-wall polymers via diferulate bridges is of importance to plant physiologists, food chemists and technologists (Hartley and Jones, 1976). Thermal stability of cell adhesion and maintenance of crispness of plant based foods, gelling properties of cereal arabinoxylans and sugar beet pectins, insolubility of cereal dietary fibers and limited cell wall degradability by ruminants are related to the formation of ferulate cross-links. Degree of cross-linking in insoluble arabinoxylans was higher than in their water-soluble counterparts (Ebringerova, 2006; Izydorczyk and Biliaderis, 1995).

Recent studies by Piber and Koehler (2005) provided evidence for a covalent linkage between arabinoxylans and proteins. Dehydroferulic acid-tyrosine dimers were isolated from wheat and rye dough preparations and identified based on mass spectrometric data.

Structural heterogeneity and polydispersity of arabinoxylans

Arabinoxylans from various cereals and different plant tissues share the same general molecular structures. However, they differ in the fine structural features, which are known to affect their physico-chemical properties (Coutin and Delcour, 1998). These differences are reflected in the degree of polymerization, ratio of arabinose to xylose residues, relative proportions and sequence of various glycosidic linkages, pattern of substitution of the xylan backbone with arabinose residues and the presence and amount of other substituents, such as feruloyl groups or glucuronic acid residues. Since, arabinoxylans are not under strict genetic control, even polymers isolated from a single plant or tissue exhibit structural microheterogeneity (Izydorczyk and Biliaderis, 1993; Saulnier et al., 2007).

The ratio of arabinose to xylose residues indicates degree of branching in these polysaccharides. Depending upon the origin of arabinoxylans, the ratio of Ara/Xyl may vary from 0.3 to 1.4, but large variations were observed among the cereals. The water-extractable arabinoxylans from wheat bran were found to have lower degree of substitution and lower molecular weights, compared to their alkali-extractable counterparts (Cleemput et al., 1993).

The ratio of Ara/Xyl indicates degree of branching in arabinoxylans. However, it does not reveal detailed structural features of these polymers. The relative amounts of unsubstituted, monosubstituted (at O-3 or O-2), and doubly substituted xylose residues, as well as the sequences of these four structural elements are better indicators of the molecular structures of cereal arabinoxylans (Cleemput et al., 1993; Delcour et al., 1999). However, the relative proportions of the four structural elements in arabinoxylan chains may be related to the arabinose to xylose ratio and same trends have been reported. For example, in water extractable rye and wheat arabinoxylans a higher Ara / Xyl ratio was associated with a higher content of 2-monosustituted and disubstituted xylose residues and a lower content of 3-monosustituted and

unsubstituted xylose residues (Izydorczyk and Biliaderis, 1993; Cyran et al., 2003).

It is generally agreed that cereal arabinoxylans are highly heterogeneous, consisting of a range of structures with different degrees and patterns of substitution. However, despite the structural heterogeneity of arabinoxylans, most studies point to a non-random distribution of arabinose residues along the xylan backbone. Bengtsson and Aman (1990) proposed that in rye arabinoxylans, the mono- and disubstituted xylose residues were present in different polymers or in different regions of the same polymer chain. It was hypothesized that the major polymer structure contained only un- and mono-sustituted xylose residues, whereas the minor structure contained un- and di-substituted xylose. Vinks and Delcour (1996), isolated a highly branched rye arabinoxylan (Ara / Xyl=1.42) with a very low proportion of 3-monosubstituted Xyl_p.

Molecular weight

The molecular weight of arabinoxylans varies depending on their origin and the method used for determination. The early studies using sedimentation techniques reported low molecular weight values (65,000 to 66,000) for water-extractable wheat arabinoxylans. However, very high molecular weight values (800,000 to 5,000,000) were reported when gel filtration chromatography was used, and the molecular weight of arabinoxylans was estimated by comparing their elution volume with that of standards with known molecular weights (Fincher and Stone, 1986).

BIOSYNTHESIS OF ARABINOXYLANS

Relatively little is known about the mechanism of arabinoxylan synthesis. Arabinoxylans are the products of synthases and glycosyl transferases and as such are secondary gene products (Basic et al., 1988). Arabinoxylans and other cell wall polysaccharides (except cellulose) are synthesized within the cell in the golgi apparatus and endoplasmic reticulum (Fig. 4). It is believed that distinct

glycosyl transferases are necessary for each of the different monosaccharide and glycosidic linkages existing in arabinoxylan chains. The attachment of feruloyl groups to arabinoxylans may occur by transacylation and the polysaccharides are feruloylated co-instantaneously with the polymerization processes within the endomembrane system.

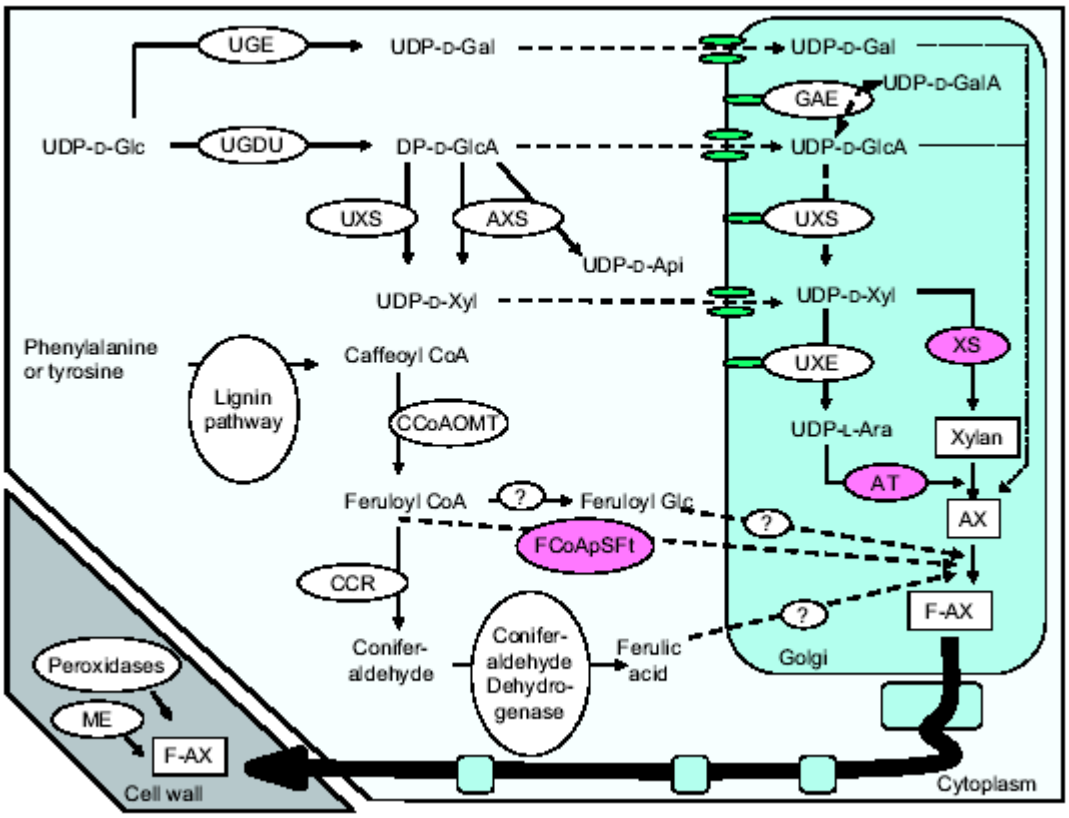


Fig. 4: Schematic representation of biosynthesis of arabinoxylans

(Saulnier et al., 2007)

Because of the numerous and complex events involved in biosynthesis of arabinoxylans, the formation of these polymers is not strictly regulated and may depend on several factors (Saulnier et al., 2007). As a result, arabinoxylans show high degree of microheterogeneity and belong to the class of polydispersed polysaccharides. Their polydispersity can be reflected in

the degree of polymerization of individual chains, in the abundance, distribution and degree of polymerization of side-chain substituents and in the degree of feruloylation and cross-linking (Delmer and Stone, 1988).

PHYSICOCHEMICAL PROPERTIES OF ARABINOXYLANS

Viscosity

AX exhibit high viscosity in aqueous solution. The viscosity of arabinoxylans depends upon their molecular weight and substitutions (Vinkx and Delcour, 1996). The high viscosity is determined by AX chain length, degree of substitution and substitution pattern (Izydorczyk and Biliaderis, 1995). Ferulic acid cross-links between individual AX molecules also influence viscosity, as they increase the molecular weight of AX and change their conformation (Dervilly et al., 2001). In addition interactions between AX and proteins in the extract would result in even higher viscosities than for AX alone (Delcour et al., 1991).

Water holding capacity

Both water soluble and insoluble pentosans are hydrophilic in nature. Although pentosans comprise only 2-3% of the flour, Bushuk (1966) reported that 23% of the water in doughs was associated with pentosans. Therefore, pentosans were found to be very important regulators of water absorption and distribution in doughs. Adding pentosans to dough immobilized free water and resulted in stiffer doughs and shorter mixing times for optimum dough development (Cyran et al., 2003; Izydorczyk and Biliaderis, 1995).

Solubility

The solubility of arabinoxylans depends primarily on the degree of substitution of arabinose on xylan backbone. Removal of arabinose residues from arabinoxylans decreases its solubility in water. Insolubility of the low Ara /

Xyl fraction was attributed to an increased aggregation of unsubstituted regions of the arabinoxylans, stabilized by hydrogen bonds (Andrewartha et al, 1979).

Oxidative cross linking

Aqueous solutions of arabinoxylans possess a unique ability to form hydrogels in the presence of free radical generating agents, such as peroxidase-H_2O_2, laccase, ammonium persufate and ferric chloride (Geissmann and Neukom, 1973). Covalent cross linking of arabinoxylan chains through dimerization of ferulic acid substituents is responsible for this unique property of arabinoxylans (Fig. 5).

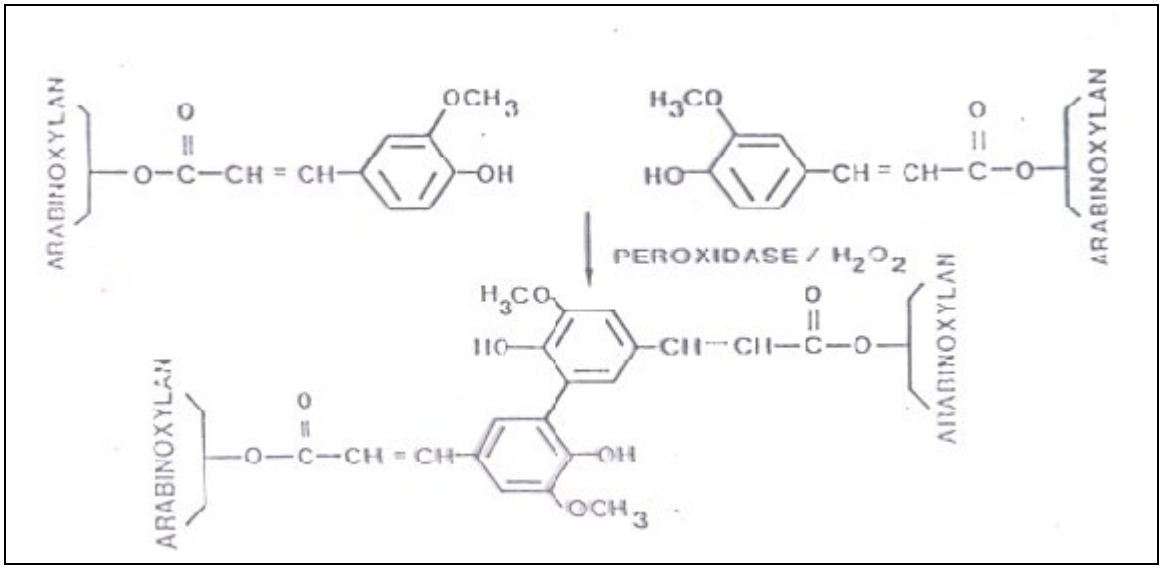


Fig. 5: Oxidative gelation of arabinoxylans via ferulic acid substituents in the presence of peroxidase/H_2O_2. (Neukom and Walder, 1978).

Both the aromatic ring and the double bond in the structure of ferulic acid serve as cross-linking sites (Izydorczyk, et al., 1990). It was shown that an ultraviolet spectrum of pentosans had a peak at 320 nm, which disappeared when oxidizing agents are added. This peak has been attributed to ferulic acid in pentosans. Disappearance of this peak implied that ferulic acid was involved in the gelation reaction (Shelton and D 'Appolonia, 1985; Yeh et al., 1980). The cross linked arabinoxylans have very high water absorption capacity (up to 100g of water per gram of dry polymer) and are not susceptible to changes in pH or electrolyte concentrations (Izydorczyk and Biliaderis, 1995). These properties, together with the macroporous texture of gels and the dietary fibre nature of arabinoxylans makes them useful for controlled delivery of therapeutic proteins (Carvajal et al, 2005).

Foam stabilization

Izydorczyck et al., (1990) observed that arabinoxylans stabilize protein films against thermal disruption. The phenomenon was accompanied by lower initial foam formation in the presence of AX. Both the phenomenon are attributed to the increased viscosity of the liquid brought about by AX.

FUNCTIONAL PROPERTIES OF ARABINOXYLANS

The role of arabinoxylans in the bread making processes and their effects on the final bread product have been studied extensively (Biliaderis et al., 1995). Addition of pentosans in the dough system improved the loaf volume and crumb structure of bread. There are differences among cereal chemists about their functional role. This diversity has been attributed to be due to difference in the method of isolation, degree of purity, composition of pentosan preparations, level of supplementation and various baking systems employed (Courtin and Delcour, 1998; Saulnier et al., 2007).

AX have been shown to be competing with other constituents of the dough for water when added to the flour. Significant increases in farinograph water absorption and dough development time have been reported (Cyran et

al., 2007; Dervilly et al., 2000). The effects were shown to be dependent on the amount of AX added, their molecular weight and on the nature of base flours. Oxidative gelation was shown to increase the hydration capacity of AX. The ability of AX to imbibe and hold water was found to increase with the cross-linking density of gel network (Izydorczyk et al., 1990). Higher than the optimum concentration of AX in the dough was found to cause viscosity build up in the dough thereby hindering and decreasing the loaf volume. Flours when treated with xylanase were found to yield sticky dough and breads of low loaf volume and soggy texture (Hilhorst et al., 2002).

AX have also been implicated in the firmness of bread crumbs. It has been shown that crumbs of bread fortified with AX were less firm than those of controls. The positive effect has been related to the increased moisture content of the samples. Water acts as a plasticizer of the gluten-starch composite matrix thereby lowering rigidity of the products. This in turn was shown to be dependent on the amount and molecular size of added polymers into bread formation (Shelton and D'Appolonia, 1985; Izydorczyk and Biliaderis, 1995).

It was shown that arabinoxylans with higher molecular weight increased the farinograph water absorption to a greater extent than their low molecular weight counterparts (Biliaderis et al., 1995). Water-soluble arabinoxylans are believed to increase viscosity of the aqueous phase of the dough and therefore to have a positive effect on the dough structure and its stability, especially during early baking processes, when a relatively high pressure is generated inside the gas cells. These processes lead to a higher loaf volume and improve crumb structure. Adding 1-2% pentosans to poor wheat flours markedly improved dough properties (Shelton and D'Appolonia, 1985; Rouau, 1993).

Another functional property of arabinoxylans may be associated with their role in bread staling. Bread staling is a complex phenomenon involving loss of aroma, deterioration of crust characteristics and increase in crumb firmness (Izydorczyk and Biliaderis, 1995; Shelton and D'Appolonia, 1985).

Biliaderis et al., (1995) measured bread staling by monitoring the crumb firmness of bread fortified with arabinoxylans.

It was shown that over a seven day storage period, the arabinoxylans-fortified breads exhibited lower crumb firmness than the controls. Kinetic studies indicated that adding pentosans decreased the amount of starch components available for crystallization and thus decreased the bread staling rate. The observed effects were also attributed to a higher moisture content of breads substituted with arabinoxylans and to the plasticizing effects of water (Shelton and D'Appolonia, 1985).

ARABINOXYLANS IN HUMAN HEALTH

Arabinoxylans as part of dietary fibre have many potential physiological effects along the entire human gastrointestinal tract (Lu et al., 2000; Plaami, 1997). These effects are dependent on complex mixture of molecular and physical properties of arabinoxylans. It is generally agreed that arabinoxylans enhance the growth of potentially health-promoting bacteria, the so-called probiotics. The degree of branching and distribution of arabinose along the xylan backbone influence the degradation of arabinoxylans. Unsubstituted xylans are known to form insoluble aggregates and hindered accessibility of the bacterial enzymes to the substrates (Crittenden, 2002).

Arabinoxylans also play a vital role in the management of diabetes in human health. Lu et al., (2000) showed that addition of arabinoxylans to enrich fiber to bread eaten at the breakfast lowered postprandial glucose and insulin responses in healthy humans. Due to their viscosity generating properties, arabinoxylans impair mixing of the food mass and can markedly affect the degree of contact of food substrates with the enzymes that digest them in the small intestine. In this way, arabinoxylans in the human diet may slowdown the rate of gastric emptying and reduce small intestine motility, which results in delayed glucose absorption. The beneficial role of arabinoxylans in the human diet may also be associated with the presence of ferulic acid covalently bound

to these polymers (Katapodis et al., 2003; Peyron et al., 2001). Recent studies revealed that ferulic acid has strong anti-inflammatory properties, inhibits chemically induced carcinogenesis in rats and plays a role as an antioxidant, inhibiting lipid peroxidation and low-density lipoprotein oxidation and scavenging of oxygen radicals (Rondini, 2004).

FLAT BREADS / *CHAPATI* IN INDIA

Apart from leavened breads, wheat has found a popular use in the form of flat breads. At present, flat breads are consumed around the world. *Chapati*, a flat and unleavened baked product made from whole wheat flour, is the staple diet of majority of population of the Indian subcontinent (Austin and Ram, 1971; Haridas Rao et al., 1986). *Chapaties* constitute an important source of dietary proteins, calories, some of the vitamins and minerals for the poorer section of the Indian population (Bajaj et al., 1990). Traditionally, wheat is milled into whole-wheat flour (*atta*), on a power driven stone mill (*chakki*), after removing 3-5% of coarse bran by sieving, the 95-97% extraction *atta* is used for the preparation of *chapaties* (Leelavathi et al., 1986).

Chapaties are the most popular unleavened flat breads in India and is consumed during almost every meal of the day in some parts of India (Fig. 6). The two most important quality parameters for *chapaties* are softness and flexibility (Murthy and Austin, 1963; Sidhu, 1995). Dough is prepared by hand mixing of flour with an optimum amount of water. The dough is rested for 15-20 min, at room temperature, depending upon the convenience of the preparer. About 60-100g of the dough is rounded between the palms and sheeted manually into a disk, 2-3 mm thick using a wooden rolling pin. It is then immediately baked on a preheated iron griddle (*tawa*) at a temperature of 230ºC for about 1 minute on each side (Sidhu et al., 1988). As *chapati*es are quite thin, they are susceptible to moisture loss as well as stalling after baking. Therefore, they are mainly prepared fresh for both lunch and dinner.

Fig. 6: Indian *chapaties* / unleavened flat breads.

It is estimated that 80% of wheat in India is consumed in the form of *chapati* (Haridas Rao et al., 1986). The quality characteristics of *chapati* are mainly governed by the quality of wheat used and the processing conditions employed for converting it into whole-flour (Haridas Rao et al., 1986; Prabhasankar et al., 2002). Traditionally, wheat is milled into whole-wheat flour (*atta*) on a power driven stone mill (*chakki*). Softness and flexibility are the important quality parameters. Creamy yellow colored *chapaties* are said to be most desired. But it is affected by wheat cultivar, flour extraction rate and processing treatments that the flour undergoes (Leelavathi et al., 1986). Flour milling conditions also influence the quality of *chapaties* (Sidhu et al., 1988). The moisture content of baked *chapati*es was highly correlated with farinograph water absorption of the flour. It was seen that finer flours with high amounts of water absorption by the dough resulted in *chapati*es with soft texture. Hence, absorption of water has been shown to be one of the important parameters, which affect the *chapati* making quality (Leelavathi et al., 1986; Sidhu et al., 1988).

SCOPE AND OBJECTIVE OF THE PRESENT INVESTIGATION

Wheat (*Triticum aestivum*) is a major cereal crop used mostly for the preparation of leavened breads all over the world. However, in India wheat is used mainly for the preparation of unleavened flat bread known as *chapati*. The textural and functional properties of wheat products are mainly attributed to the presence of proteins and pentosans in wheat. Arabinoxylans, which are often referred to as pentosans, are the major non-starch polysaccharides in wheat grains, constituting 1.5-2.5% of the endosperm. The pentosans are known to play an important role in water balance of dough, rheological properties of dough, retrogradation of starch and bread making quality.

Pentosans vary in different varieties of wheat both quantitatively and qualitatively. Their solubility pattern varies depending on their structure. The pentosans, which are present, as arabinoxylans are known to vary in the ratio of arabinose to xylose. The pentosans have a xylan backbone where in xylopyranose residues are linked linearly by β (1-4) linkages. The backbone is further branched at O-2 and/or O-3 by arabinofuranosyl residues. Arabinoxylans are known to have highly branched regions with more open regions. A variation in structures of pentosans brings about differences in their physico-chemical, solubility and functional properties. These components may also affect dough and gluten properties by interacting with the gluten proteins.

Changes in the quantity and properties of pentosans are reflected in changes in dough physico-chemical properties and hence final product quality. Unravelling the underlying structure-function relationships of pentosans is therefore of great importance.

There have been very few studies to relate the wheat *chapati* making quality with respect to structure-function relationship of pentosans in Indian wheat varieties. Hence, the objective of the present study was to undertake a detailed investigation on the structure–function relationships of pentosans in Indian wheat varieties varying in their *chapati* making quality.

OBJECTIVE

To study carbohydrate profile and examine structure-function relationship of pentosans in Indian wheat varieties having differences in *chapati* making quality.

Chapter 2
Materials and Methods

GENERAL

1. All the results are average of minimum three experiments.
2. All extractions are done using double glass distilled water.
3. Centrifugation of samples was done either using Remi (RC8) or Sigma (202C) centrifuge.
4. Dialysis was carried out using dialysis bags of 12,000 d cut off (Hi-media).
5. Evaporation of the samples was carried out using Buchi rotavapor with a water bath temperature of 35-40^{0}C.
6. Lyophylization was carried out using Vertis Freeze Mobile (12SL).
7. Colorimetric and spectrometric readings of test solution with appropriate blanks were taken using Shimadzu double beam spectrometer (UV-1600A).

2.1. MATERIALS

General Chemicals

Methyl iodide, sodium azide, sodium borodeuteride, sodium borohydride, potassium bromide, DPPH (1,1 Diphenyl-2-picryl hydrazyl), DMSO (dimethyl sulphoxide), carbazole, Coomassie brilliant blue G-250, deuterium oxide, ruthenium red, were from Sigma Chemical Company, St. Louis, USA. Folin phenol reagent (2N), bovine serum albumin and hydrogen peroxide were procured from Sisco Research laboratories, Mumbai, India. Trifluoroacetic acid (Spectroscopy grade) was procured from Spectrochem, Mumbai, India. Sodium hydride (99%) was from Aldrich Chemical Company, Milwaukee, USA.

Gel matrices and ion exchange resins

Sepharose CL-2B and Amberlite IR-120-P were from Pharmacia Fine Chemicals, Uppsala, Sweden. Sep-Pak C_{18} cartridges were from Waters Associates, Millford, USA.

Dextran standards

T-series dextran standards (T-10, T-20, T-40, T-70, T-500, T-2000) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Enzymes

Glucoamylase (E.C. 3. 2. 1. 3) was from *Aspergillus niger*, horse radish peroxidase (E.C. 1. 11. 1. 7) and glucose oxidase (E.C. 1. 1. 3. 4) from *Aspergillus niger*, Termamyl (E.C. 3. 2. 1.2) from *Bacillus licheniformis* were from Sigma Chemical Company, St. Louis, USA.

Sugar standards

Rhamnose, fucose, xylose, arabinose, glucose, fructose, galactose, mannose, maltose, inositol, sucrose, raffinose and stachyose were from ICN Pharmaceutical Inc, Cleveland, USA.

Phenolic acid standards

Phenolic acid standards such as gallic acid, caffeic, p-coumaric, ferulic, gentisic, protocatechuic, syringic and vanillic acids, butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) were from Sigma Chemical Company, St. Louis, USA.

GLC columns

OV-225 (1/8" x 6ft) 3% on chromosorb W (80-190 mesh) was from Pierce Chemical Company, Rockford, USA. SP-2330 (0.32 mm X 30M) was obtained from Supelco, Tokyo, Japan.

HPLC columns

Shimpack C_{18} column (ϕ 4.6 mm X 250 mm) was obtained from Shimadzu, Corporation, Tokyo, Japan. E-linear (ϕ 7.8 mm X 300 mm) and E-1000 (ϕ 3.9 mm X 300 mm) gel permeation columns were obtained from Waters Associates, USA.

All chemicals and solvents used for HPLC and GLC were HPLC grade. All other analytical reagents were obtained from E-Merck, SRL Mumbai, India. Triple distilled and degassed water was used for HPLC analysis.

Wheat varieties

DWR-162 variety of wheat (*Triticum aestivum* L.) was obtained from University of Agricultural Sciences, Dharwad and MACS-2496, GW-322, HD-2189 varieties were procured from Agharkar Research Institute, Pune, India. Wheat was milled into whole flour in a commercial disc mill. The whole-wheat flour obtained was cooled and stored at 4ºC until use.

2.2. METHODS

2.2.1. Chapati making quality

2.2.1.1. Preparation of chapati

Chapaties were prepared from whole wheat flour according to Haridas Rao et al., (1986) with slight modifications. *Chapati* dough was prepared by mixing 200 g flour and water equivalent to *chapati* water absorption in a Hobart mixer (Model N-50) at speed 1 for 3 min. The dough was rested for 10 min. The dough was then divided into pieces of 50 g each and hand sheeted to a thickness of 1.5 mm using a specially designed aluminium platform and a detachable aluminium frame to maintain uniform thickness. The sheeted dough was cut into a circular shape of 12 cm diameter using a die. The sheeted and cut dough was baked on a hot plate maintained at 215°C for 70 s on side 1 and 85 s for side 2. The *chapati* was then transferred to a heated gas tandoor (370°C) in such a way that side 1 was placed on the grill and heated for 10 s. The puffed *chapati* from the tandoor was cooled and evaluated.

2.2.1.2. Sensory evaluation of *chapati*

Sensory evaluation of *chapaties* was done by a panel of eight trained judges using a 10 point scale. The product was evaluated for its appearance, tearing strength, pliability, taste, aroma and chewiness, according to Haridas Rao et al., (1986). The panelists were asked to give the scores on the basis of the quality description given against each sensory parameter evaluated. The *chapaties* having higher scores were considered to have better quality.

2.2.2. Studies on carbohydrates

2.2.2.1. Isolation of free sugars (Suhasini et al., 1997)

Whole wheat flours (10g) were suspended in aqueous ethanol (70%, 2 h, thrice) and stirred continuously at room temperature. It was then centrifuged and the pooled extracts (free sugars) were deionized by passage through Dowex-50 (H^+) and Dowex-1 (OH^-) resins to remove cationic as well as anionic

contaminants. The purified sugars were concentrated by flash evaporation. The deionised samples dissolved in known volume of distilled water were analyzed by HPLC.

2. 2. 2. 2. Extraction of polysaccharides

a. Water soluble polysaccharides (Salimath and Tharanathan, 1982)

The alcohol insoluble residue was cooked with water at boiling water bath temperature for 1 h to gelatinize starch and then Termamyl (1 mL) was added at intervals to digest starch. It was digested till it shows negative test to iodine solution. The contents were cooled and subjected to glucoamylase digestion at 60º C for 2 h. After the digestion, samples were centrifuged and the supernatant (water soluble polysaccharides) was dialyzed and lyophilized.

b. Barium hydroxide extracted polysaccharides (Gruppen et al., 1991)

The water-insoluble residue was extracted twice with saturated barium hydroxide solution (250 mL) containing sodium borohydride (260 mM) for 16 h. The contents were centrifuged and pooled samples were dialyzed against sodium acetate buffer (0.2 M, pH 5) followed by water and lyophilized. The residue was washed with water till it gave neutral pH and then dried by solvent exchange.

c. Sodium hydroxide extracted polysaccharides (Salimath and Tharanathan, 1982)

The barium hydroxide-insoluble residue was extracted with sodium hydroxide (10%) under nitrogen atmosphere for 6 h in dark. The contents were centrifuged. To the supernatant, acetic acid (50%) was added at ice cold temperature till the pH of the solution reaches 4.5. The precipitate was separated by centrifugation (4000 rpm, 10 min). The precipitate (hemicellulose A) was washed with water to neutral pH and dried by solvent exchange. The supernatant (hemicellulose B) was dialyzed against water and lyophilized. The

alkali-insoluble residue (AIR) was washed with water till neutral pH and dried by solvent exchange.

2.2.2.3. Isolation of total dietary fiber (Asp, et al., 1983)

The flour was first extracted with petroleum ether for 30 min to remove fat. The defatted sample (1.0 g), was suspended in phosphate buffer (25 mL, pH 6.0, 0.1 M) followed by the addition of Termamyl (0.1 mL) and kept in a boiling water bath for 15 min to digest starch. The contents were cooled and water (20 mL) was added and the pH was adjusted to 1.5 with 4 N HCl. Proteins were removed by digesting with pepsin (100 mg) at 40^{o}C for 1 h. Once again water (20 mL) was added and the pH adjusted to 6.0 with 4 M NaOH. To this, pancreatin (100 mg) was added, and incubated at 40^{o}C for 1 h. Finally the contents were cooled and the pH was adjusted to 4.5 with 4 N HCl and filtered through a dried and weighed crucible containing celite (0.5 g).

a. Insoluble dietary fiber

The residue retained in the crucible was washed with ethanol (95%, 20 mL) followed by acetone (20 mL). The crucible was kept in an oven (105^{o}C) till the weight became constant and the final weight was taken accurately (D_1). The crucible was then incinerated at 550^{o}C for 5 h and once again its weight (I_1) was recorded.

b. Soluble dietary fiber

The volume of the filtrate was adjusted to 100 ml and the soluble fibers were precipitated by adding 4 volume of warm ethanol (60^{o}C). The precipitate was filtered through celite, dried and weighed after drying at 105^{o}C (D_2) followed by incineration at 550^{o}C (I_2).

C. Blank was prepared as above without sample.

D. Soluble and insoluble dietary fiber contents (%) were calculated by using following formula:

$$\% \text{ Insoluble fiber} = \frac{D_1 - (I_1 - B_1)}{W} \times 100$$

$$\% \text{ Soluble fiber} = \frac{D_2 - (I_2 - B_2)}{W} \times 100$$

2.2.2.4. Hydrolysis of polysaccharides

2.2.2.4.1. Trifluoroacetic acid hydrolysis

Sample (10-15 mg) was taken in 1 mL of trifluroacetic acid (2 N) and the tube was sealed. Hydrolysis was carried out at 100^{o}C for 6-8 h in an oven. After the hydrolysis, acid was removed by flash evaporation at water bath temperature of 40^{o}C and co-distilled with water (1 mL, x3).

2.2.2.4.2. Sulphuric acid hydrolysis (Salimath and Tharanathan, 1982)

Polysaccharide (10-15 mg) was suspended in water and was hydrolyzed by prior solubilization with 72% sulphuric acid at ice-cold temperature followed by dilution to 8% acid and heating in a boiling water bath at 100^{o}C for 10-12 h. The above mixture was neutralized with barium carbonate (solid), filtered through Whatman No.1 filter paper and deionized with regenerated Amberlite IR 120 H^+ resin and concentrated.

Regeneration of Amberlite IR - 120 H^+ resin

The Amberlite resins were washed with water until fines, color and other impurities are removed. Nylon cloth was used to successive washes by filtration, and then regenerated by suspending in HCl (2 N) for 1 h at room temperature with intermittent shaking. The resin was then filtered through nylon cloth and washed with water till the filtrate gave neutral pH.

2.2.2.4.3. Hydrolysis of permethylated polysaccharides

The permethylated polysaccharide was hydrolyzed with formic acid (2 mL, 90%) at 100ºC for 2 h in a boiling water bath. Excess acid was removed by codistilling with methanol (1 mL, x 5). The samples were again hydrolysed by TFA (1 mL, 2N) in sealed tubes at 100ºC in an oven for 6 h. The acid was removed by codistilling with water (1mL, x 3).

2.2.2.5. Analytical methods

2.2.2.5.1. Total sugar estimation (Dubois et al., 1956)

The sample (0.5 mL) containing 5-25 µg of sugars was mixed with phenol (0.3 mL, 5%) and concentrated sulphuric acid (1.8 mL) was added directly on to the top of solution. It was cooled to room temperature (10 min) and absorbance was read at 480 nm in a spectrophotometer. Standard graph was generated using 0-25 µg of glucose.

2.2.2.5.2. Uronic acid estimation by carbazole method (Dische, 1947)

Sample (0.5 mL) containing 10-50 µg of uronic acid was mixed with 3 mL concentrated sulphuric acid (slowly through the sides of the tubes by keeping the tubes in ice cold temperature). It was then heated for 20 min in water bath. After cooling the tubes to room temperature, 0.1 mL of carbazole (recrystallised, 0.1 % in ethanol) was added and kept in dark for 2 h. The absorbance was read at 530 nm in a spectrophotometer. Standard graph was generated using 0-50 µg of glucuronic acid.

2.2.2.5.3. Glucose estimation by glucose oxidase method (Dahlqvist, 1964)

To a 0.5 mL of sample digested with glucoamylase, 3 mL of glucose-oxidase reagent was added. After mixing well, the sample was incubated in a water bath at 37 ºC for 1 h and absorbance was read at 420 nm. Standard graph was prepared by using D-glucose.

2.2.2.5.4. Determination of starch (Hassid and Neufeld, 1964)

Sample (0.5 - 1 g) was taken in a conical flask and dispersed in 50 mL water. Termamyl (0.1 mL) was added and then kept in boiling water bath for 10 min. After cooling, acetate buffer (pH 4.6, 0.05 M) was added and equilibrated at 60 ºC for 2 h. To this glucoamylase (50 mg) was added and incubated in a shaking water bath at 60ºC for 2 h. The solution was filtered and made up to 100 mL and the liberated glucose was determined by glucose oxidase method (Section 2.2.2.5.3**)**. The glucose value was multiplied by factor, 0.9 to get starch content.

2.2.2.5.5. Protein content (Bradford, 1976)

Coomassie brilliant blue G-250 (10 mg) was dissolved in ethanol (5 mL, 95%) and phosphoric acid (10 mL, 85 %) was added. The solution was made upto 100 mL with water and filtered through Whatman No.1 filter paper.

To the sample (0.2 mL) Bradford reagent (0.8 mL) was added and mixed well. Absorbance was read at 595 nm. Protein content was determined by referring to the standard graph using BSA (2-10 µg in 0.2 mL).

2.2.2.5.6. Determination of total proteins by Kjeldahl method (Hawk, 1965)

Sample (0.5-1 g) was digested with 20 mL of concentrated H_2SO_4 in the presence of digestion mixture (98 parts K_2SO_4 + 2 parts $CuSO_4$) which acts as a catalyst. It was digested till the solution became clear in the Kjeldahl flask. Glass beads were added to prevent bumping. The contents of the flask were cooled and the volume was made up to 100 mL with water in a volumetric flask. The mixture (5 mL) was made alkaline by adding 10 mL of sodium hydroxide (40 %). It was steam distilled into a container containing 10 mL of boric acid with methyl red indicator. The solution in the flask was titrated against HCl (0.01 N) till bluish green colour was obtained. Simultaneously a blank was processed as above without sample. Titre value of the blank was deducted from test values.

Ammonium sulphate (5 mL, 1M) was used as the standard. It was steam distilled in a similar way as mentioned above for the sample mixture.

(%) Protein was calculated using the formula:

[Titre value of sample/ Titre value of standard] x [Dilution factor/ Volume of solution taken (5 mL)] x [Factor (6.25)/ Weight of sample x 1000]

2.2.2.5.7. Ferulic acid content (Fauch et al., 1963)

The sample (10 mg) was treated with 1N NaOH (5 mL) at room temperature for 4 h under nitrogen. The mixture was acidified to pH 3 with HCl (2 N) and extracted three times with equal volume of ethyl acetate. The extracts were evaporated to dryness in a rotary evaporator and the residue dissolved into a known amount of methanol. The absorbance was measured at 320 nm and compared with a standard solution of ferulic acid (10-50 µg).

2.2.2.5.8. Phenolic acid content (Singleton and Rossi, 1965)

The total phenolic content of the samples was determined colorimetrically using the Folin-Ciocalteu method. Test sample (0.1 mL) was mixed with 2 mL of 2% sodium carbonate solution, after 3 min, 0.1mL of 50 % of Folin-Ciocalteu phenol reagent was added to the mixture. After 30 min of incubation at room temperature, absorbance was measured at 765 nm against blank using a spectrophotometer. Standard graph was generated using 0-50 µg of gallic acid. The phenolic content of each extract was expressed as micrograms of gallic acid equivalents (GAE) per gram of whole wheat flour.

2.2.3. Phenolic acids and antioxidant properties

2.2.3.1. Isolation of free phenolic acids (Suresh et al., 2006)

Whole-wheat flour of each variety (2 g each) was extracted with 70% ethanol (50 mL, ×3, 2 h) and the supernatants were obtained by centrifugation

and concentrated, and pH was adjusted to 2 with HCl (4 N). Phenolic acids were separated by ethyl acetate phase separation (50 mL, × 4) and the pooled fractions were treated with anhydrous sodium sulphate, filtered and evaporated to dryness. Phenolic extracts were taken in methanol for analysis.

2.2.3.2. Isolation of bound phenolic acids (Nordkvist et al., 1984)

Whole-wheat flour (2 g) was extracted with 70% ethanol (50 mL × 2) and hexane (50 mL, × 2) to remove free phenolic acids and fat, respectively. The dried samples were extracted with (1 M) sodium hydroxide (50mL, × 2) containing 0.5% sodium borohydride under nitrogen atmosphere in dark for 2 h at room temperature and then centrifuged. The combined supernatant (bound phenolic acids) was acidified with HCl (4 N) to pH 1.5 and phenolic acids were extracted with ethyl acetate (x3). The extracts were combined and dried with anhydrous sodium sulphate overnight. It was then filtered and evaporated to dryness. Phenolic acids were taken in methanol and analyzed by HPLC.

2.2.3.3. Antioxidant activity

a. Measurement of reducing power (Yen and Chen, 1995)

The reducing power of free and bound phenolic acid extracts of the whole-wheat flour from wheat varieties was determined. The phenolic acid extracts (10-50 µg/mL) were mixed with an equal volume (2.5 mL) of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide (2.5 mL). The mixture was incubated at 50ºC for 20 min. An equal volume (2.5 mL) of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and 0.5 mL of 0.1% ferric chloride and the absorbance was recorded at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

b. Scavenging effect of extracts on DPPH radical (Suresh et al., 2006)

An aliquot of 200 μL of different phenolic fractions (2-10 μg, GAE) of the sample and standard antioxidants (2-10 μg) were mixed with 100 mM Tris-HCl buffer (800 μL, pH 7.4) and then added to 1 mL of 500 μM DPPH in ethanol (final concentration of 250 μM). The mixture was shaken vigorously and left for 20 min at room temperature in the dark. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The capability to scavenge the DPPH radical was calculated using the following equation.

Scavenging effect (%) = (1 - absorbance of sample at 517 nm / absorbance of control at 517 nm) x 100.

IC_{50} (the concentration required to scavenge 50% of free radicals under experimental conditions) values were calculated from the dose response curves. Synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) were used for comparison.

2.2.4. Chromatography methods

2.2.4.1. Paper chromatography

Whatman No 1 paper was used to run descending paper chromatography. For the separation of neutral sugars, solvent system consisting of n-butanol, pyridine and water in the ratio of 6:4:3 was used. The paper was run for 24 h, air-dried and then sprayed with aniline-phthalate reagent or silver nitrate reagent.

a. Aniline phthalate reagent (Partridge, 1949)

Pthalic acid (1.66 g) was dissolved in water saturated butanol (95 mL butanol + 5 mL water). To this 1.0 mL of aniline was added and mixed well. The air-dried chromatogram was dipped in the reagent, air dried and then placed in an oven for 5 min at 110^{o}C.

b. Silver nitrate reagent (Trevelyan, 1950)

The air dried chromatogram was dipped in silver nitrate solution (1 mL saturated $AgNO_3$ in water, diluted to 6 mL with water + 200 mL acetone). After drying, it was dipped in methanolic potassium hydroxide (1 volume of 10 % aqueous potassium hydroxide + 5 volumes of methanol). When black spots appeared in the region of sugars, the chromatogram was washed with sodium thiosulphate (0.05 M) solution to clear the background colour. Finally the chromatogram was washed with water and dried.

2.2.4.2. Gas Liquid Chromatography

Preparation of alditol acetates (Sawardekar et al., 1967)

The neutralized and deionised sample was concentrated to about 0.5 mL and sodium borohydride (20-30 mg) was added and the test tubes were stoppered and taped with adhesive plaster around to hold the stoppers and were left over night. Next day, excess borohydride was destroyed with acetic acid (2 M). The excess borate and other salts were removed by co-distillation with methanol (1mL, x4) and then evaporated to dryness. Dry and distilled acetic anhydride and pyridine (0.5 mL, each) were added and kept in an oven at 100^{o}C for 2 h after tightly stoppering the tubes. Excess reagent was removed by co-distillation with water (1 mL, x3) and toluene (1 mL, x3). After thorough drying, the contents were taken in chloroform and filtered through glass wool and dried by passing nitrogen gas. They were taken in chloroform for analysis.

Operating conditions for GLC

The alditol acetates were detected in gas liquid chromatography (Model-CR 4A) fitted with flame ionization detector (FID) using OV-225 column at column temperature 200^{o}C, detector 250^{o}C and injector temperature of 250^{o}C. Nitrogen (40 mL/min) was the carrier gas (Fig. 7).

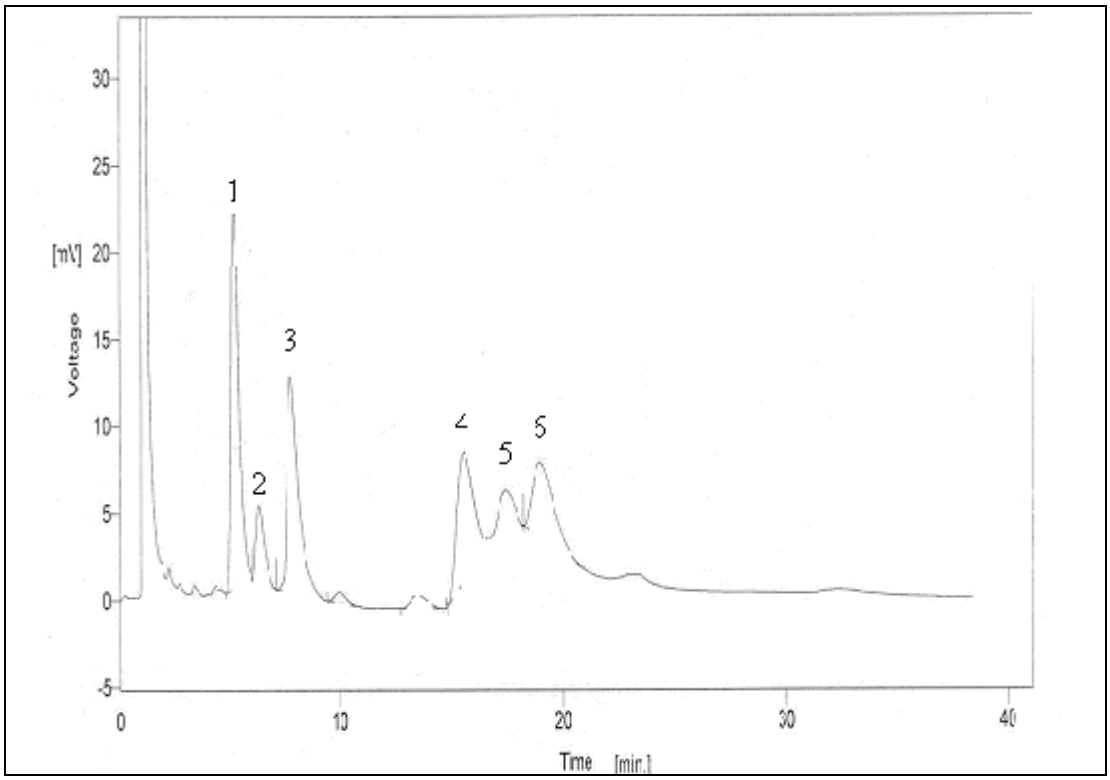


Fig. 7: GLC separation of alditol acetates of sugars on OV-225 column.

1. Rhamnose/Fucose, 2. Arabinose, 3. Xylose,

4. Mannose, 5. Galactose, 6. Glucose.

2.2.4.3. Gas Liquid Chromatography-Mass spectrometry (GLC-MS)

GLC-MS analysis was carried out on Shimadzu (Model-QP5000) by using SP-2380 capillary column. The temperature gradient of 180-200ºC with an increase of 4ºC/ min was maintained for the analysis. Ionization potential was 70 eV and mass range (m/z) was 40-400. Helium was used as carrier gas.

2.2.4.4. High performance liquid chromatography (HPLC) (McGinnis and Fang, 1980)

The free sugars were resolved and identified by high performance liquid chromatography (C-R4 A, Shimadzu) using a micro-bondapack amino column (4.1 mm x30 cm) and eluted with acetonitrile and water (75:25) solvent system at flow rate of 1 ml/min. Glucose, fructose, galactose, maltose, sucrose, raffinose and stachyose were used as reference sugars. The area under each peak was measured to quantify individual sugars.

2.2.4.5. High performance size exclusion chromatography (HPSEC)

(Gruppen et al., 1993)

E-Linear (7.8 mm x 30 cm) connected in series with E-1000 (3.9 mm x 30 cm) column were used. Degassed triple distilled water was the mobile phase used. Flow rate was maintained at 0.6 mL/min. Oven temperature was 50 ºC and RI setting was 8 x 10^{-6} RIU. Calibration curve was prepared using dextran standard (5 to 10 µL) of different molecular weights (T-40, T-70, T-150, T-500 and T-2000).

2.2.4.6. Sepharose CL-2B column chromatography (Izydorczyk and Biliaderis, 1993)

Purified polysaccharides (10 mg) were dissolved in 1mL of water containing 0.05% sodium azide and were analyzed on a precalibrated (with T-series dextran standards as T_{10}, T_{20}, T_{40}, T_{70},T_{500}, T_{2000}) Sepharose CL-2B column (1.6 x 92 cm) using triple distilled and degassed water containing 0.05% sodium azide as eluant. Fractions (3 mL) were collected using an LKB Bromma

2211 fraction collector at 18 mL/h flow rate and total sugar contents were analyzed by phenol-sulphuric acid method (Section 2.2.2.5.1).

2.2.4.7. Reverse phase chromatography (Wulf and Nagel, 1976)

Phenolic acid composition of free and bound forms were analyzed by using HPLC (model LC-10A, Shimadzu corporation, Tokyo, Japan) analysis on a reverse phase Shimpak C_{18} column (4.6 × 250 mm) using a diode array UV-detector (operating at 280 and 320 nm). A solvent system consisting of water: acetic acid: methanol (isocratic; 80: 5:15, v/v/v) was used as mobile phase at a flow rate of 1 mL / min. Standard phenolic acids were used for identification and quantification of phenolic acids present in the sample.

2.2.5. Purification of polysaccharides (Chanliaud et al., 1995)

Polysaccharide (1.0 g) was dissolved in water (50 mL). The pH of the solution was adjusted to 3.0 with HCl (1 N). Polysaccharides were precipitated by adding two volumes of ethanol (95%). It was kept at 4ºC for 6 h and then centrifuged. The precipitate so obtained was dissolved in acetate buffer (pH 4.5, 0.1 M) and subjected to glucoamylase digestion at 60ºC for 4 h. After digestion, the polysaccharides were precipitated by two volume of ethanol and kept at 4ºC overnight. Residue was collected by centrifugation. It was uniformly dispersed in water and dialyzed and lyophilized.

2.2.6. Structural analysis

2.2.6.1. Methylation analysis (Hakomori, 1964)

a. Preparation of methyl sulphinyl carbanion (MSC)

Sodium hydride (99%, 500 mg) was taken in a reaction vial and repeatedly (4-5 times) washed with petroleum ether (dried with sodium wire) and finally dried by passing dry nitrogen gas. Dimethyl sulphoxide (DMSO) was added in small portions over a period of time. It was kept at 37ºC for 12 h with occasional venting of hydrogen, formed during the reaction. The prepared MSC gave red blood colour with triphenyl methane.

b. Methylation

Purified polysaccharide (10 mg) was dissolved in dry, distilled DMSO (0.5 mL) with stirring and occasional ultrasonication. MSC (1 mL) was added to the above solution and the mixture was stirred at room temperature for 3-4 h. After the reaction, the mixture was tested for excess reagent and was made sure that the test was positive for triphenyl methane test. Iodomethane (1 mL) was added to the reaction mixture at ice cold temperature with the help of syringe. The reaction mixture was stirred over night.

c. Purification of methylated polysaccharides (Waeghe et al., 1983)

Purification was done by using Sep-Pak C_{18} cartridge. The cartridge was activated by flushing ethanol (40 mL) followed by acetonitrile (2 mL) and water (4 mL). The methylated reaction mixture was diluted with equal amount of water and passed through the cartridge. More polar contaminants of the reaction such as DMSO and sodium iodide were eluted with water (2 mL, x 4). Less polar contaminants were eluted with 2 mL of the following; acetonitrile-water 3:17 (v/v, x4), acetonitrile- water 1:4 (v/v, x4), 100% acetonitrile (x4), 100% methanol (x4) and 95% ethanol (x4). The cartridge was flushed with solvent mixtures at a rate of about 1-2 drops/ sec. The fractions eluted were tested on the silica gel TLC strip by charring with 5% sulphuric acid in ethanol (v/v). The fractions giving positive test for carbohydrates were pooled and concentrated by flash evaporation. The methylated samples were eluted in 100% acetonitrile and 100% methanol.

d. Derivatization of purified methylated samples

The methylated polysaccharides were hydrolyzed by formic acid and TFA (section 2.2.2.4.3) and reduced using sodium borodeuteride ($NaBD_4$) in D_2O and acetylated (section 2.2.4.2).

e. GLC and GLC-MS of permethylated samples

GLC-MS analysis was done as described (Section 2.2.4.3). The column temperature was maintained at 180ºC.

2.2.6.2. Periodate oxidation (Avigad, 1969)

To 5.0 mL of aqueous solution of the polysaccharide, sodium meta periodate (5.0 mL, 20 mM) was added. The reaction mixture was mixed well and kept at 4^{o}C in dark. At regular intervals, 0.5 mL aliquots were withdrawn and mixed with saturated sodium bicarbonate (1.0 mL), sodium arsenate (2.0 mL, 0.005 M) and potassium iodide (0.2 mL, 20%). The reaction mixture was kept aside for 15 min in the dark and titrated against iodine solution (standardized, 0.01 N) using 1-2 drops of starch solution (2.0%) as indicator. Simultaneously, a blank was run by taking 0.5 mL water in place of the sample. The consumption of periodate was calculated using the formula:

$$\text{Periodate oxidation} = \left(E - \frac{(V_1 - V_2) \times C}{1000} \right) \times \frac{M}{G}$$

Where, E = Actual moles of periodate taken,

V_1 = Titre value of the blank,

V_2 = Titre value of the sample,

C = Concentration of standardized iodine solution,

M = Molecular weight of sugar

G = Weight of sample taken in grams.

2.2.6.3. Formic acid liberation (Brown et al., 1948)

Aliquot (1mL) was withdrawn from the above reaction mixture after the periodate consumption became constant. Ethylene glycol (1mL) and methyl red

indicator (2 drops, 0.02% in ethanol) were added to it and titrated against sodium hydroxide (0.01 N). Change in the color of the solution from pink to yellow indicated the end point. A reagent blank was prepared in the same way with ethylene glycol and the difference in acidity between the blank and sample represented the formic acid liberation.

2.2.6.4. Smith degradation (Abdel-Akher et al., 1952)

To the polysaccharide solution (10 mg in 5mL) sodium meta periodate (5 mL, 20 mM) was added and kept at 4°C for 48 h. Reaction was stopped by the addition of ethylene glycol (0.1mL) and the oxidized polysaccharide sample was reduced with sodium borohydride (100 mg) at room temperature for 16 h. Excess borohydride was destroyed by using acetic acid (2 N) and the solution was dialyzed. The sample was hydrolyzed with sulphuric acid (2 mL, 0.5 N) at boiling water bath temperature for 4 h. Derivatised and analyzed by GLC.

2.2.6.5. ^{1}H NMR Spectroscopy (Hoffmann et al., 1992)

Purified polysaccharide (10 mg/mL in D_2O) was taken in sample probe (ф 5mm x 15cm) and resonance spectrum was recorded in a Bruker 500-MHz NMR (Bruker, Biospin, Switzerland) spectrometer. Deuterium resonance was used as reference.

2.2.6.6. ^{13}C NMR spectroscopy (Hoffmann et al., 1992).

^{13}C NMR spectra were recorded on a Bruker 500 MHz (Bruker, Biospin, Switzerland) spectrometer. The samples were exchanged twice with D_2O (99%). The samples (20 mg) were dissolved in D_2O (1 mL) for recording the spectra.

2.2.6.7. Infra red spectroscopy (Kacurakova et al., 1994).

Polysaccharide sample (1 mg) was blended with potassium bromide (100 mg) and the pellet prepared by using a palletizer. IR spectra were recorded between 4000-400 cm^{-1} (4 cm^{-1} resolution) using a Perkin-Elmer 2000 spectrometer (Norwalk, USA).

2.2.6.8. Optical rotation (Saavendra et al., 1988)

Aqueous solution of polysaccharide (0.5 – 1.0 %) was used to measure the optical rotation in a Perkin - Elmer (Model 243) polarimeter. Optical rotation was calculated using the formula:

$$\text{Optical rotation } [\alpha]_D = \frac{100\,\theta}{lc}$$

Where, θ = Angle of rotation of plane polarized light,

l = The path length (1cm),

c = Concentration (%) of the polysaccharide solution.

2.2.7. Textural profile analysis of wheat dough (Manu and Prasad Rao, 2008)

Wheat doughs were prepared by adding optimum amount of distilled water (based on their farinograph water absorption value) to the whole-wheat flour (after 30 min of resting period) and were subjected to textural profile analysis using a universal Textural measuring system (Lloyds, LR5K, Fareham, Hampshire, UK). Dough was cut into cylindrical pieces of 3 cm diameter and 1 cm height and measured for dough hardness (peak force during the first compression cycle or first bite), cohesiveness (ratio of the positive force area during the second compression to that of the first compression) and adhesiveness (negative force area for the first bite). Triplicate measurement was taken for each variety. Textural profile analysis was measured with cross head speed of 60 mm/min, load cell of 5 kg, compression of 50% of sample height and probe diameter of 3.5 cm.

2.2.8. Relative viscosity

Relative viscosity of purified pentosans, with respect to water was determined as described earlier (Muralikrishna et al., (1987). Polysaccharide fractions were dissolved in water and the viscosity was determined in an Ostwald viscometer.

2.2.9. UV spectroscopy (Patil et al., 1975b)

UV absorption spectra of polysaccharide solution taken in quartz cuvette were recorded between 200-400 nm using a UV-visible spectrophotometer (Shimadzu, Kyoto, Japan).

2.2.10. Statistical Analysis

All the results were average of three determinations. Statistical significance was assessed by ANOVA and post-hoc Duncan's Multiple Range Test ($p<0.05$) using SPSS 10 for windows (SPSS, Chicago, IL, USA).

Chapter 3
Results and Discussion

3. RESULTS AND DISCUSSION

3.1. Carbohydrate composition of pentosans isolated from wheat varieties having differences in *chapati* making quality (Revanappa et al., 2007)

Wheat is a major cereal in India and is consumed mainly in the form of unleavened flat bread known as *chapati* (Prabhasankar et al., 2002). *Chapati* is usually prepared from whole-wheat flour and the desired quality parameters in *chapati* are greater pliability, soft texture, light creamish brown colour, slight chewiness and baked wheat aroma (Haridas Rao et al., 1986). Pentosans (Arabinoxylans) are the major non-starch polysaccharides in wheat and consist of xylan backbone with branches of arabinose residues in various linkages. Pentosans are known to play an important role in water balance of dough (Courtin and Delcour, 2002), rheological properties of dough (Michniewicz et al., 1991; Shogren et al., 1987), retrogradation of starch (Gudmundsson et al., 1991) and bread making quality (Delcour et al., 1991). Variations in the amount, chemical composition and structures of pentosans bring about differences in their physico-chemical, solubility and functional properties (Gruppen et al., 1993; Courtin and Delcour, 1998). Pentosans in wheat are known to be best extracted with barium hydroxide (Gruppen et al., 1991). Alkali-soluble pentosans (hemicelluloses A and B) have also generated much interest (Salimath and Tharanathan, 1982; Subba Rao and Muralikrishna, 2006). In the present study, pentosans are isolated from whole-wheat flours of different wheat varieties differing in *chapati* making quality by using various extractants and determined their carbohydrate composition and studied structure-function relationship of pentosans.

3.1.1. *Chapati* making quality

Sensory evaluation scores of *chapaties* prepared from four wheat varieties are given in Table 1. *Chapaties* prepared from DWR-162 and GW-322 had appealing light brown colour. On the other hand MACS-2496, had relatively

dark colour. Tearing strength data indicated that GW-322 followed by DWR-162 had desirable tearing strength. *Chapaties* made from GW-322 and DWR-162 was highly pliable. Highest sensory scores for taste and aroma were recorded for GW-322 and DWR-162. MACS-2496 and HD-2189 recorded significantly lower scores for taste due to bland taste. Chewiness properties showed that DWR-162 and GW-322 had higher scores. These *chapaties* were neither tough nor hard to chew and had optimum chewing properties. As shown by high overall quality scores, varieties DWR-162 and GW-322 yielded highly acceptable *chapaties*. MACS-2496 and HD-2189 yielded lower overall quality scores (Hemalatha et al., 2007).

3.1.2. Free sugars in whole-wheat flours

Glucose, fructose, sucrose, maltose and raffinose were the free sugars detected in all the wheat varieties (Table 2). Sucrose and maltose were the predominant free sugars. Free sugars play a vital role in baking and malting process (Nirmala et al., 2000). Free sugars of the seeds form the source of energy for the biochemical activities during initial stages of germination and also contribute to the malting quality (Malleshi et al., 1986; Suhasini, et al., 1997). Free sugar contents were higher in MACS-2496 and HD-2189 (poor *chapati* making quality) compared to DWR-162 and GW-322 wheat varieties (good *chapati* making quality).

Table 1. Sensory evaluation of *chapaties* prepared from different wheat Varieties

Wheat variety	Appearance (10)	Tearing strength (10)	Pliability (10)	Taste and aroma (10)	Chewiness (10)	Overall quality (50)
DWR-162	8.8	8.3	8.3	8.3	8.3	42.0
GW-322	8.2	8.6	8.6	8.3	8.0	41.7
MACS-2496	7.3	7.4	7.2	6.2	7.1	35.2
HD-2189	7.3	7.7	7.6	6.5	6.9	36.0

Table 2. Composition of soluble sugars in alcoholic extracts of the whole-wheat flours

Wheat variety	Glucose	Fructose	Sucrose	Maltose	Raffinose	Total sugar (%)
DWR-162	0.09	0.08	0.62	0.56	0.03	1.38
GW-322	0.05	0.07	0.42	0.62	0.04	1.20
MACS-2496	0.07	0.09	0.54	0.68	0.06	1.44
HD-2189	0.10	0.06	0.68	0.72	0.02	1.58

3.1.3. Contents of total protein in wheat varieties

The wheat variety MACS-2496 had the highest protein content of 17%. Other wheat varieties viz., DWR-162, GW-322 and HD-2189 had the protein content of 14.6, 15.5, and 14.1%, respectively. This revealed that the wheat varieties used in the present investigation had relatively high protein content. Protein content as high as 21% and as low as 6.5% have also been reported in wheat varieties (Hoseney, 1994).

3.1.4. Starch contents in wheat varieties

Starch is the most abundant carbohydrate found in all the cereals including wheat. Starch content of wheat has been reported to be in the range of 63-72 % (Hoseney, 1994; Pomeranz, 1989). In the present study, the starch content was highest in HD-2189 (67.9%), while, MACS-2496 had the lowest starch content (61.4 %). DWR-162 and GW-322 had the starch content of 66.2 and 65.3%, respectively. Physical properties of starch, such as gelatinization, retrogradation and gelation, strongly influence the quality of wheat flour products (Mahadevamma and Tharanathan, 2001; Sasaki et al., 2004).

3.1.5. Carbohydrate composition of DWR-162

Carbohydrate profile of DWR-162 variety of wheat flour and its isolated fractions are given in Table 3. Wheat flour and its various polysaccharide fractions were rich in carbohydrates and uronic acid content ranged from 2.6-5.7%. Sugar analysis of the flour indicated glucose, predominantly, with small amounts of arabinose and xylose and minor quantities of rhamnose/fucose and mannose. The ratio of arabinose to xylose in the flour was 1.26. Water-soluble polysaccharides were rich in glucose, which could be arising from β-glucans, resistant starch and unhydrolysed starch molecules (Fincher and Stone, 1986). Arabinose, xylose, rhamnose/fucose and mannose were also observed in minor amounts. Polysaccharides extracted with barium hydroxide were found to be rich in pentosans (78.1%) and had moderate amount of glucose (22%). This polysaccharide could be a complex mixture of arabinoxylans and β-glucans.

Presence of β-glucans in arabinoxylan fractions is reported earlier (Cleemput et al., 1993). The ratio of arabinose to xylose in barium hydroxide extract was 1.68, indicating that it might be due to higher degree of branching in these pentosans. Higher degree of branching was reported in some Canadian wheat flours of variable bread making quality (Izydorczyk and Biliaderis, 1995).

Hemicellulose A contained xylose predominantly (66.4%), with small amounts of arabinose and glucose. This fraction may contain mainly xylan-type of polysaccharides and ratio of arabinose to xylose was low (0.14). High amounts of arabinose, xylose and glucose were observed in hemicellulose B. Presence of glucose in this fraction may be due to the presence of β-glucans and resistant starch and unhydrolysed starch molecules. The alkali-insoluble residue still contained moderate amounts of arabinose and xylose, suggesting strong association of arabinoxylans with cellulose. Strong association of cellulose with non-starch polysaccharides was also reported in redgram (Swamy et al., 1991) and wheat bran (Brillout and Mercier, 1981).

Table 3. Carbohydrate composition (%) of whole-wheat flour (DWR-162) and its isolated fractions

Fractions	Yield	Total sugar	Uronic acid	Rha/ Fuc	Ara	Xyl	Man	Gal	Glc	Ara/Xyl ratio
Flour	(100)	75.6	5.7	1.10	7.30	5.75	2.96	-	82.78	1.26
WSP	11.5	87.5	3.4	0.74	1.97	1.26	2.47	-	93.5	1.56
BE	3.6	78.1	3.0	-	49.0	29.0	-	-	22.0	1.68
Hem A	1.0	88.1	2.6	1.72	9.87	66.49	1.26	-	20.62	0.14
Hem B	1.0	80.0	2.8	-	26.66	38.87	2.48	-	31.97	0.68
AIR	3.5	73.4	3.6	-	29.20	17.55	2.07	-	51.15	1.66

WSP: Water-soluble polysaccharides, BE: Barium hydroxide extract,
Hem A: Hemicellulose A, Hem B: Hemicellulose B,
AIR: Alkali-insoluble residue, Rha: Rhamnose,
Fuc: Fucose, Ara: Arabinose,
Xyl: Xylose, Man: Mannose,
Gal: Galactose, Glc: Glucose.

3.1.6. Carbohydrate composition of GW-322

Carbohydrate composition of wheat variety GW-322 that exhibited good *chapati* making quality revealed that flour and its various polysaccharide fractions were rich in carbohydrate content (Table 4). Uronic acid content ranged from 2.1-6.7%. Glucose was the major sugar and a small amount of arabinose and xylose with minor quantities of mannose and rhamnose / fucose were present in the flour. The ratio of arabinose to xylose was found to be 1.32 in whole-wheat flour. The water-soluble polysaccharide was rich in glucose (83%) and had a small amount of arabinose and xylose. Water-soluble extracts are known to contain high amounts of β-glucans with associated pentosans. The barium hydroxide extract was rich in arabinose, xylose (with a total amount of 83%) and contained small amounts of glucose (13.7%). The ratio of arabinose to xylose was 1.14, indicating a higher degree of branching in these pentosans. Izydorczyk and Biliaderis (1995) have reported A/X ratio ranging from 1.1 to 1.2 in Canadian wheat varieties. Large variations in the degree of branching of pentosans were also found among European wheat flours of different bread making property (Cleemput et al., 1993).

Both hemicelluloses A (79.2%) and B (63%) were rich in xylose, with small amounts of arabinose, glucose and uronic acid. These hemicellulosic fractions (hemicellulose A and B) may contain mixture of xylan-type polysaccharide with small amounts of arabinoxylan type of polysaccharides. The associated glucose might be coming from β-glucans and resistant starch formed during gelatinization of starch. The alkali-insoluble residue was mainly cellulosic in nature with associated small amounts of arabinoxylans.

Table 4. Carbohydrate composition (%) of whole-wheat flour (GW-322) and its isolated fractions

Fractions	Yield	Total sugar	Uronic acid	Rha/ Fuc	Ara	Xyl	Man	Gal	Glc	Ara/Xyl ratio
Flour	(100)	76.3	3.5	0.44	5.56	4.20	0.80	-	89.00	1.32
WSP	12.2	83.0	4.7	0.46	6.98	9.18	1.40	-	81.94	0.76
BE	4.2	80.2	2.1	-	44.50	38.90	-	2.90	13.70	1.14
Hem A	0.3	88.0	6.7	-	4.36	79.25	-	-	16.39	0.05
Hem B	0.5	90.0	4.5	-	13.50	62.90	-	-	23.60	0.21
AIR	3.2	74.0	3.8	-	15.6	11.80	2.20	-	70.40	1.32

Abbreviations as in Table 3.

3.1.7. Carbohydrate composition of MACS-2496

The wheat variety, MACS-2496 exhibited poor *chapati* making quality and the carbohydrate composition of the flour and its fractions are shown in Table 5. Wheat flour was rich in carbohydrates (70.8%) and the uronic acid content was 4.7%. Glucose was the major sugar and a small amount of arabinose and xylose were observed in the flour. The water-soluble polysaccharide contained glucose, mainly, with small amounts of arabinose and xylose and high amount of glucose (86.5%) may be due to the presence of β-glucan type polysaccharide and resistant starch along with unhydrolysed starch molecules. β-Glucan content in wheat is reported to be 0.8% on dry weight basis (Henry, 1987).

The barium hydroxide extracted arabinoxylans contained principally, arabinose and xylose in the ratio of 0.8, indicating lower degree of branching. The ratio of arabinose to xylose in wheat endosperm may vary from 0.5 to 0.7 (Cleemput et al., 1993) but it is lower than wheat bran (Brillouet and Mercier, 1981). The presence of small amounts of glucose may be due to resistant starch that may be formed during gelatinization of starch. The alkali-soluble polysaccharides were rich in carbohydrates. High amounts of xylose (42.5%) and glucose with small amount of arabinose and uronic acids were observed in hemicellulose A. This fraction may contain xylan-type polysaccharide with small amount of arabinoxylans and β-glucan type polysaccharides. Hemicellulose B was rich in pentosans (69%) and had moderate amounts of glucose. Alkali-insoluble residue still contained considerable amount of arabinose and xylose (24%).

Table 5. Carbohydrate composition (%) of whole-wheat flour (MACS-2496) and its isolated fractions

Fractions	Yield	Total sugar	Uronic acid	Rha/ Fuc	Ara	Xyl	Man	Gal	Glc	Ara/Xyl ratio
Flour	(100)	70.8	4.7	0.82	6.34	7.86	1.03	-	83.95	0.80
WSP	10.9	72.0	2.0	0.96	5.69	4.99	1.83	-	86.53	1.14
BE	2.9	76.0	3.0	-	41.0	51.20	-	-	7.80	0.80
Hem A	1.2	73.8	6.0	1.33	4.48	42.50	-	-	51.69	0.10
Hem B	1.4	80.0	4.0	-	21.44	47.84	-	-	30.72	0.44
AIR	4.5	65.8	4.1	-	10.98	13.12	2.66	0.31	72.93	0.83

Abbreviations as in Table 3.

3.1.8. Carbohydrate composition of HD-2189

Wheat variety HD-2189 was identified as poor *chapati* making variety, and was selected to study the nature of pentosans. The flour contained carbohydrates as major component with small amounts of uronic acid (Table 6). Sugar composition of the flour showed glucose, mainly, with small amounts of arabinose, xylose and mannose along with minor quantities of rhamnose/fucose. The water-soluble polysaccharides had predominantly glucose, which might be coming from β-glucans, resistant starch, and unhydrolysed starch molecules. Arabinose, xylose and glucose were the major sugars observed in barium hydroxide extract. A/X ratio was found to be lower (0.65) in these polysaccharides, indicating that it may be due to lower degree of branching. Izydorczyk and Biliaderis (1993) reported that degree of branching (A/X ratio) ranged between 0.58–0.96 in some of the Canadian wheat varieties of diverse technological characteristics. Vinkx and Delcour, (1996) reported A/X ratio as low as 0.21 for rye arabinoxylans.

Hemicellulose A contained xylose, predominantly (59.7%), with moderate amounts of glucose along with small amounts of arabinose. This fraction may be a mixture of xylan type polysaccharide with small amounts of arabinoxylan type of polysaccharides. Arabinose, xylose and glucose were the major sugars found in hemicellulose B fraction. Alkali-insoluble residue contained mainly, glucose (80%), with small amounts of arabinose and xylose.

Table 6. Carbohydrate composition (%) of whole-wheat flour (HD-2189) and its isolated fractions

Fractions	Yield	Total sugar	Uronic acid	Rha/ Fuc	Ara	Xyl	Man	Gal	Glc	Ara/Xyl ratio
Flour	(100)	76.2	4.0	0.32	3.80	4.50	3.20	-	88.18	0.84
WSP	14.5	89.3	3.0	0.43	2.60	3.00	0.36	-	93.61	0.86
BE	5.5	85.0	1.6	-	26.70	40.68	-	1.20	31.40	0.65
Hem A	0.5	95.5	3.3	-	5.00	59.78	0.80	-	34.40	0.08
Hem B	0.8	84.0	1.9	-	30.60	24.60	2.00	0.40	42.40	1.24
AIR	3.2	72.7	2.6	-	10.00	8.10	1.90	-	80.00	1.23

Abbreviations as in Table 3.

Wheat varieties, GW-322 and DWR-162 revealed good *chapati* making characteristics, while, MACS-2496 and HD-2189 had poor *chapati* making quality (Table 1). The wheat varieties studied showed differences in the nature of constituent sugars and hence suggested variations in the nature of polysaccharides in wheat varieties having differences in *chapati* making characteristics. Barium hydroxide soluble polysaccharides and hemicelluloses A and B were rich in pentosans. Arabinoxylans of barium hydroxide extracted polysaccharides might be highly branched in wheat varieties, DWR-162 and GW-322 having good *chapati* making quality as indicated by high Ara / Xyl ratio, where as those of MACS-2496 and HD-2189 wheat varieties (poor *chapati* making characteristics) showed lower degree of branching as revealed by low Ara / Xyl ratio.

Arabinoxylans in different varieties of wheat are known to vary and have relation to bread making quality. The contents of arabinose and xylose were reported to be higher in varieties of wheat that have good tandoori roti making quality (Saxena et al., 2000). Some amount of glucose in these fractions may originate from β-glucans (Mares and Stone, 1973; Henry, 1987) or α-glucan polymers that might have entrapped within the insoluble polysaccharide matrix (Smith and Hartley, 1983) or may be as a result of incomplete removal of starch by amylase treatment. The ratio of Ara / Xyl, indicative of degree of branching of arabinoxylans, may play an important role in the physico-chemical properties of these constituents and branching is known to affect conformation of these biopolymers in solutions (Andrewartha et al., 1979). The alkali-insoluble residues (AIR) are mainly cellulosic in nature in poor varieties (MACS-2496 and HD-2189) and appear to be more tightly bound to pentosans in good *chapati* making varieties (DWR-162 and GW-322) (Revanappa et al., 2007).

3. 2. Phenolic acid profile and antioxidant properties of wheat varieties

Whole-wheat flour contains several phytochemicals that could be important dietary antioxidants and among these compounds, free and bound phenolic acids seem to have the potential health benefits (Baublis et al., 2000; Adom et al., 2002). Epidemiological evidence has associated the consumption of whole-grains and their products with reduced incidence of chronic diseases such as cardiovascular disease, diabetes and cancer (Yu et al., 2002; McCallum et al., 1990). These health benefits have been attributed in part to the unique phytochemicals of whole wheat flours. Antioxidants are believed to contribute to health benefits through several possible mechanisms, such as directly reacting with and quenching free radicals, chelating transition metals, reducing peroxides and stimulating antioxidative enzyme defense (Rice-Evans et al., 1996; Robbins, 2003). Synthetic antioxidants have been in use as food additives since long time but safety concerns and reports on their involvement in chronic diseases have restricted their use in foods (Onyeneho and Hettiarachchy, 1992). Therefore, exploitation of potential, economical, indigenous sources of antioxidants based on natural origin is focus of research since last decade. Wheat variety with a high level of antioxidant activity should have potential for use as an excellent dietary source of antioxidants for disease prevention and health promotion (Chandrica et al., 2007). However, many factors such as genotypes and growing conditions have been reported to have a significant effect on the phenolic acids and antioxidant properties of wheat (Yu et al., 2003; Zielinski and Kozlowska, 2000).

Apart from phenolic acids, whole-wheat flour is also one of the rich source of soluble and insoluble dietary fibre (Adom et al., 2003; Nandini and Salimath, 2001). Dietary fibres are known to be beneficial against a variety of diseases, including colon cancer and diabetes (Plammi, 1997). Dietary fibre includes cellulose, pectin, hemicellulose and other polysaccharides (Esposito et al., 2005). Chemical structure, solubility and lignification are some of the

properties of dietary fibre of great importance for fermentability (Bourguin et al., 1996). Cross linking of polysaccharides in the presence of ferulic acid will have an important influence on fibre solubility and ultimately on resistance to microbial degradation (Bunzel et al., 2001).

Phenolic acids exist in free and bound forms in cereals (Sosulski et al., 1982; Krygier et al., 1982). Free phenolic acids are found in the outer layers of kernel (pericarp, testa and aleurone), whereas most of the phenolic acids in cereals primarily occur in the bound form as conjugates with sugars, fatty acids, or proteins (White and Xing, 1997). It is essential to consider the contribution from the bound phenolic acid to total phenolic content and antioxidant activity (Chandrica et al., 2006; Serpen et al., 2008). The literature on the proportion of free and bound phenolic acids in Indian wheat varieties is limited. Free and bound phenolic acids were isolated from different Indian wheat varieties and have been characterized colorimetrically as well as by HPLC methods and analyzed their antioxidant activities using *in-vitro* methods. Both soluble and insoluble dietary fibre contents and their sugar composition were also analyzed in these wheat varieties.

3.2.1. Phenolic acid contents in wheat varieties

Phenolic acid contents of wheat varieties are presented in Table 7. Free phenolic contents were the highest in MACS-2496 (260.2 µg/g) followed by HD-2189 (246.5 µg/g), while, GW-322 had the lowest amount of free phenolic acid (196.6 µg/g). Bound phenolic contents was also the highest in MACS-2496 (680.4 µg/g) and lowest in GW-322 (514.4 µg/g). On an average, bound phenolic acid content was nearly 2-3 folds higher than free phenolic acid contents in all the wheat varieties. Bound phenolic acid content was significantly more than the free phenolic acid content among various cereals (Adom et al., 2003; Chandrica et al., 2006; Serpen et al., 2008).

Phenolic acid content and antioxidant activity of wheat fractions had correlated well in Canadian wheat varieties (Beta et al., 2005; Zhou et al., 2004)

and provided evidence that the predominant source of antioxidants is derived from phenolic compounds in wheat. A significant correlation was also found between total phenolics and antioxidant activity of other plant products (Velioglu et al., 1998). Both free and bound phenolic contents were higher in MACS-2496 followed by HD-2189. The bound phenolic acids were higher than the free phenolic acids in all the wheat varieties, indicating that major phenolic acids in whole-wheat were not extractable by aqueous ethanol but released upon alkaline or acid hydrolysis (Kim et al., 2006).

Table 7. Free, bound and total phenolic acid contents (µg/g of flour) of wheat varieties

Wheat varieties	Free phenolic acid	Bound phenolic acid	Total phenolic acid
DWR-162	216.4 ± 8.5	530.4 ±14.8	746.8 ± 19.3
GW-322	196.6 ± 7.6	514.6 ±12.6	711.2 ± 20.2
MACS-2496	260.2 ± 12.8	680.4 ±16.8	940.6 ± 25.6
HD-2189	246.5 ± 11.5	646.4 ±15.2	892.9 ± 22.7

Values are mean ± standard deviation (n = 3).

3.2.2. Phenolic acid composition analysis using HPLC

Free and bound phenolic acid composition was further analyzed by HPLC. The identities of phenolic acids were confirmed based on the retention times of standard phenolic acids. Ferulic, p-coumaric and vanillic acids were found to be the major phenolic acids in free form, where as syringic and protocatechuic acids were present in small amounts (Table 8). These results are consistent with the earlier reports on various cereals (Hatcher and Kruger, 1997; Moore et al., 2005; Subba Rao and Muralikrishna, 2002). HD-2189 variety had the highest amount of ferulic acid (39.4 µg /g) in free form followed by MACS-2496 (29.6 µg /g) and was lowest in GW-322 (23.5 µg /g). Ferulic acid was the predominant phenolic acid in bound form, along with small amount of p-coumaric acid in all the wheat varieties (Table 8). These results are in accordance with those of earlier reports on wheat phenolics (Velioglu et al., 1998). Wheat varieties such as MACS-2496 (395.4 µg /g) and HD-2189 (368.5 µg /g) had higher contents of ferulic acids in bound form. Phenolic acids in cereals primarily occur in the bound form, as these are strongly associated with cell wall polysaccharides (White and Xing, 1997). Ester linkages of ferulic acid with arabinoxylans and their role in the formation of arabinoxylan structures have been widely described (Ishii, 1997). Ferulic acid and its dimers play an important role in the formation and functional properties of dietary fiber (Renger and Steinhart, 2000). Ferulic acid content was positively associated with scavenging of free radicals and total phenolic acid content and hence may be used as potential marker of wheat antioxidants (Sindhu and Emilia, 2004; Zhou et al., 2004).

Table 8. Free and bound phenolic acid composition of wheat varieties

Wheat variety	Free phenolic acids (µg/g of flour)					Bound phenolic acids (µg/g of flour)	
	Proto	Van	Syr	p- CA	FA	p-CA	FA
DWR-162	3.4	14.9	7.6	21.4	25.4	18.6	286.0
GW-322	2.4	12.6	6.9	16.4	23.5	14.8	310.0
MACS-2496	3.2	10.4	5.4	23.5	29.6	18.4	395.4
HD-2189	2.9	11.5	6.1	26.8	39.4	22.0	368.5

Proto: Protocatechuic acid, Van: Vanillic acid, Syr: Syringic acid, p-CA: para Coumaric acid, FA: Ferulic acid.

3.2.3. Antioxidant activities of free and bound phenolic fractions

The antioxidant activity of phenolic fractions was evaluated by determining their reducing power and free radical scavenging activity.

3.2.3.1. Reducing power of phenolic acids

The reducing power of free and bound phenolic extracts from whole-wheat flour of different wheat varieties is shown in Figs. 8 and 9, respectively. Increase in absorbance of the reaction mixture indicated increased reducing power of the phenolic extracts. Results indicate concentration dependent increase in the activity of free and bound phenolics in different wheat varieties. Free phenolic extracts of HD-2189 and MACS-2496 had higher reducing capability compared to other varieties (Fig. 8). Bound phenolic fractions have exhibited higher reductive capabilities than the free phenolics (Fig. 9). Higher activity might be due to the presence of higher levels of ferulic acid in bound forms along with other phenolic constituents (Chandrika et al., 2006). Wheat varieties such as MACS-2496 and HD-2189 had higher reducing power in bound phenolic extracts (Fig. 9). DWR-162 had the lowest reducing power in both free and bound phenolic extracts. The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity (Meir et al., 1995; Shimada et al., 1992; Suresh et al., 2006).

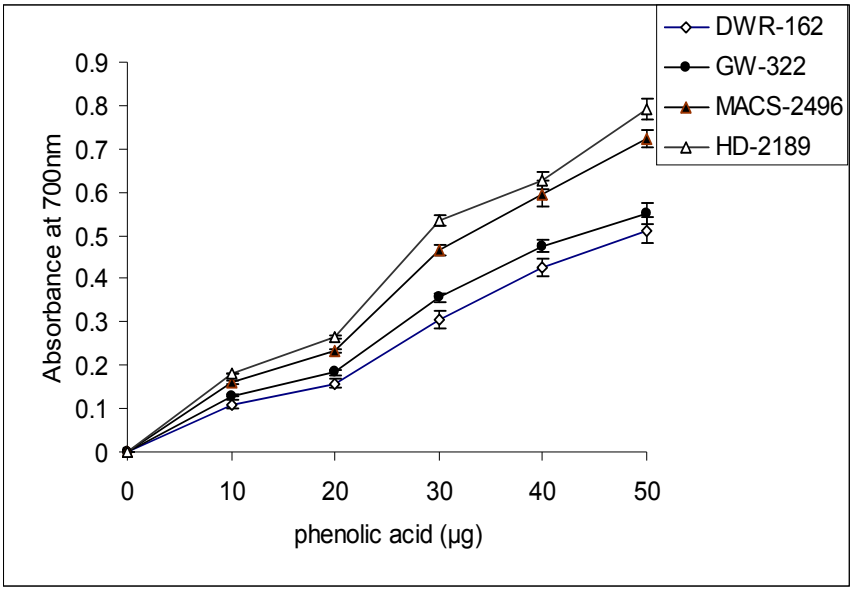


Fig. 8. Reducing power of free phenolic extracts of wheat varieties.

Values are represented as mean ± SD (n=3).

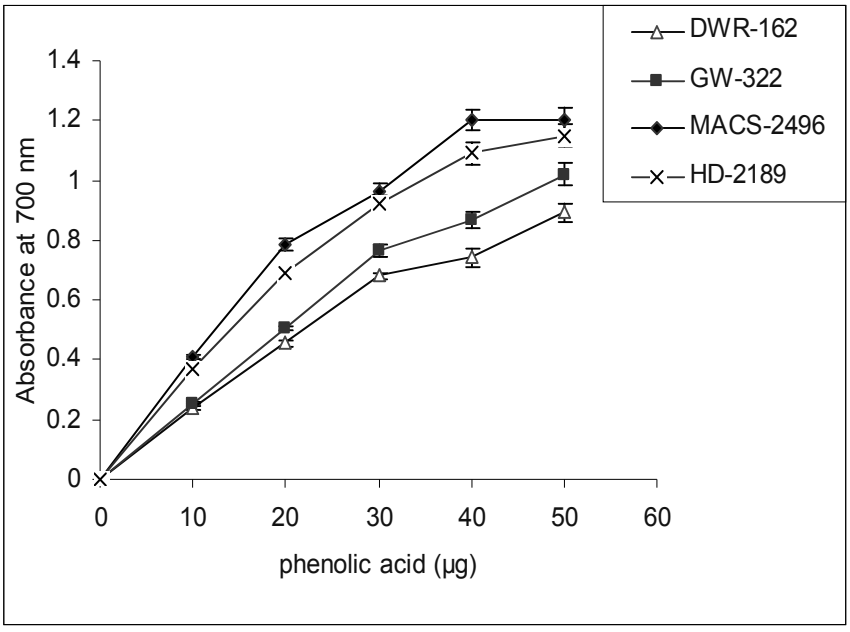


Fig. 9. Reducing power of bound phenolic extracts of wheat varieties.

Values are represented as mean ± SD (n=3).

Antioxidant action of reductones is based on the breaking of free radical chain reaction by donating a hydrogen atom or reacting with certain precursors of peroxides to prevent peroxide formation (Liu and Yao, 2007; Suresh et al., 2006). The phenolic fractions (mainly bound forms) from wheat varieties examined in this study demonstrated strong reducing capacity thereby acting as efficient reductones.

3.2.3.2. Free radical scavenging activity

Free radicals are involved in the process of lipid peroxidation and are considered to play a fundamental role in several chronic diseases, such as diabetes, cancer and cardiovascular diseases and are also implicated in aging process (Halliwel et al., 1992; White and Xing, 1997). Therefore, it was considered important to assess the free radical scavenging efficacy of the wheat phenolic extracts. The DPPH radical scavenging activities of free and bound phenolic extracts from whole-wheat flour of different wheat varieties along with the reference standards BHA and BHT are shown in Figs. 10 and 11, respectively. Dose dependent increase in free radical scavenging activity was observed in both free and bound phenolic extracts. In general, bound phenolic extracts were better free radical scavengers than free phenolic extracts in all the wheat varieties (Fig. 11). The results are also expressed as IC_{50} values (Table 9). IC_{50} value is the concentration of an antioxidant required to quench 50% radicals in the reaction mixture under the experimental conditions. A lower IC_{50} value is associated with a higher antioxidant activity.

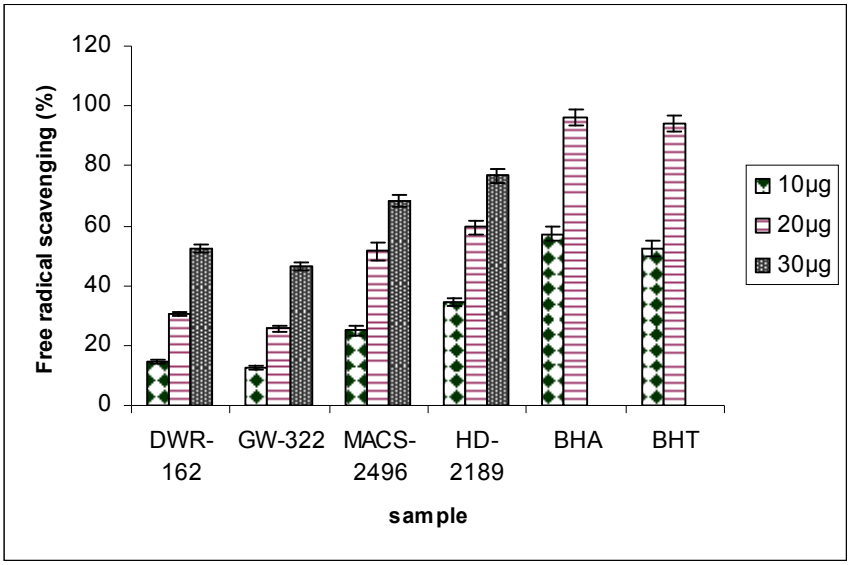


Fig. 10: Free radical scavenging activity of free phenolic extracts.

Values are represented as mean ± SD (n=3).

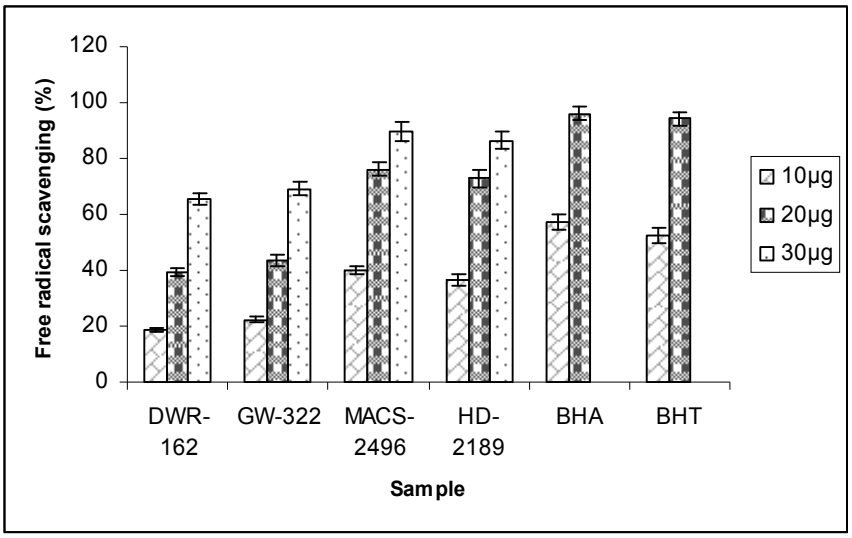


Fig. 11: Free radical scavenging activity of bound phenolic extracts.

Values are represented as mean ± SD (n=3).

HD-2189 wheat variety had the highest free radical scavenging activity, followed by MACS-2496 and it was lowest in GW-322 (Fig. 10, Table 9) in free phenolic extracts. Higher scavenging activities in these varieties could be due to higher levels of free ferulic acids along with other phenolic constituents such as syringic and vanillic acids. Similar results are previously reported on cereal phenolic antioxidants (Maillard and Berset, 1995). Bound phenolic extracts from MACS-2496 and HD-2189 had higher scavenging activities (Fig 11, Table 9). However, free radical scavenging activities of free and bound phenolic acid mixture was found to be significantly lower than that of synthetic antioxidants such as BHA (IC_{50}: 8.42 µg /ml) and BHT (IC_{50}: 10.23 µg /ml). Similar observations were also made in the case of barley and finger millet phenolic acid mixtures (Subba Rao and Muralikrishna, 2002).

Table 9. Comparative IC_{50} values of free and bound phenolic extracts of wheat varieties

Wheat variety	Free phenolic acids (µg)	Bound phenolic acids (µg)
DWR-162	29.86 ± 0.96	24.63 ± 0.46
GW-322	32.64 ± 1.10	22.40 ± 0.92
MACS-2496	23.20 ± 0.64	12.84 ± 0.36
HD-2189	19.86 ± 0.86	14.20 ± 0.42

Values are mean ± SD (n = 3).

The antioxidant activities of bound phenolic acid mixture, in the present study were found to be higher compared to that of free phenolic acid mixture. These results are in agreement with earlier reports on barley varieties (Maillard and Berset, 1995). The presence of higher amounts of ferulic acid with other phenolic constituents might result in higher antioxidant activities of the bound phenolic fractions, since, ferulic acid exhibits strong antioxidant and antinflammatory activities (Klepacka and Fornal, 2006; Serpen et al., 2008; Sindhu and Emilia, 2004).

3.2.4. Dietary fibre contents and composition in wheat varieties

Whole grains of cereals are a rich source of fermentable carbohydrates including dietary fibre, resistant starch and non-digestible oligosaccharides (Plaami, 1997). Dietary fibre is mainly concentrated in the outer layers of the grain (Esposito et al., 2005; Hartmann et al., 2005). Analysis of dietary fibre from whole-wheat flours of different varieties is given in Table 10. Whole-Wheat flours contain significant amounts of dietary fibres in these wheat varieties. Variations in the contents of insoluble (IDF) and soluble dietary fibres (SDF) were observed among these wheat varieties. Both soluble and insoluble dietary fibres were rich in carbohydrate contents. The content of total dietary fibre (sum of IDF and SDF) was highest in MACS-2496 (14.2%) followed by HD-2189 (13.2%). Analysis of sugar composition by GLC revealed the presence of arabinose and xylose as the major sugars in both the soluble (SDF) and insoluble dietary fibres (IDF). This demonstrates that arabinoxylans (pentosans) are the major contributors to the SDF and IDF contents (Bunzel et al., 2001; Renger and Steinhart, 2000).

Table 10. Contents and carbohydrate composition (%) of dietary fibres from whole wheat flours of different varieties

Sample	Yield (%) of defatted flour	Total sugar (%)	Sugars identified (%)					
			Ara	Xyl	Man	Gal	Glc	A/X
DWR-162								
IDF	10.2	79.3	43.4	42.6	0.4	1.0	12.6	1.01
SDF	1.7	86.5	39.0	44.0	1.4	8.6	7.0	0.89
GW-322								
IDF	10.0	84.2	40.0	42.0	0.8	1.2	16.0	0.95
SDF	1.5	92.3	40.2	46.0	1.6	6.2	6.0	0.87
MACS-2496								
IDF	12.0	75.4	38.4	43.6	1.8	2.2	14.0	0.88
SDF	2.2	80.4	34.0	45.0	1.0	11.5	8.5	0.75
HD-2189								
IDF	11.2	78.2	39.0	48.4	1.8	0.4	11.4	0.80
SDF	2.0	84.6	30.4	44.0	1.8	12.2	10.6	0.69

Abbreviations as in Table 3.

IDF, insoluble dietary fibre; SDF, soluble dietary fibre.

Small amounts of mannose and galactose were present in insoluble dietary fibres. Moderate amounts of galactose and glucose were observed in soluble dietary fibres, indicating that arabinogalactan and β-glucan type of polysaccharides could exist in soluble dietary fibres (Looseveld et al., 1998; Hartmann et al., 2005). Chemical structure, solubility and lignifications are some of the properties of dietary fibres of great importance and also fermentability of dietary fibres. Cross linking of polysaccharides in the presence of ferulic acid will have an influence on fibre solubility and ultimately on resistance to microbial degradation (Hartley et al., 1990). The amount of dietary fibres and their chemical nature brings about variations in the functionality of dietary fibre and their fermentation characteristics leading to short chain fatty acids (Bourguin et al., 1996).

The role of whole-wheat products in nutrition and health has been scientifically well documented. Whole grains are recognized sources of several physiologically active components (Adom et al., 2003). Dietary antioxidants that could scavenge free radicals may play an important role in preventing chronic diseases (Shahidi et al., 1992; Sun and Tang, 2005). There has been recent interest in the use of natural antioxidants for improved quality and stability of food products because of concerns over safety and negative consumer perception for synthetic antioxidants (Botterweck et al., 2000). Whole-wheat flour is a rich source of natural antioxidants, containing unique mixture of phenolic acids, dietary fibres and other phytochemicals, suggesting its use in nutraceuticals and functional food industries (Adom et al., 2002).

The present results, indicated variations in the contents and composition of phenolic acids and dietary fibres, among the wheat varieties differing in *chapati* making quality. Bound phenolic fractions had more reducing power and free radical scavenging activity compared to free phenolic fractions in all the wheat varieties, indicating bound phenolics are the major source of antioxidants in wheat. The present results also revealed significant contribution of bound phenolic acids to the total phenolic contents of whole-wheat flours. Wheat

varieties thus studied differed in their antioxidant activities both in free and bound forms, indicating potential effect of genotypes on the antioxidant activities. Wheat varieties such as MACS-2496 and HD-2189 had higher phenolic antioxidants (free and bound forms) and dietary fibre contents and could be exploited as potent dietary source for nutraceuticals and functional foods.

3.3. Structural studies of purified pentosans (arabinoxylans) from wheat varieties differing in *chapati* making quality

Structural features of arabinoxylans in different varieties of wheat are known to vary and have relation to bread-making quality (Cleemput et al., 1993; Izydorczyk and Biliaderis, 1995). To establish molecular basis of arabinoxylan functionality on *chapati* making characteristics, it is necessary to gain more insight into the structural diversity of the arabinoxylans from wheat varieties differing in *chapati* making quality. Arabinoxylans from wheat varieties having differences in *chapati* making qualities were purified and their fine structural features are elucidated by a combination of methods. Comparisons are made between good and poor *chapati* making varieties for their fine structural features of arabinoxylans.

3.3.1. Arabinoxylans (pentosans) from barium hydroxide extracted polysaccharides

Barium hydroxide is one of the best extractants of arabinoxylans (Gruppen et al., 1993). Polysaccharides extracted with barium hydroxide were purified as described in Materials and Methods (Chanliaud et al., 1995). The purified arabinoxylans showed increased amount of total sugars, while, uronic acid content was decreased when compared to native samples (Table 11). Sugar analysis by GLC revealed the predominant presence of arabinose and xylose. This accounted to around 95% of the sugars in all the wheat varieties studied. They also contained galactose and glucose in minor amounts.

Table 11. Carbohydrate composition (%) of native and purified barium hydroxide extracted pentosans from whole-wheat flours of different varieties

Samples	Total sugar	Uronic acid	Ara	Xyl	Man	Gal	Glc	Ara/ Xyl
DWR-162								
Native	78.1	3.0	49.0	29.0	-	-	22.0	1.68
Purified	88.2	2.6	46.6	48.6	1.0	1.3	2.5	0.96
GW-322								
Native	80.2	2.1	44.5	38.9	-	2.9	13.7	1.14
Purified	90.4	0.8	45.4	50.0	-	1.6	3.0	0.91
MACS-2496								
Native	76.0	3.0	41.0	51.2	-	-	7.8	0.80
Purified	89.4	2.4	40.4	56.8	-	1.6	1.2	0.71
HD-2189								
Native	85.0	1.6	26.7	41.0	-	1.8	30.5	0.65
Purified	91.5	1.0	41.5	54.6	-	1.2	2.7	0.76

Abbreviations as in Table 3.

Galactose might be coming from galctoarabinoxylans or arabinogalactans, which has been reported in members of graminae family (Aspinall, 1980). Trace amounts of mannose in DWR-162 may be coming from glucomananns, which has been found in various cereal cell walls (Fincher and Stone, 1986). Small amounts of glucose present in these purified arabinoxylans could be due to β-glucan and unhydrolysed starch. Differences were observed in A/X ratio among wheat varieties studied which indicates differences in degree of branching. Accordingly, arabinoxylans from good *chapati* making varieties DWR-162 and GW-322 showed higher A/X ratio compared to poor *chapati* making varieties - MACS-2496 and HD-2189.

In order to determine the apparent molecular size, HPSEC of purified arabinoxylans from all the four varieties was carried out. The elution of arabinoxylans on E-linear and E-1000 columns, connected in series, was carried out using deionized water and was monitored with a refractive index detector. Purified arabinoxylans from all the varieties were eluted as a single broad peak before T_{2000} dextran standard (Fig. 12), indicating that they are homogeneous and their apparent molecular weight exceeded 2 million. Average molecular weight of 16 X 10^6 has been reported for barium hydroxide extracted polysaccharides from wheat flour based on dextran standards (Gruppen et al, 1993). High molecular weights are reported for both water-soluble and water-insoluble arabinoxylans obtained from wheat (Gruppen et al., 1992), barley (Dervilly et al., 2002), sorghum (Verbruggen, et al., 1995) and rye (Cyran et al., 2003) by HPSEC using both pullulan and T-series dextran standards, further validating our results obtained in the present investigation.

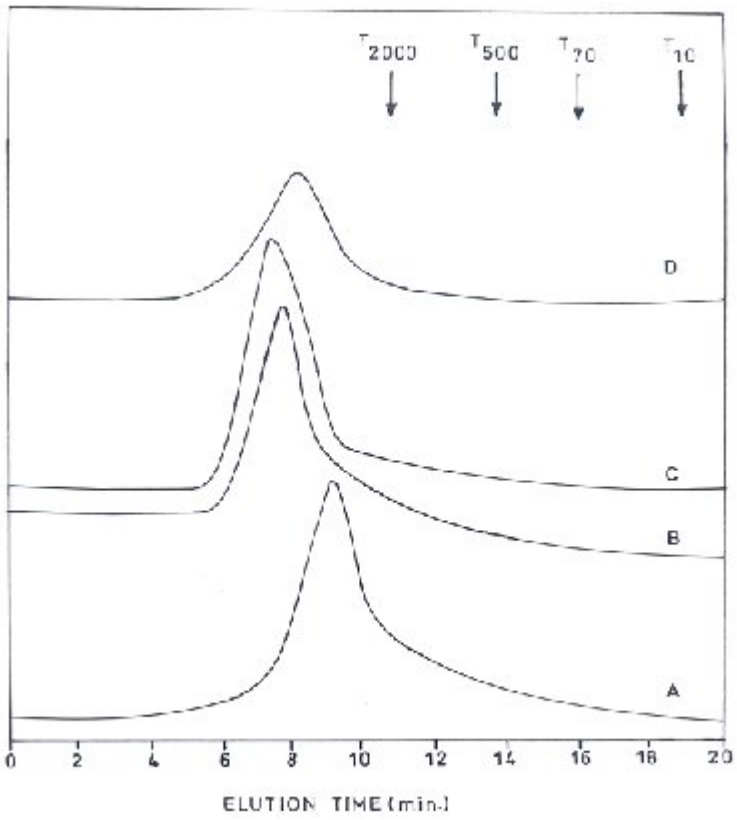


Fig. 12. HPSEC profile of purified barium hydroxide extracted arabinoxylans from wheat varieties.

(A; DWR-162, B; GW-322, C; MACS-2496, D; HD-2189).

Earlier studies have shown that alkali-soluble pentosans were excluded from Sepharose CL-4B column indicating apparent molecular weight exceeding 5 million (Gruppen et al., 1993; Nandini and Salimath, 2003). Since arabinoxylans are branched, gel filtration chromatography often results in over estimation of molecular weights using dextran standards (Patil et al., 1975a).

Methylation analysis

Methylation analysis was performed to determine the nature of glycosidic linkages between monosaccharide residues of arabinoxylans. The samples were methylated by the method of Hakomori (1964). They were hydrolyzed, reduced with sodium borodeuteride and their permethylated alditol acetate derivatives were prepared. Their identities and relative proportion of sugars were determined by GLC and GLC-MS (Table 12). The mass spectra and fragmentation schemes are given in Figs. 13 and 14. Methylation analysis revealed xylose residues in the main chain with 1, 4 linkages (Table 12). Substitution by arabinose on xylan backbone was evidenced by the presence of 2-Me-Xyl (monosubstituted) and Xyl (di-substituted) among the methylated species. Similar results are reported for wheat (Dervilly et al., 2000), sorghum (Woolard et al., 1976), rice and ragi (Shyamprasad Rao and Murali Krishna, 2007) and rye (Vinkx and Delcour, 1996) arabinoxylans. Arabinose is mainly terminally linked, as these arabinose residues might be present as short side chains on the xylan backbone and they also provide a site for the covalent attachment of ferulic acid (Courtin and Delcour, 2002).

Table 12. Methylation analysis of purified barium hydroxide extracted arabinoxylans (%) from different wheat varieties

Mode of linkage	Alditol acetates	DWR-162	GW-322	MACS-2496	HD-2189
$Ara_f \rightarrow 1$	2,3,5-Me_3-Ara	31.6	29.2	22.6	26.0
$5 \rightarrow Ara_f \rightarrow 1$	2,3-Me_2-Ara	3.7	3.2	4.3	2.7
$2,3,5 \rightarrow Ara_f \rightarrow 1$	Ara	6.0	8.0	9.4	10.0
$Xyl_p \rightarrow 1$	2,3,4 –Me_3-Xyl	2.0	1.6	3.7	4.6
$4 \rightarrow Xyl_p \rightarrow 1$	2, 3- Me_2 –Xyl	21.5	23.0	36.0	32.0
$3,4 \rightarrow Xyl_p \rightarrow 1$	2- Me-Xyl	19.0	18.4	14.8	14.2
$2,3,4 \rightarrow Xyl_p \rightarrow 1$	Xyl	16.2	16.6	9.2	10.5
	2, 3- Me_2 –Xyl 2- Me-Xyl +Xyl	0.60	0.65	1.50	1.30
	Xylose 2- Me-Xyl	0.85	0.90	0.62	0.73

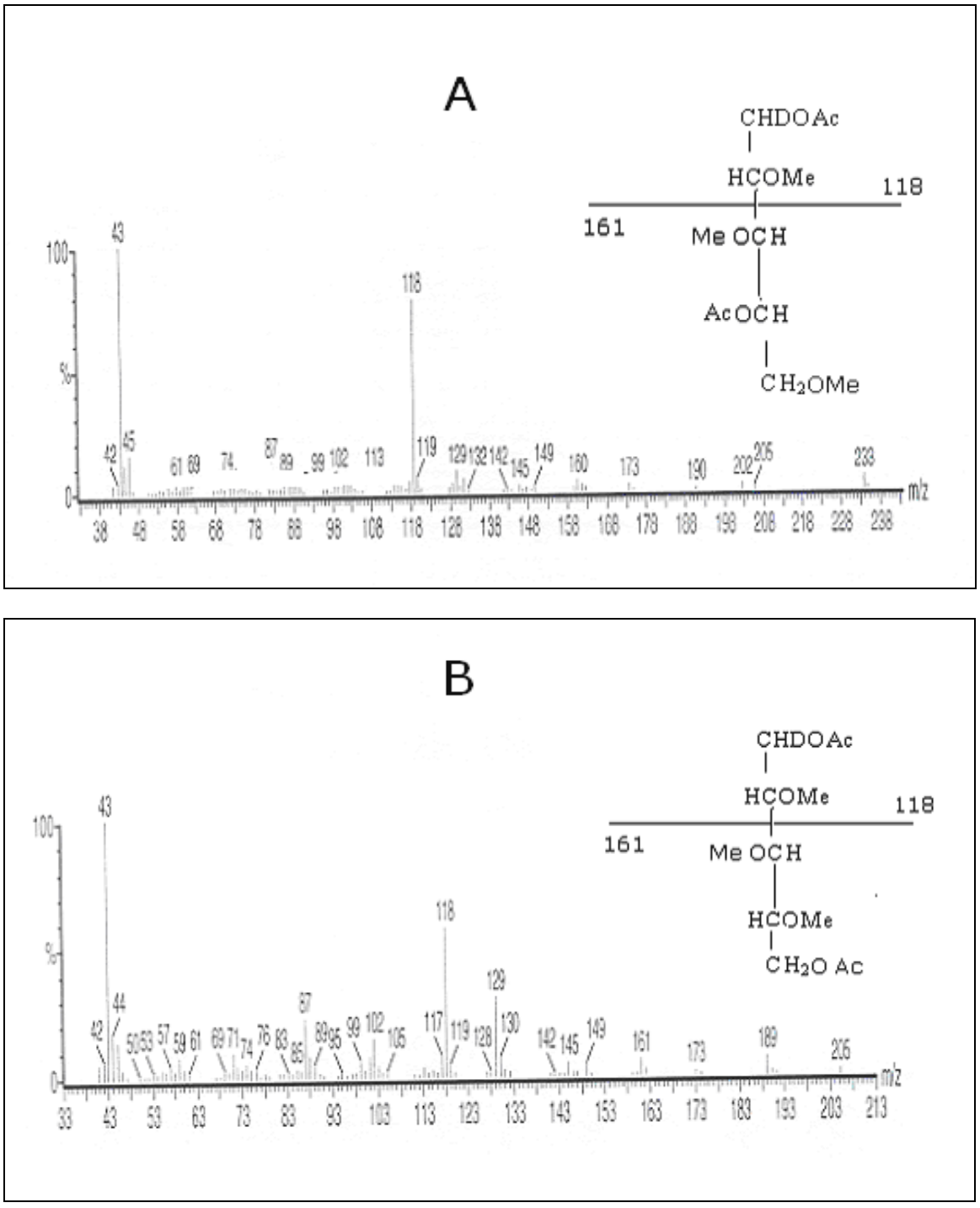
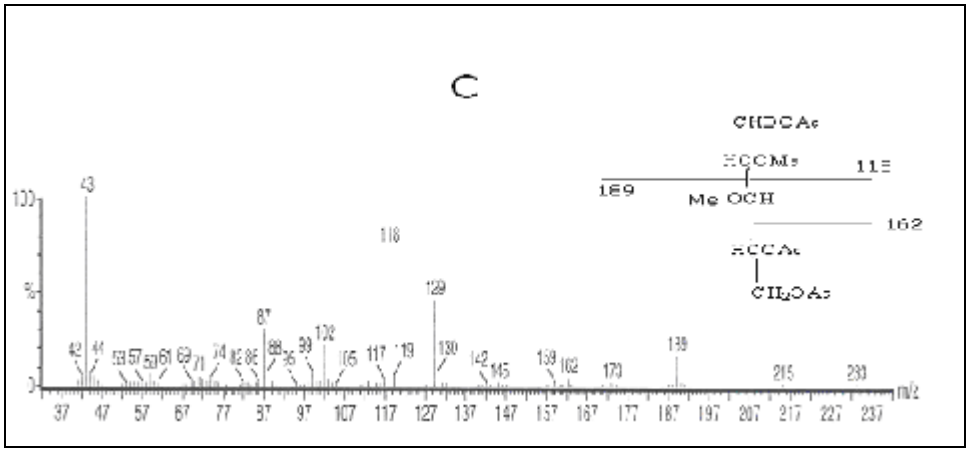
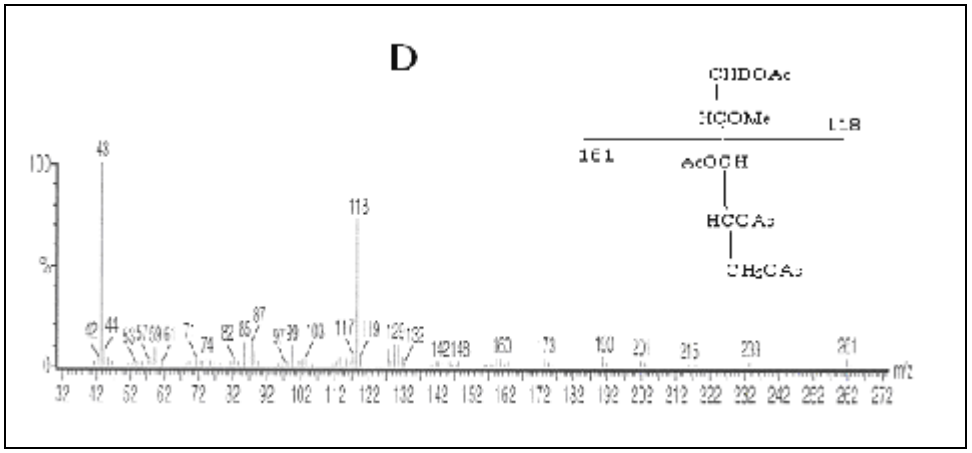


Fig.13: Mass spectra and fragmentation scheme of partially methylated alditol acetates of (A) 2, 3, 5-Me$_3$-pentitol and (B) 2,3,4-Me$_3$-pentitol.





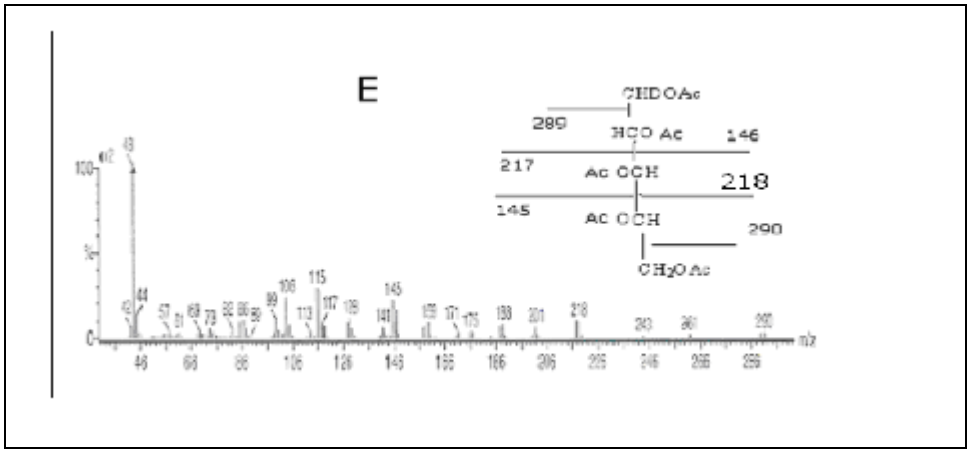


Fig. 14: Mass spectra and fragmentation scheme of partially methylated alditol acetates of (C) 2, 3-Me_2-pentitol (D) 2-Me -pentitol and (E) Pentitol.

Most of the xyloses in purified arabinoxylans from MACS-2496 and HD-2189 were unsubstituted as evidenced by the presence of large amounts of 2,3-Me_2-Xyl. Singly and doubly substituted xyloses were present in higher amounts in arabinoxylans of DWR-162 and GW-322, compared to MACS-2496 and HD-2189 as evidenced by the presence of 2-Me-Xyl and xylose residues, respectively.

Arabinoxylans from DWR-162 and GW-322 revealed high amounts of disubstituted xylose residues. Doubly substituted xyloses have been reported in arabinoxylans from wheat (Dervilly et al, 2000), rice (Shibuya and Misaki, 1978) and sorghum (Verbruggen et al., 1995). The ratio of doubly to singly substituted xyloses was more in case of GW-322 followed by DWR-162. Arabinose was present mainly as terminal sugar (2, 3, 5-Me_3-Ara). Main chain arabinosyl residues (2, 3-Me_2-Ara) were observed notably in all the wheat varieties. Short arabinosyl chains are reported in arabinoxylans from rice and wheat (Shibuya et al. 1983; Saulnier et al., 2007). Arabinitol occurs among methylated species due to fully branched arabinosyl residues and was observed in all the wheat varieties. The presence of arabinitol has been reported in branched arabinoxylans (Subba Rao and Muralikrishna, 2004). AX from rice (Yui et al., 1995) and sorghum (Nandini and Salimath, 2002; Verbruggen et al., 1995) are highly branched than those from wheat, rye and barley (Dervilly et al., 2001; Izydorczyk and Biliaderis, 1995).

^{13}C NMR of arabinoxylans

^{13}C NMR spectroscopy was carried out on D_2O exchanged samples of purified arabinoxylans from barium hydroxide extracted polysaccharides of wheat varieties. Signals were assigned based on the data available in the literature (Hoffmann et al., 1991; Gruppen et al., 1993). Partial structure of arabinoxylans is shown in Fig. 15 and assignment of signals is shown in Tables 13 and 14 and ^{13}C NMR spectra are shown in Figs. 16-19.

Arabinoxylans from all the wheat varieties showed signals characteristic of arabinose and xylose residues. The chemical shifts in ppm of arabinoxylans from DWR-162 and GW-322 are given in Table 13 and the NMR spectrum of AX from DWR-162 and GW-322 are given in Figs. 16 and 17, respectively. Signals due to anomeric carbon atoms of xylose residues were observed in the region between 101.01- 103.2 ppm and are characteristic features of β-linked xylopyranose (Table 13, Figs. 16 and 17). The multiplicity of signals at this region indicates that xyloses are present in different modes of branching. This substantiates methylation analysis (Table 12). The signals due to anomeric carbon atoms of α-linked arabinofuranose were observed at 108.4-109.2 ppm. The multiplicity of peaks corresponding to C_1 of arabinose residues denotes the anomeric carbon of differently linked arabinosyl residues which undergo a slight shift depending on their mode of linkage. The signals at 108.4 ppm was attributed to C_1 of arabinose linked by O-3 of monosubstituted xylose (sugar D) and O-3 of disubstituted xylose (sugar E), where as the signal at 109.0 ppm was assigned to arabinose residue linked by O-2 of doubly branched xylose residue (sugar F). Signal due to ring carbon atoms of singly branched xylose residues were observed between 73.2-81.0 ppm (sugar A). Those of main chain xylose residue gave signals due to ring carbon atoms between 75.4-76.42 ppm (sugar C). Signals due to ring carbon atoms of arabinose residues were observed between 80-84.9 ppm (sugar D). Chemical shifts due to C_5 of xylopyranose was observed at 63.2-64.9 ppm, whereas that of arabinofuranose was observed at 61.0-62.4 ppm. Apart from the above signals, a characteristic signal was observed at 99.4 ppm, which could be due to C_1 of glucuronic acid residue. Signal due to C_6 of glucuronic acid was not clearly visible (Oraphin et al., 2004; Izydorczyk and Biliaderis, 1995).

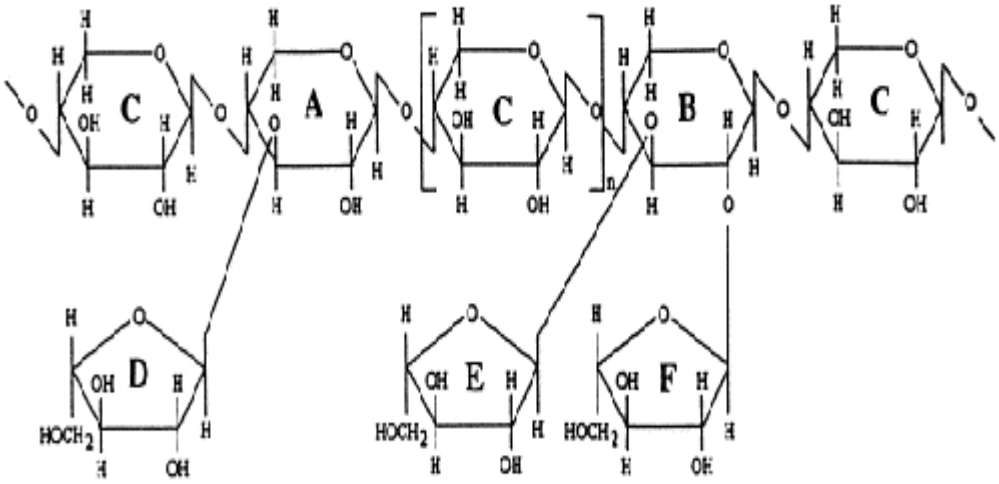


Fig. 15: Partial (one of the probable) structures of arabinoxylans (Identification of ^{13}C NMR signals are shown in Tables 13 and 14).

Table 13. Assignment of ^{13}C NMR signals of purified barium hydroxide extracted arabinoxylans obtained from DWR-162 and GW-322 wheat varieties

Sugar	Chemical shifts (ppm)									
	DWR-162					**GW-322**				
	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5
A	103.3	73.3	78.2	76.4	63.0	101.5	73.3	80.4	76.0	64.9
B	101.0	72.4	-		63.0	101.0	72.4	-	-	64.9
C	103.3	72.6	75.4	77.0	63.0	101.5	72.6	76.4		64.9
D	109.0	82.0	80.4	84.4	61.0	108.4	80.9	80.4	84.2	62.0
E	108.4	81.0	-	84.1	61.0	108.4	80.7	-	84.2	62.0
F	109.0	80.7	-	84.0	61.0	109.1	80.9	-	84.2	62.0

Table 14. Assignment of ^{13}C NMR signals of purified barium hydroxide extracted arabinoxylans obtained from MACS-2496 and HD-2189 wheat varieties

Sugar	Chemical shifts (ppm)									
	MACS-2496					**HD-2189**				
	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5
A	103.34	73.5	80.45	75.98	64.9	103.2	73.5	80.0	76.00	62.9
B	101.0	72.0	-	-	64.9	101.0	72.06	-	-	62.9
C	103.34	72.4	76.52	76.52	64.9	103.2	72.41	-	79.95	62.9
D	108.4	80.4	80.0	84.2	62.0	108.0	81.2	80.96	84.2	60.8
E	107.3	80.4	-	84.2	62.0	108.4	80.92	-	84.2	60.8
F	108.4	80.4	-	84.2	62.0	110.0	80.42	-	84.2	60.8

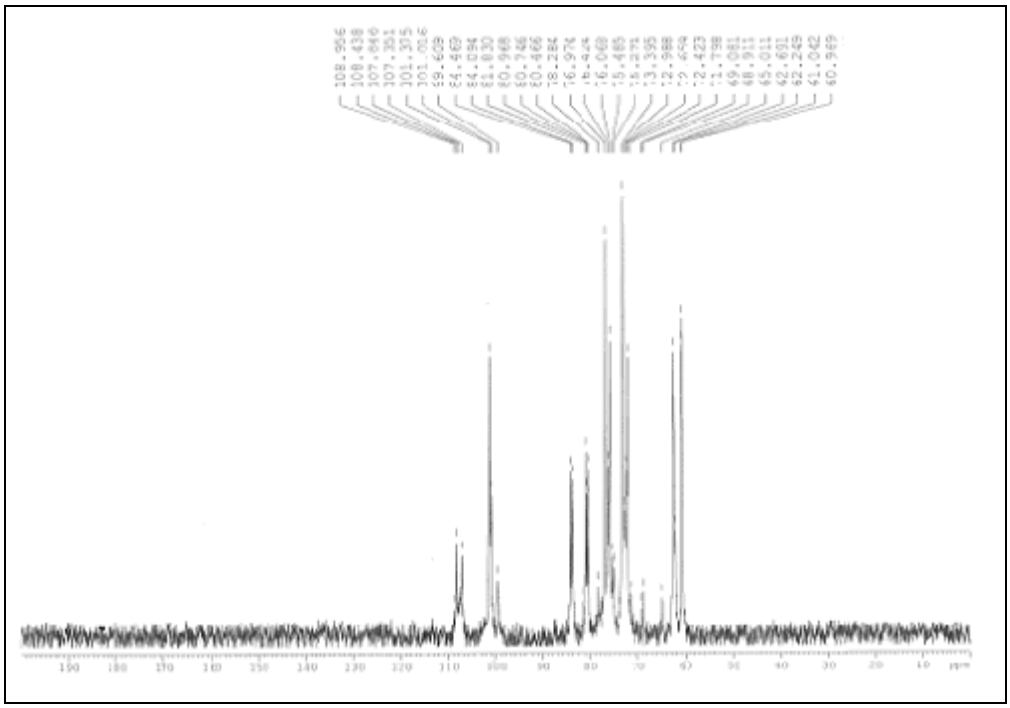


Fig. 16: ^{13}C NMR spectrum of purified barium hydroxide extracted arabinoxylans from DWR-162 wheat variety.

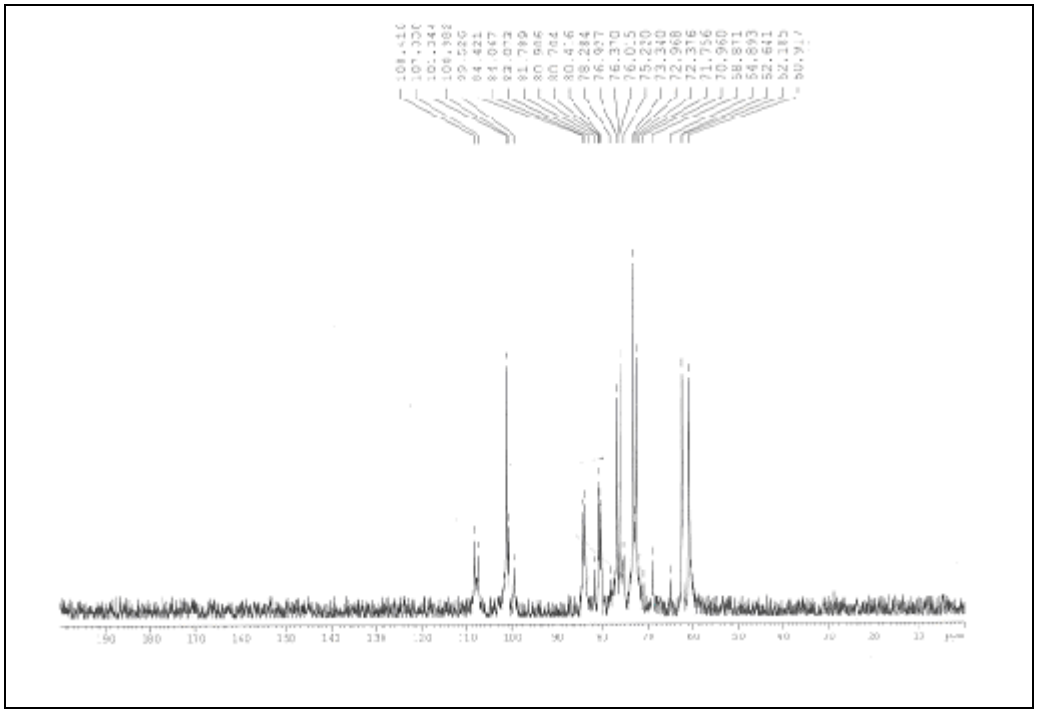


Fig. 17: ^{13} C NMR spectrum of purified barium hydroxide extracted arabinoxylans from GW-322 wheat variety.

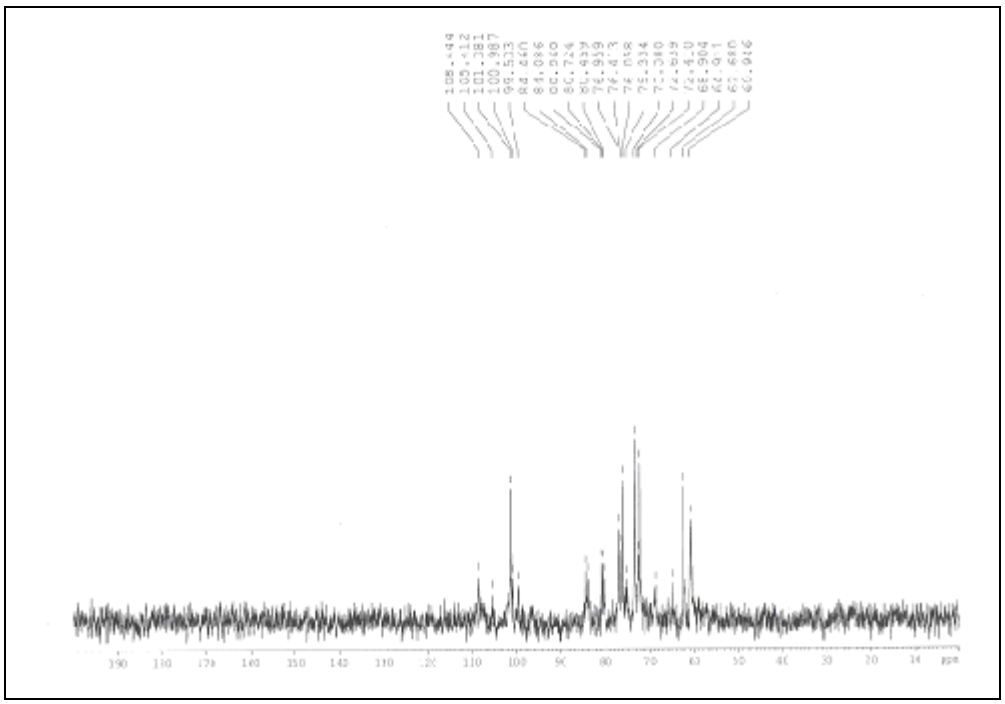


Fig. 18: ^{13} C NMR spectrum of purified barium hydroxide extracted arabinoxylans from MACS-2496 wheat variety.

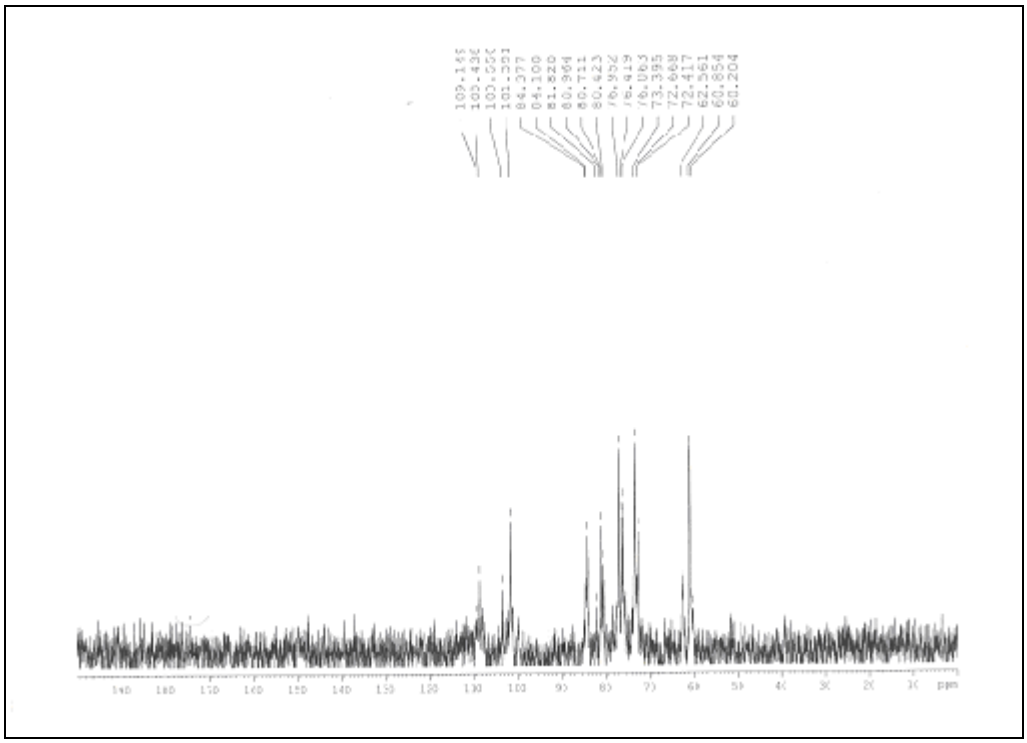


Fig. 19: ^{13}C NMR spectrum of purified barium hydroxide extracted arabinoxylans from HD-2189 wheat variety.

^{13}C NMR spectra of arabinoxylans from MACS-2496 and HD-2189 showed signals due to anomeric carbon atoms, characteristic of β-linked xylopyranose in the region of 101.0-103.3 ppm (Table 14. Figs. 18 and19). The signals due to anomeric carbon atoms of α-linked arabinofuranose were observed at 107.3-108.4 ppm. Signals due to ring carbon atoms of singly branched xylose residues were observed between 73.5-80.4 ppm (sugar A). The chemical shifts due to ring carbon atoms vary depending on the mode of substitution. The chemical shifts due to the ring carbon atoms of arabinoses were observed between 80.42 to 84.46 ppm. The signals due to other ring carbon atoms assigned are given in Table 14.

^{13}C NMR spectroscopy is a powerful molecular probe for detecting differences in branching pattern of arabinoxylans. From the relative peak intensities of the anomeric region of arabinose residues for arabinoxylans from the wheat varieties, it could be observed that O-3 substituted xylose residues (sugar A) were present in higher amounts in arabinoxylans of DWR-162 and GW-322 than MACS-2496 and HD-2189 varieties (Izydorczyk and Biliaderis, 1993). These results further substantiate methylation analysis (Table 12).

^{1}H NMR spectra of arabinoxylans

^{1}H NMR analysis of arabinoxylans from wheat varieties indicated signals around 5.36, 5.30 and 5.20 ppm corresponding to anomeric protons of α-L-arabinofuranose substituted at O-3 (monosubstituted) and at both O-3 and O-2 (disubstituted) of xylose residues, respectively (Figs. 20 and 21).

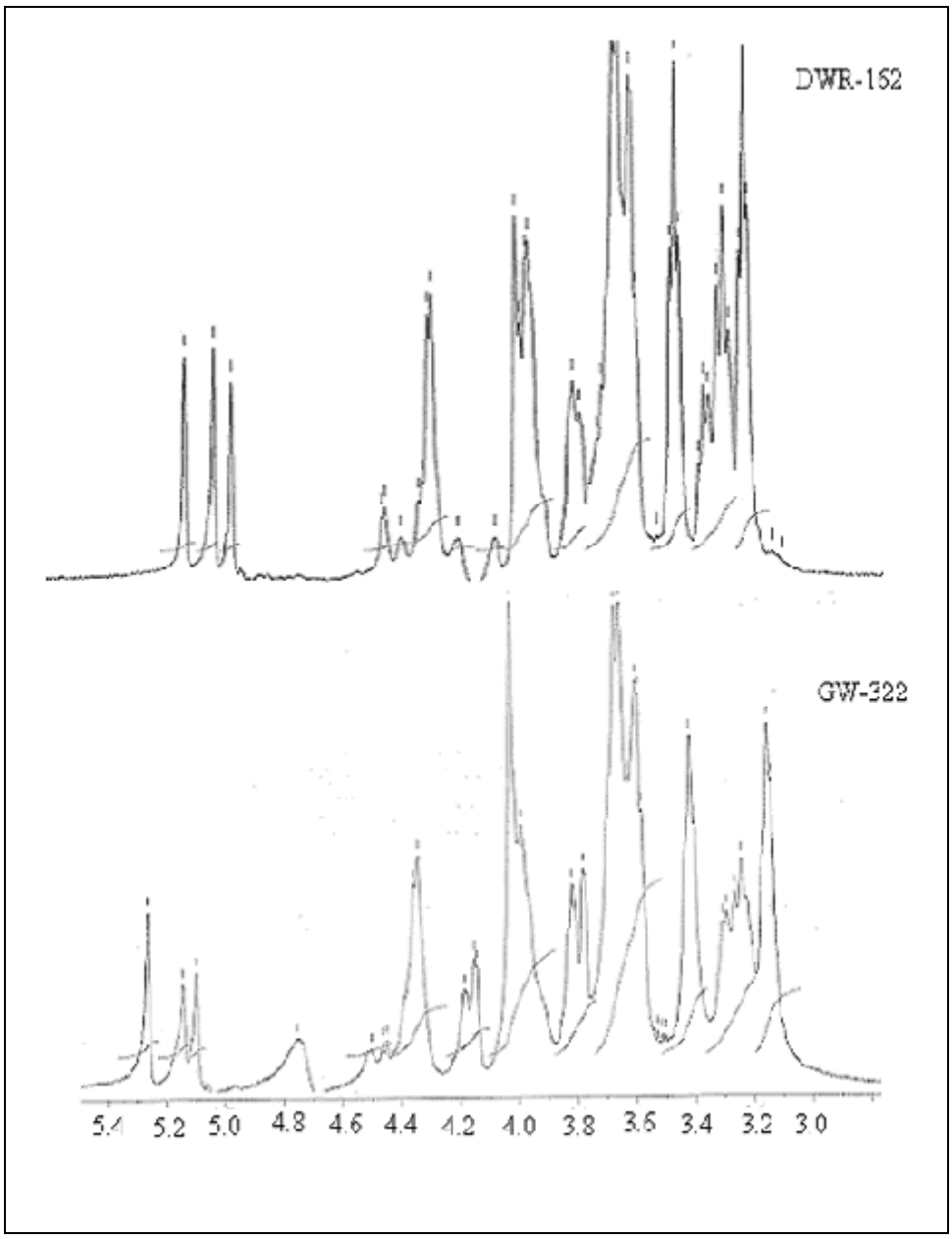


Fig. 20: ^{1}H NMR spectra of purified barium hydroxide extracted arabinoxylans from DWR-162 and GW-322 wheat varieties.

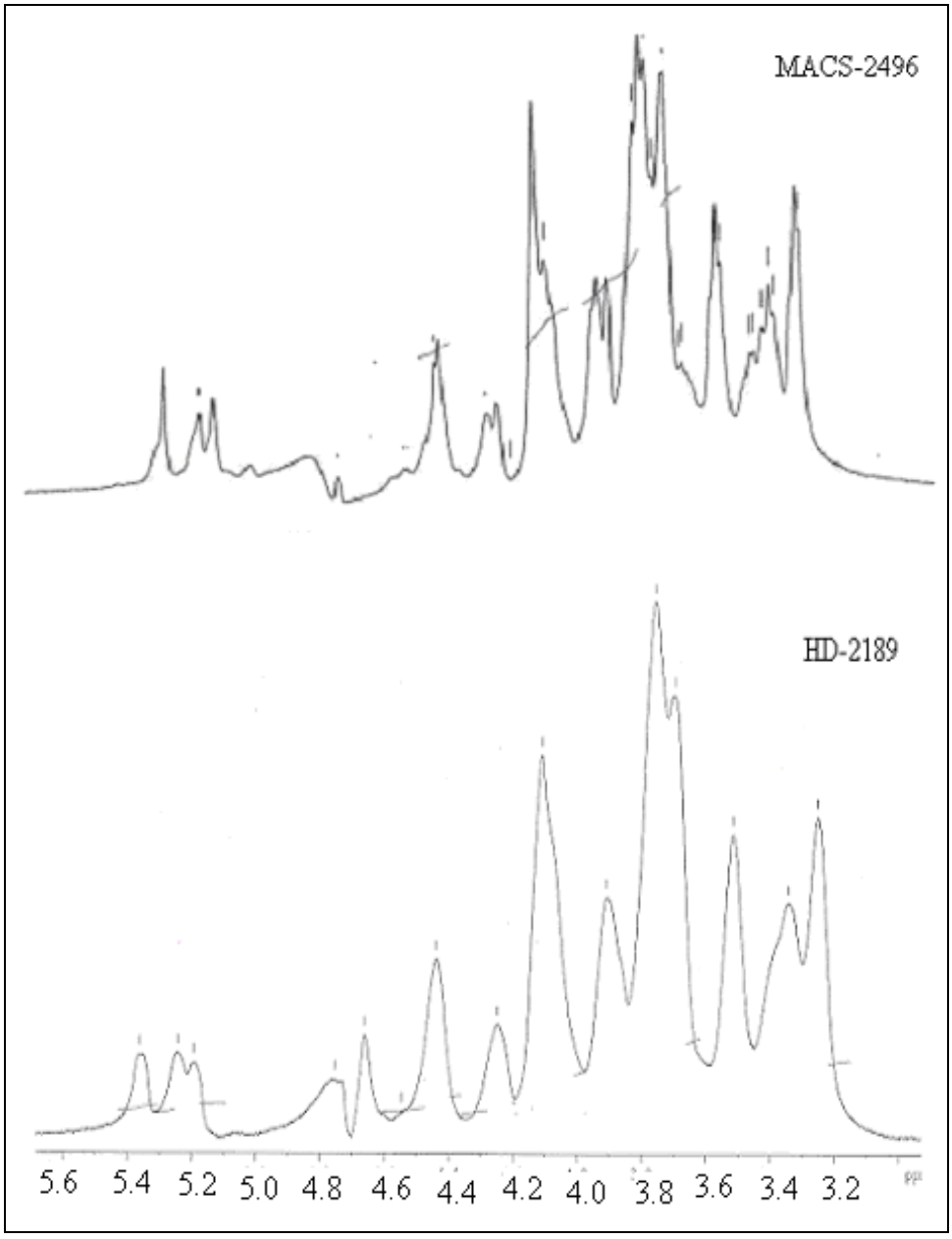


Fig. 21: ^{1}H NMR spectra of purified barium hydroxide extracted arabinoxylans from MACS-2496 and HD-2189 wheat varieties.

These anomeric proton signals (arabinofuranose) are more pronounced in arabinoxylans of DWR-162 and GW-322 indicating higher arabinose substitution on xylan backbone. Signals obtained around 4.40- 4.60 ppm were due to anomeric protons of β-D-xylopyranose substituted at C-2 and C-3 (disubstituted). The signals for other protons of arabinose and xylose were obtained in the region of 3.20-4.30 ppm and were in close proximity with the signals obtained for wheat arabinoxylans (Hoffmann et al., 1992). The unresolved signals or shoulders down stream of the peak at 5.24 ppm resulted from two consecutive disubstituted xylose residues in the arabinoxylans (Bengtsson and Aman, 1990) of HD-2189 and indicated that it contained both isolated and paired disubstituted arabinoxylans. The presence of an unresolved peak at around 5.34 ppm represented the presence of monosubstituted xylose adjacent to disubstituted xylose. It was prominent in arabinoxylans from DWR-162 and GW-322 wheat varieties (Hoffmann et al., 1992). These results further substantiate the methylation results (Table 12).

Optical rotation

Optical rotation values of purified arabinoxylans from DWR-162, GW-322 and MACS-2496 and HD-2189 were found to be -56º, -62º, -68º and -70º, respectively (Table 15). High negative values indicated that the xylan backbone of arabinoxylans was mainly made up of β-linkages. The range of optical rotation for neutral xylans has been reported between -37º and -108º (Woolard et al., 1976; Nandini and Salimath, 2003). Optical rotation of -107º and -105º has been reported for two arabinoxylans from rye (Vinkx and Delcour, 1996). Theses results are in agreement with the values reported in the literature for other cereal arabinoxylans (Subba Rao and Muralikrishna, 2004).

Periodate oxidation and Smith degradation

Periodate oxidation studies were carried out using sodium metaperiodate (0.02M) to estimate the degree of substitution. Wheat varieties, DWR-162 and GW-322 consumed nearly 0.5 moles of periodate per mole of anhydrosugar, whereas MACS-2496 and HD-2189 had consumed nearly 0.7 moles of periodate per anhydrosugar (Table 15). These results indicated that arabinoxylans had higher degree of substitution in DWR-162 and GW-322 compared to MACS-2496 and HD-2189 wheat varieties. These results were further substantiated by the major products obtained as glycerol, xylose and arabinose, in Smith degraded samples (Table 16). Glycerol might have originated from the side chains of arabinose (Medcalf et al., 1968; Shyamprasad Rao and Muralikrishna, 2007). Smith degradation analysis of glucuronoarabinoxylans from sorghum husk showed high amount of glycerol and xylose (Woolard et al., 1976). Gruppen et al., (1993) reported that most of the branched residues are present as isolated units of blocks of two contiguous substituted xylose residues. Similarly, Smith degradation analysis of rice and ragi arabinoxylans showed high amounts of glycerol and xylose indicating high degree of branching (Shyamprasad Rao and Muralikrishna, 2007). Trace amount of formic acid liberated in these polysaccharides, indicated low amount of three consecutive hydroxyl groups in the sugars (Subba Rao and Muralikrishna, 2004).

Table 15. Moles of periodate consumed and optical rotation and ferulic acid contents of arabinoxylans isolated from barium hydroxide

Wheat variety	Moles of periodate consumed/ anhydrosugar	$[\alpha]_D$	Ferulic acid (µg/g)
DWR-162	0.52	−56.20	420.8
GW-322	0.56	−62.50	490.4
MACS-2496	0.72	−68.30	314.6
HD-2189	0.70	−70.42	290.9

Table 16. Analysis of Smith degradation products (%) obtained for purified arabinoxylans from barium hydroxide extract

Wheat variety	Glycerol	Arabinose	Xylose
DWR-162	45.6	8.4	46.0
GW-322	40.0	10.6	49.4
MACS-2496	52.6	15.4	32.0
HD-2189	48.8	10.2	41.0

Arabinoxylans from DWR-162 and GW-322 wheat varieties had high amounts of bound ferulic acid compared to MACS-2496 and HD-2189 varieties having poor *chapati* making characteristics (Table 15). Ferulic acid plays a significant role in cross-linking between arabinoxylans and other cell wall components. Feruloyl groups esterified with arabinoxylans are involved in the oxidative gelation phenomenon of pentosan solutions (Courtin and Delcour, 2002; Saulnier, et al., 2007). Cross-linked arabinoxylans absorb high amount of water and thus influence water distribution in dough (Izydorczyk and Biliaderis, 1995). In dough, such cross-linking was suggested to be responsible for the improvement of physico-chemical properties (Izydorczyk et al., 1990).

FT-IR spectra of arabinoxylans

The FT-IR spectra of arabinoxylans from wheat varieties are shown in Fig. 22. The spectra obtained from wheat varieties were characteristic of arabinoxylans (Kacurkova et al., 1994). Signals observed at around 1430 and 2930 cm^{-1} are due to CH_2 and CH stretching vibrations, respectively. The prominent signal observed around 3300 cm^{-1} represents the hydroxyl stretching vibrations of polysaccharides and water involved in the hydrogen bonding (Kacurkova et al., 1998). The prominent signal around 1040 cm^{-1} is attributed to C-O, C-C or C-O-H bending vibrations in arabinoxylans. This signal shows variations in spectral shape with the increase in branches of O-3 on the xylan backbone.

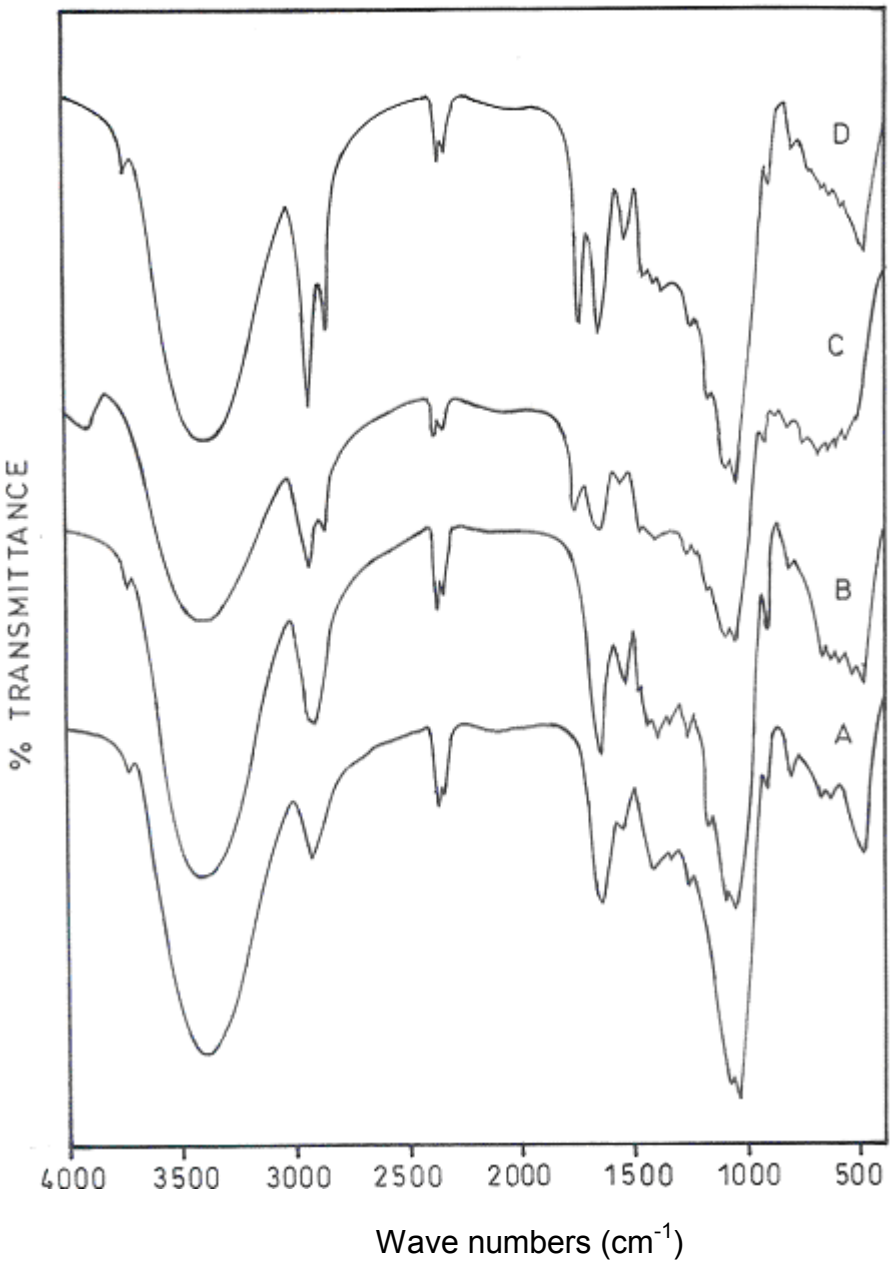


Fig. 22: FT-IR spectra of barium hydroxide extracted arabinoxylans from wheat varieties (A; DWR-162, B; GW-322, C; MACS-2496, D; HD-2189).

The signal at 1400 cm^{-1} is due to C-C, C-O and C-O-H bending vibrations. Signal at this region shows variations, depending on the amount of substitution at O-2 and O-3 positions. The intensity of signals in this region decreased (coupled with the loss of peak multiplicity) with increased substitution (Kacurkova et al., 1998). A signal due to associated water was observed at 1654 cm^{-1}. IR spectra (DWR-162 and DWR-322) of arabinoxylans indicated high arabinose substitution at C-3 of xylan residue (low intensity shoulder at 990 and 1160 cm^{-1}) and the loss of peak multiplicity in the region 1100-1000 cm^{-1} is typical and is characteristic of highly substituted arabinoxylans (Kacurkova et al., 1994; Subba Rao and Muralikrishna, 2004). The spectral shape of arabinoxylans from MACS-2496 and HD-2189 were similar in nature. From the spectral shape, it can be inferred that arabinoxylans from DWR-162 and GW-322 were more branched than arabinoxylans from MACS-2496 and HD-2189, because the spectral shape around 1000-1500 cm^{-1} are relatively smoother for arabinoxylans from DWR-162 and GW-322 (Kacurkova et al, 1998; Subba Rao and Muralikrishna, 2004)).

The structural features of arabinoxylans purified from different wheat varieties were thus elucidated by a combination of methods such as methylation, ^{13}C NMR, ^{1}H NMR, periodate oxidation, Smith degradation and optical rotation measurements. Arabinoxylans consisted of a xylan backbone in which xylose residues are in the pyranose form, linked by β- (1-4) linkages. Arabinose residues in the furanose form were branched through O-3 and O-2 and O-3 of xylose residues and were present in α-linkages. Thus xyloses are in different modes of substitution, unbranched, singly branched and doubly branched. Arabinoxylans from DWR-162 and GW-322 (good *chapati* making quality) are more branched, compared to MACS-2496 and HD-2189 (poor *chapati* making quality) wheat varieties and could be responsible to bring about differences in *chapati* making characteristics.

3.3.2. Pentosans from hemicellulose A

The carbohydrate composition of native and purified pentosans from hemicellulose A is given in Table 17. The carbohydrate content of purified samples was higher in most of the fractions of hemicellulose A (Hem A) compared to native samples (Table 17). Uronic acid content was highest in HD-2189 (4.7%) followed by MACS-2496 (4.3%). Sugar composition analysis indicated that these fractions are rich in xylose residues along with moderate levels of arabinose and glucose in all the wheat varieties. Native fractions had substantial amounts of glucose, a part of which may be coming from undigested starch molecules and other associated polysaccharides (Dupont and Selvendran, 1987). Small amounts of galactose might be due to arabinogalactans, the presence of which has been reported in the members of graminae (Izydorczyk and Biliaderis, 1993). The lower arabinose to xylose ratio in all these fractions might be due to the presence of higher un-substituted xylan chains or xylan type of polysaccharides. Wheat varieties such as DWR-162 and GW-322 had higher arabinose to xylose ratio.

Table 17. Carbohydrate composition of native and purified pentosans from hemicellulose A of whole-wheat flour of different varieties

Samples	Total sugar	Uronic acid	Ara	Xyl	Gal	Glc	Ara / Xyl
DWR-162							
Native	88.1	2.6	10.8	66.5	1.2	21.5	0.16
Purified	90.0	3.7	19.2	74.6	1.0	5.2	0.25
GW-322							
Native	88.0	6.7	4.3	79.3	-	16.4	0.05
Purified	92.5	3.1	19.5	68.2	1.3	7.0	0.28
MACS-2496							
Native	73.8	6.0	4.8	50.5	-	44.7	0.11
Purified	86.0	4.3	11.2	78.0	2.4	8.4	0.14
HD-2189							
Native	95.5	3.3	5.0	60.4	-	34.6	0.08
Purified	94.5	4.7	12.8	76.5	1.5	9.2	0.16

Abbreviations as in Table 3.

Methylation analysis of purified pentosans from Hem A

Methylation and subsequent hydrolysis revealed that the xylose residues were present in three forms; unsubstituted, monosubstituted and disubstituted (Table 18). Methylation analysis revealed the presence of xylose residues in the main chain with 1, 4 linkages. Most of the xylose residues were unsubstituted as evidenced by the presence of large amounts of 2, 3-Me_2-Xyl. This was more in MACS-2496 and HD-2189 varieties which are having poor *chapati* making characteristics. Small amounts of terminal xylose residues were present in the pyranose form, as indicated by the presence of 2, 3, 4-Me_3-Xyl. The xylan backbone was substituted by arabinofuranosyl residues at O-3 position, as indicated by the presence of 2-Me-Xyl. Doubly substituted xylose residues were also found in minor amounts in all the wheat varieties as indicated by the presence of xylitol among the methylated species. Disubstituted xylose residues have been reported in wheat (Saulnier et al., 2007), rice (Yui, et al., 1995) and sorghum (Nandini and Salimath, 2002). Small amounts of arabinosyl residues were present as terminal sugars, as indicated by the presence of 2, 3, 5-Me_3-Ara. Main chain arabinosyl residues (2, 3-Me_2-Ara) were also observed in minor amounts in all the wheat varieties. Short arabinosyl chains have been reported in branched arabinoxylans (Izydorczyk and Biliaderis, 1993). Variations were observed in the degree of branching as indicated by the ratio of unbranched to branched xyloses. The ratio was higher in MACS-2496 and HD-2189 indicating that pentosans in these varieties are less branched compared to DWR-162 and GW-322 wheat varieties.

Table 18. Methylation analysis of purified pentosans (%) from hemicellulose A

Mode of linkage	Alditol acetates	DWR-162	GW-322	MACS-2496	HD-2189
$Ara_f \rightarrow 1$	2,3,5-Me_3-Ara	10.7	10.2	8.7	8.2
$5 \rightarrow Ara_f \rightarrow 1$	2, 3-Me_2-Ara	4.5	4.3	1.5	2.2
$2,3,5 \rightarrow Ara_f \rightarrow 1$	Ara	5.8	5.2	4.6	3.6
$Xyl_p \rightarrow 1$	2,3,4 –Me_3-Xyl	11.7	12.5	10.8	12.2
$4 \rightarrow Xyl_p \rightarrow 1$	2, 3- Me_2 –Xyl	52.6	51.3	60.2	58.5
$3,4 \rightarrow Xyl_p \rightarrow 1$	2- Me-Xyl	10.2	11.6	7.8	8.7
$2,3,4 \rightarrow Xyl_p \rightarrow 1$	Xyl	3.5	4.0	2.0	3.0
$4 \rightarrow Glc_p \rightarrow 1$	2,3,6-Me_3	0.8	0.5	3.2	2.3
$3 \rightarrow Glc_p \rightarrow 1$	2,4, 6-Me_3	0.2	0.4	1.2	1.3
	2, 3- Me_2 –Xyl + 2- Me-Xyl +Xyl	3.8	3.28	6.14	5.0

Abbreviations as in Table 3.

The hemicellulosic polymers from the cell walls of wheat bran sequentially extracted with alkali showed both high and low degrees of branching (Schooneveld-Bergmans et al., 1999). In addition to xylopyranose and arabinofuranose, glucopyranosyl residues linked at C-3 and C-4 were also detected, suggesting that glucose in pentosan fractions from hemicellulose A might originate from β-(1→3) and (1→4) glucans and their concentration was higher in MACS-2496 and HD-2189 which are poor *chapati* making varieties (Izydorczyk and Biliaderis, 1995).

Periodate oxidation and Smith degradation

The arrangement of branching in pentosans was investigated by the periodate oxidation of pentosans. Periodate consumed per mole of anhydrosugar was lowest in GW-322 (0.5) followed by DWR-162 (0.54, Table 19) indicating higher degree of branching in these polysaccharides, which is also evident from their higher arabinose content (Table 17). Highly branched arabinoxylans obtained from sorghum were shown to consume about 0.64 mol of periodate over 24 h of oxidation (Nandini and Salimath, 2003). Trace amount of formic acid was released. Glycerol, arabinose and xylose were the Smith degradation products identified (Table 20). The products obtained by the Smith-degradation further substantiated periodate oxidation results. Smith degradation analysis of the glucurono-arabinoxylans from sorghum showed high amounts of glycerol and xylose (Woolard et al., 1976). Arabinoxylans from native and malted ragi showed high amount of glycerol and xylose (Shyamprasad Rao and Muralikrishna, 2007).

Table 19. Optical rotation, moles of periodate consumed and ferulic acid contents (µg / g) of pentosans purified from hemicellulose A

Wheat variety	$[\alpha]_D$	Moles of periodate consumed / anhydrosugar	Ferulic acid (µg / g)
DWR-162	−100.21	0.54	240.0
GW-322	−84.54	0.50	220.5
MACS-2496	−66.33	0.65	205.6
HD-2189	−80.42	0.68	210.4

Table 20. Analysis of Smith degradation products (%) obtained for purified arabinoxylans from hemicellulose A

Wheat variety	Glycerol	Threitol	Arabinose	Xylose
DWR-162	34.0	2.0	15.0	50.0
GW-322	30.0	3.0	19.0	48.0
MACS-2496	28.0	1.2	8.0	63.0
HD-2189	26.0	1.0	5.0	68.0

Optical rotation measurements were also determined and were -100.2° and -84.5° for DWR-162 and GW-322 respectively, while MACS-2496 and HD-2189 had -66.3° and -80.4°, respectively (Table 19). High negative optical rotation indicates preponderance of β-linkages in the xylan backbone. These results are in agreement with the optical rotation values reported in the literature for arabinoxylans (Nandini and Salimath, 2003; Shyamprasad Rao and Muralikrishna 2007).

The ferulic acid content (µg/g) of purified pentosans is given in Table 19. DWR-162 had highest ferulic acid content (240 µg/g) followed by GW-322 (220.5 µg/g). The values reported here are low compared to other pentosans reported in literature (Izydorczyk and Biliaderis, 1993). In wheat, ferulic acid is esterified to arabinose residues of cell wall arabinoxylans. Ferulic acid may be involved in cross-linking of cell wall polysaccharides in wheat through ester and ether bonds (Izydorczyk and Biliaderis, 1995).

Pentosans from Hem A of wheat varieties consisted of mainly xylan backbone in β-(1, 4) linkages to which small amounts of arabinose residues were attached at O-3 and /or O-2 and O-3 positions by α-linkages. Pentosans from wheat varieties such as DWR-162 and GW-322 had higher degree of xylan substitution by arabinosyl residues compared to MACS-2496 and HD-2189. It is generally accepted that differences in AX structure, even relatively small, can result in changes in conformation and intermolecular association, which may have an impact on functionality of these polysaccharides (Ordaz-Ortiz et al., 2005).

Poor *chapati* making varieties had high amounts of glucose compared to good varieties. High amounts of glucose could be arising from β-glucans which is strongly associated with the arabinoxylans. The strong association between alkali-extractable arabinoxylans and β-glucans is believed to be caused by their non-covalent interactions between them and also with other cell wall materials such as cellulose and lignin (Maes and Delcour, 2002). Their insoluble nature

may be due to ferulic acid cross-links occurring between arabinoxylan chains (Fincher and Stone, 1986).

3.3.3. Pentosans from hemicellulose B

The carbohydrate composition of native and purified pentosans from hemicellulose B is given in Table 21. The carbohydrate content was higher in all the purified fractions of hemicellulose B compared to native samples of all wheat varieties (Table 21). Uronic acid content was highest in DWR-162 followed by MACS-2496. Sugar composition analysis indicated that these fractions are rich in pentosans (arabinose and xylose) and glucose was the major contaminating sugar observed in all the fractions. Native fractions had a substantial amount of glucose, a part of which may be coming from undigested starch molecules and other associated polysaccharides such as xyloglucans and glucans (Dupont and Selvendran, 1987). Galactose and mannose were observed in GW-322 and HD-2189 in small amounts.

Table 21. Carbohydrate composition (%) of native and purified pentosans from hemicellulose B of whole-wheat flour of different varieties

Samples	Total sugar	Uronic acid	Ara	Xyl	Man	Gal	Glc	Ara / Xyl
DWR-162								
Native	80.0	2.8	26.5	39.0	2.5	-	32.0	0.67
Purified	90.2	3.2	34.2	61.2	-	-	4.6	0.55
GW-322								
Native	90.0	4.5	13.5	63.0	-	-	23.5	0.21
Purified	94.7	2.6	35.0	58.6	1.4	1.0	4.0	0.59
MACS-2496								
Native	80.0	4.0	21.6	48.0	-	-	30.4	0.44
Purified	88.5	2.8	30.2	65.6	-	-	4.2	0.46
HD-2189								
Native	84.0	1.9	30.6	24.6	2.0	0.4	42.4	1.24
Purified	90.6	2.3	25.6	64.2	1.8	2.4	6.0	0.39

Abbreviations as in Table 3.

Galactose might be due to galactoarabinoxylans and arabinogalactans, the presence of which has been reported in members of graminae (Looseveld et al., 1998; Dupont and Selvendran, 1987). Mannose may be due to glucomannans, which has been found in various cell walls. The lower arabinose to xylose ratio in all these fractions might be due to the presence of mixture of xylan and arabinoxylan type of polysaccharides (Saulnier et al., 2007). Presence of glucose in small amounts along with arabinose and xylose, indicate them to be glucuronoarabinoxylan and xyloglucan type of polysaccharides (Dupont and Selvendran, 1987). Arabinoxylans may be more branched in GW-322 and DWR-162 wheat varieties having good *chapati* making quality as indicated by higher Ara / Xyl ratio.

Methylation analysis

The pentosans from hemicellulose B were methylated by the method of Hakomori (1964) to study the nature of glycosidic linkages. Methylation analysis revealed that xylose residues are present in three forms; unsubstituted, monosubstituted and disubstituted (Table 22). Methylation analysis revealed the xylose residues in the main chain with 1, 4 linkages. Most of the xylose residues were unsubstituted as evidenced by the presence of large amounts of 2, 3-Me_2-Xyl. This was more in MACS-2496 and HD-2189 varieties which are having poor *chapati* making characteristics. Small amounts of terminal xylose residues were present in the pyranose form as indicated by the presence of 2, 3, 4-Me_3-Xyl. The xylan backbone was substituted mainly by arabinofuranosyl residues at O-3 position, as indicated by the presence of higher amounts of 2-Me-Xyl in GW-322 variety followed by DWR-162.

Table 22. Methylation analysis of purified pentosans (%) from hemicellulose B

Mode of linkage	Alditol acetates	DWR-162	GW-322	MACS-2496	HD-2189
$Ara_f \rightarrow 1$	2,3,5-Me_3-Ara	16.7	18.2	14.7	16.0
$5 \rightarrow Ara_f \rightarrow 1$	2,3-Me_2-Ara	1.5	1.0	2.7	1.2
$2,3,5 \rightarrow Ara_f \rightarrow 1$	Ara	9.8	10.0	9.6	7.6
$Xyl_p \rightarrow 1$	2,3,4 –Me_3-Xyl	3.5	3.2	3.8	3.2
$4 \rightarrow Xyl_p \rightarrow 1$	2, 3- Me_2 -Xyl	42.6	40.6	50.4	49.0
$3,4 \rightarrow Xyl_p \rightarrow 1$	2- Me-Xyl	16.2	17.6	12.8	14.0
$2,3,4 \rightarrow Xyl_p \rightarrow 1$	Xyl	9.7	9.4	6.0	9.0
-	2, 3- Me_2 -Xyl 2- Me-Xyl +Xyl	1.64	1.50	2.6	2.0
-	Xylose 2- Me-Xyl	0.60	0.53	0.47	0.64

Abbreviations as in Table 3.

Doubly substituted xylose residues were also found in all the wheat varieties as indicated by the presence of xylitol among the methylated species. This was present in higher amounts in arabinoxylans from hemicellulose B of DWR-162 followed by GW-322. Disubstituted xylose residues have been reported in wheat (Saulnier et al., 2007), rice (Yui, et al., 1995) and sorghum (Nandini and Salimath, 2002). Most of the arabinosyl residues were present as terminal sugars, indicated by the presence of 2, 3, 5-Me_3-Ara. Main chain arabinosyl residues (2, 3-Me_2-Ara) were also observed in minor amounts in all the wheat varieties. Short arabinosyl chains have been reported in branched arabinoxylans (Izydorczyk and Biliaderis, 1995). Variations were observed in the degree of branching as indicated by the ratio of unbranched to branched xyloses. The ratio was higher in arabinoxylans from hemicellulose B of MACS-2496 and HD-2189 indicating that arabinoxylans in these varieties are less branched compared to DWR-162 and GW-322 wheat varieties. However, the ratio of disubstituted to monosubstituted xylose was higher in HD-2189 and lower in MACS-2496. The hemicellulosic polymers from the cell walls of wheat bran sequentially extracted with alkali showed both high and low degree of branching (Schooneveld-Bergmans, et al., 1999).

^{1}H-NMR spectroscopy of pentosans from Hem-B

^{1}H NMR spectra of the hemicellulose B from wheat varieties are shown in Figs. 23 and 24. Chemical shifts were assigned by comparison with previously reported literature data (Gruppen et al., 1992; Hoffmann et al., 1992).

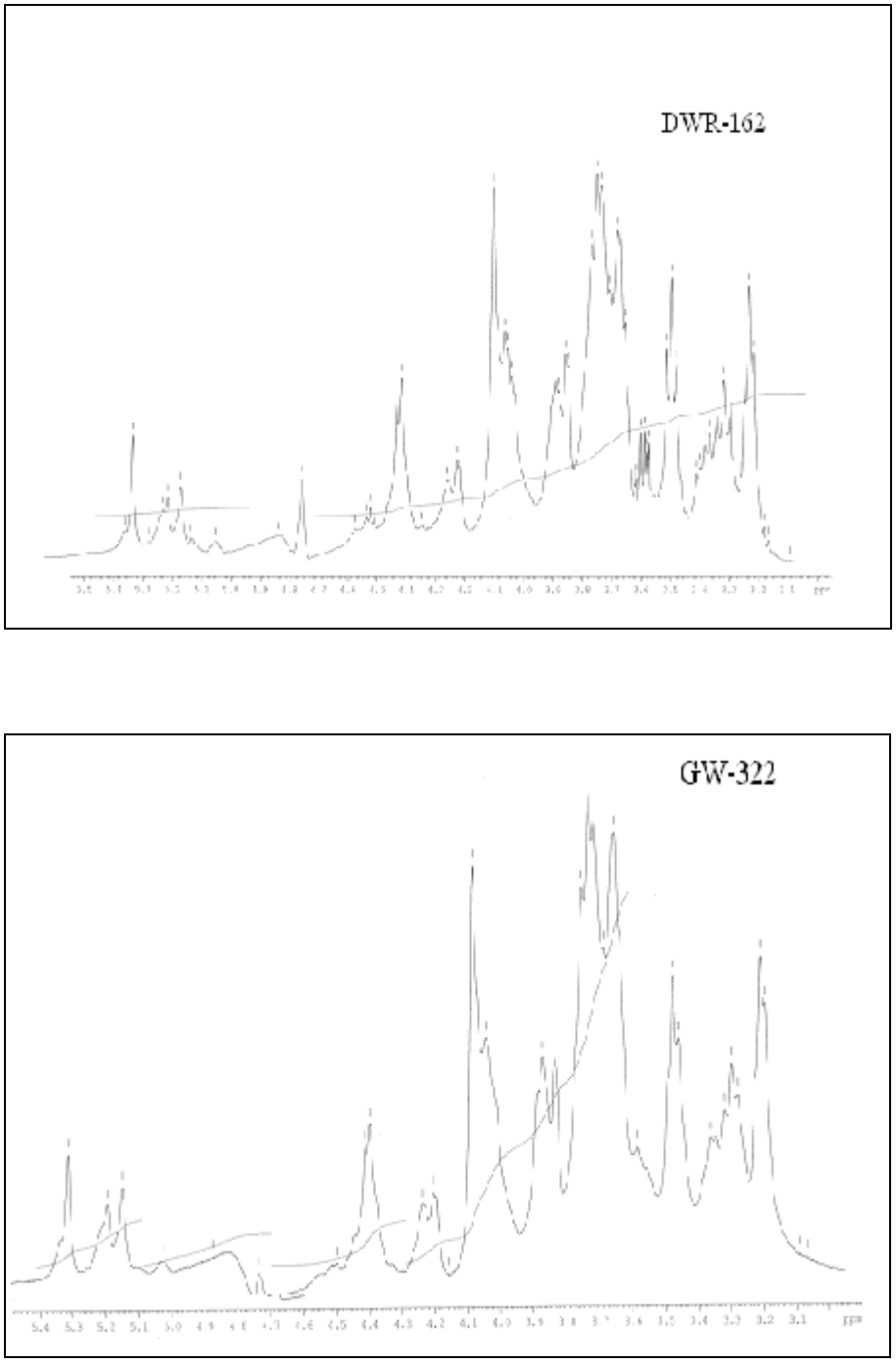


Fig. 23: ^{1}H NMR spectra of purified arabinoxylans from hemicellulose B from DWR-162 and GW-322 wheat varieties.

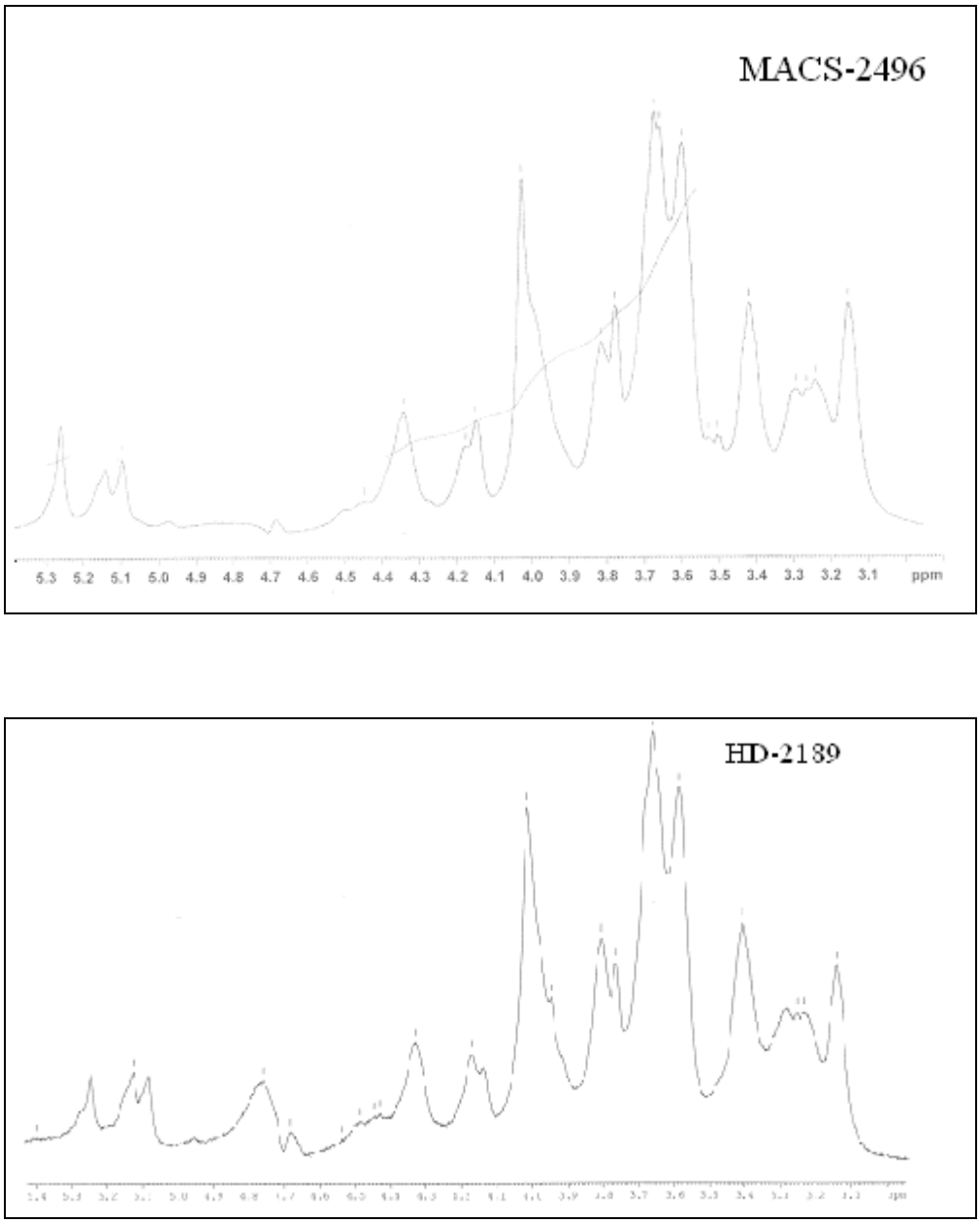


Fig. 24: ^{1}H NMR spectra of purified arabinoxylans from hemicellulose B from MACS-2496 and HD-2189 wheat varieties.

Proton NMR analysis of hemicellulose B fractions from wheat varieties revealed signals for anomeric protons of terminal α-D-arabinofuranosyl residues around 5.2-5.4 ppm and of β-D-xylopyranosyl residues at 4.4-4.7 ppm (Hoffmann et al., 1992). Signals around 5.35, 5.29 and 5.26 ppm are due to anomeric protons of arabinose residues substituted at O-3 (mono-substituted) and at both O-3 and O-2 (di-substituted) of xylose residues, respectively. The unresolved signals at the left side of the peaks at 5.32 ppm (DWR-162 and GW-322) are probably the result of two neighboring di-substituted xylose residues in the chain (Cleemput et al., 1993). There were low levels of mono-substituted xylose residues (δ 5.34 ppm) in HD-2189 as revealed by low intensity of peak. The small unresolved peaks around 5.38 ppm can be attributed to mono-substituted xylose adjacent to di-substituted xyloses and were observed in DWR-162 and GW-322. Apart from known arabinose peaks, the spectra of DWR-162 and GW-322 contained a variety of unidentified peaks, which revealed complexity of the polysaccharides.

Mono-substituted xylose residues adjacent to disubstituted xylose residues as suggested by Cleemput et al., (1993) was probably not present in MACS-2496 and HD-2189, because the shoulder down field of the signal of the arabinose (5.36 ppm) on the mono-substituted xylose was absent. The proton NMR spectra from these wheat varieties showed complex structure of the polysaccharides and further substantiated methylation analysis.

FT-IR study of pentosans

FT-IR spectra of arabinoxylans (pentosans) from Hem B of different wheat varieties are illustrated in Fig. 25. All the four spectra were characteristic of arabinoxylans (Kacurakova et al., 1994). Not much difference in the spectra between good and poor varieties could be observed. The absorption at 1635 cm^{-1} was principally associated with water, since hemicelluloses have a strong affinity for water and in the solid state these macromolecules may have disordered structures which can be easily hydrated (Kacurakova et al., 1994; Oraphin et al., 2004). Bands due to $–CH_2$ stretching vibrations were observed

around 1420 cm^{-1}. The prominent band around 1048 cm^{-1} was attributed to the C-O, C-C stretching or C-OH bending in arabinoxylans. The sharp band at 897 cm^{-1} corresponding to the C-1 group frequency or ring frequency was characteristic of β-glycosidic linkages between the sugar units (Robert et al., 2007).

The bands which appeared between 3000 and 2500 cm^{-1} represents the C-H stretching modes. The prominent band around 3380 cm^{-1} is attributed to the hydroxyl stretching vibrations of the polysaccharides and water involved in hydrogen bonding (Kacurakova et al., 1994; Nandini and Salimath, 2003). The band observed at 1045 cm^{-1} was attributed to C-O, C-C and C-O-H bending vibrations. This band shows variation in spectral shape depending on the branches at O-2 and O-3 positions (Kacurakova et al., 1994).

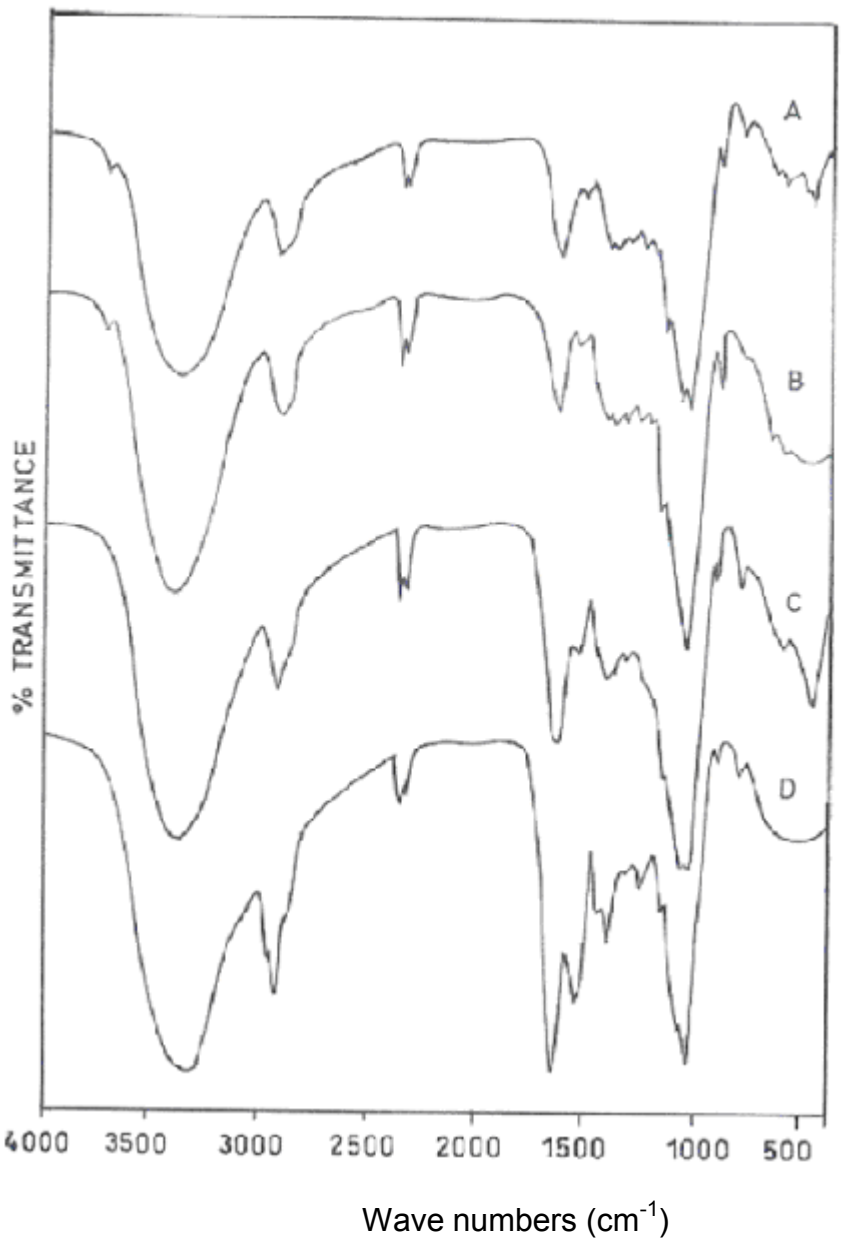


Fig. 25: FT-IR spectra of arabinoxylans from hemicellulose B of wheat varieties (A; DWR-162, B; GW-322, C; MACS-2496, D; HD-2189).

Periodate oxidation and Smith degradation

Periodate consumed per mole of anhydrosugar was lowest in DWR-162 (0.64) followed by GW-322 (0.66, Table 23) indicating higher degree of branching in these polysaccharides, which is also evident from their higher arabinose content (Table 21). Similar to this, highly branched arabinoxylans obtained from sorghum were shown to consume about 0.64 mol of periodate (Nandini and Salimath, 2002). Trace amounts of formic acid was released. Glycerol, arabinose and xylose were the major Smith degradation products (Table 24) identified. The products obtained by the Smith-degradation further substantiated periodate oxidation results. Smith degradation analysis of the glucurono-arabinoxylans from sorghum showed high amount of glycerol and xylose (Woolard et al., 1976). Similarly arabinoxylans from native and malted ragi showed high amount of glycerol and xylose (Shyamprasad Rao and Muralikrishna, 2007). Gruppen et al., (1993) reported that most of the branched residues were present as isolated units of blocks of two contiguous substituted xylose residues. Smith degradation analysis of hemicellulose B showed high amount of xylose (DWR-162 and GW-322), indicating their higher degree of substitution.

Optical rotation values were -70.2° and -76.5° for DWR-162 and GW-322, respectively, while MACS-2496 and HD-2189 had -67.3° and -65.5°, respectively (Table 23). Negative optical rotation value indicates preponderance of β-linkages in the xylan backbone. These results are in agreement with the optical rotation values reported in the literature for arabinoxylans (Nandini and Salimath, 2002; Shyamprasad Rao and Muralikrishna, 2007).

Table 23. Moles of periodate consumed, optical rotation and ferulic acid contents of purified pentosans from hemicellulose B

Wheat variety	Moles of periodate consumed / molecule of anhydrosugar	$[\alpha]_D$	Ferulic acid (µg/g)
DWR-162	0.64	−70.2	596.0
GW-322	0.66	−76.5	640.0
MACS-2496	0.80	−67.3	566.5
HD-2189	0.76	−65.5	545.4

Table 24. Analysis of Smith degradation products (%) obtained from purified pentosans from hemicellulose B

Wheat variety	Glycerol	Threitol	Arabinose	Xylose
DWR-162	38.0	2.4	11.0	48.6
GW-322	33.2	2.8	12.5	51.5
MACS-2496	43.8	5.8	8.4	42.0
HD-2189	45.8	4.5	10.2	39.5

The ferulic acid content (µg/g) of purified arabinoxylans is given in Table 23. GW-322 had highest ferulic acid content (640 µg/g) followed by DWR-162 (596 µg/g). In wheat, ferulic acid is esterified to arabinose residues of cell wall arabinoxylans. Ferulic acid may be involved in cross-linking of cell wall polysaccharides in wheat through ester and ether bonds. Apart from cross-linking, they may also be involved in plant defense mechanism and may have beneficial role as potent antioxidants (Izydorczyk and Biliaderis, 1995).

Pentosans in different varieties of wheat are known to vary and have relation to bread / *chapati* making quality. Pentosans from hemicellulose B of wheat varieties showed the basic structure of xylan backbone in β-(1, 4) linkages to which arabinose residues were attached at O-3 and /or O-2 and O-3 positions by α-linkages. Pentosans had higher degree of arabinose substitution on xylan backbone in DWR-162 and GW-322 (good *chapati* making) compared to MACS-2496 and HD-2189 (poor *chapati* making) varieties. These structural differences could be responsible for bringing about variations in *chapati* making quality.

3.3.4. Structure-function relationship of pentosans

Chapati is the most popular unleavened flat bread in India and is consumed during almost every meal of the day in north India and about a time each day in south India. Softness and flexibility are the most important quality parameters of *chapati* (Sidhu et al., 1988). Wheat varieties, GW-322 and DWR-162 revealed good *chapati* making characteristics, while, MACS-2496 and HD-2189 had poor *chapati* making quality. The structural diversity of AX has been studied with the aim of relating the structural features to specific functional properties. The structural features of arabinoxylans purified from different wheat varieties were elucidated by a combination of methods. Arabinoxylans consists of a xylan backbone in which xylose residues are in the pyranose form linked by β-(1-4) linkages. Arabinose residues in the furanose form were branched through O-3 and O-2, and O-3 of xylose residues and were present in α-

linkages. Thus, arabinoses are in different modes of substitution. AX from barium hydroxide extract were having higher degree of substitution compared to AX from Hem A and Hem B. Pentosans from Hem A were mainly xylan type in nature along with small amount of arabinoxylans. AX from Hem B had moderate degree of branching. AX exhibit different physico-chemical characteristics such as water solubility, viscosity, gelling and hydration properties (water retention or absorption), which are the basis of their functional properties in different processes and food systems (Courtin and Delcour, 2002; Saulnier et al., 2007). The hydration properties of arabinoxylans have strong impact on the functional properties of AX, as they can modify the distribution of water among different components of food, which is very much essential for cereal end products (Meuser and Suckow, 1986). Structural features such as chain length, presence of side-chain groups and their distribution will modify the water solubility of AX (Fincher and Stone, 1986). Arabinoxylans differ not only among cereals but also in different varieties of the same cereals. Even different extractants are known to extract arabinoxylans of different structural features (Izydorczyk and Biliaderis, 1995). AX from different cereals have the same basic chemical structure, but they differ in the substitution pattern of the xylan backbone which influences the capacity of AX to interact with each other and with other polysaccharides (Andrewartha et al., 1979). Degree of substitution of arabinoxylans could influence solubility of arabinoxylans, viscosity and distribution of water in dough and leads to changes in textural and chemical properties of dough (Izydorczyk and Biliaderis, 1995). Arabinoxylans from European wheat cultivars of variable bread making quality was found to be heterogeneous and have a relation to bread making quality (Cleemput et al, 1993). The contents of arabinose and xylose were reported to be higher in varieties of wheat that have good tandoori roti making quality (Saxena et al., 2000). Since the arabinoxylans have the capacity to retain water, flat breads made out of these doughs would have better palatability and pliability (Haridas Rao et al., 1986; Nandini and Salimath, 2003). Water absorption capacity of

wheat dough becomes important during *chapati* making, because the dough is left for at least half an hour to swell. Water acts as plasticizer of the gluten-starch composite matrix and lowers the rigidity of the products. Arabinoxylans which are in low /unsubstituted forms leads to insoluble complex and results in low amount of water absorption (Izydorczyk and Biliaderis, 1995) and bring about changes in physico-chemical properties of dough which might in turn be responsible for poor *chapati* making characteristics (MACS-2496 and HD-2189), while, higher branched arabinoxylans (DWR-162 and GW-322) retain gas and water, roll well and may provide soft texture and pliability (Saxena et al., 2000). Low substituted AX has a strong tendency to form aggregates (Ebringerova et al., 1990). Pentosans from good roti making qualities of sorghum and bajra varieties are highly branched in nature (Nandini and Salimath, 2001). The ability of arabinoxylans to imbibe and hold water was found to increase with the cross-linking density of the gel network (Izydorczyk and Biliaderis, 1995). Arabinoxylans from DWR-162 and GW-322 (good *chapati*-making quality) are highly branched, compared to MACS-2496 and HD-2189 (poor *chapati*-making quality) wheat varieties. Ferulic acid association with pentosan components has been known to be involved in oxidative gelation reaction (Neukom and Markwalder, 1978; Ford and Hartley, 1989) and interaction with other cell wall components. Ferulic acid content was more in good *chapati* making varieties compared to poor varieties.

Thus, results presented on wheat arabinoxylans from varieties differing in *chapati* making quality, indicates differences in structural features of arabinoxylans between the good and poor varieties. In particular, the differences in branching pattern, the way of substitution of xylan backbone by arabinose residues and ferulic acid contents, are most likely responsible for the variation in *chapati* making properties. Higher branching pattern of arabinoxylans along with ferulic acid substitution (DWR-162 and GW-322) could be the reasons for the good *chapati* making characteristics of these wheat varieties.

3.4.Changes in textural quality and arabinoxylan characteristics of whole- wheat dough by the incorporation of peroxidase

The physico-chemical characteristics of wheat flour greatly affect the quality of dough and *chapati* (Haridas Rao et al., 1986; Prabhasankar et al., 2002). The textural properties of dough are important, as they affect both the machinability of the dough and quality of the end product (Indrani and Rao, 2007). The acceptability of *chapati* can be related to properties of dough during preparation, rheological characteristics and final sensory qualities such as colour and texture (Prabhasankar, 2002). Soft texture is a desirable character of *chapati* that makes it easy to fold and forms into a scoop for picking and holding vegetable curry during consumption (Dhaliwal et al., 1996). Texture of *chapati* has been quantified using a number of laboratory instruments, notably the Instron Universal Testing Machine (Gujral and Pathak, 2002).

To improve dough handling properties, several enzymes have been tried to improve the functional properties (Mc Cleary, 1986; Rouau and Moreau, 1993). Among these, peroxidases and xylanases are important in improving dough physico-chemical properties (Hilhorst et al, 2002; Primo-Martin and Martinez-Anaya, 2003). Addition of xylanase and peroxidase has pronounced beneficial effect on both the quality of the dough and the baked products, which is mainly attributed to changes in the properties of arabinoxylans (Hilhorst et al., 1999; Selinheimo et al., 2006). An adverse effect of adding xylanse is dough stickiness, which is related to the excessive breakdown of the arabinoxylans (Van Oort, 1996). Dough stickiness was reduced with the supplementation of oxidative enzymes like peroxidases *in vitro* (Hilhorst et al., 2002).

In vitro combination of peroxidase and hydrogen peroxide catalyses the gelation of arabinoxylans via formation of diferulic acid linkages (Schooneveld-Bergmans et al., 1999). In dough, such cross linking was suggested to be responsible for the improvement of dough properties (Van Oort, 1996). Further, peroxidase has been suggested to cross-link arabinoxylans to side chains of

amino acids of proteins (Neukom and Markwalder, 1978). Thus, peroxidase action in dough has been attributed to the formation of inter-chain bonds between arabinoxylans or by the coupling of arabinoxylans to gluten proteins and therefore their properties would be expected to change (Rouau, 1993).

Most work on cross-linking of pentosans through various oxidative agents has been carried out in *in vitro* studies i.e. oxidizing agents were added to the pentosans extracted from flour (Izydorczyk et al., 1990). In the present study, different amounts of horse raddish peroxidase in the presence of fixed concentration of hydrogen peroxide were added to the whole-wheat flour, to study their effects on textural profile and changes in the dough constituents, mainly pentosans. The study was carried out on DWR-162 variety of wheat which has good *chapati* making quality.

3.4.1. Effect of peroxidase on textural properties

Textural characteristics of dough influences baking properties and play an essential role in determining the global acceptability of the food by consumers. Good quality dough will have certain degree of hardness and cohesiveness and low adhesiveness. Textural profile analysis of whole wheat dough of DWR-162 variety is given in Table 25.

Table 25. Effect of peroxidase on textural profile analysis (TPA) of wheat dough made from DWR-162 variety

Treatments	Hardness (N)	Adhesiveness (N)	Cohesiveness (dimensionless)
Control	$4.59^{a} \pm 0.08$	$0.68^{a} \pm 0.05$	$0.40^{a} \pm 0.02$
0.05% H_2O_2	$5.34^{b} \pm 0.26$	$0.54^{b} \pm 0.04$	$0.44^{a} \pm 0.01$
0.1mg peroxidase +0.05% H_2O_2	$5.57^{b} \pm 0.28$	$0.57^{b} \pm 0.04$	$0.46^{a} \pm 0.01$
0.5mg peroxidase + 0.05% H_2O_2	$6.02^{c} \pm 0.07$	$0.54^{b} \pm 0.02$	$0.43^{a} \pm 0.01$
1mg peroxidase + 0.05 H_2O_2	$7.16^{d} \pm 0.28$	$0.52^{b} \pm 0.02$	$0.48^{a} \pm 0.03$
2 mg peroxidase + 0.05 H_2O_2	$5.38^{b} \pm 0.29$	$0.48^{b} \pm 0.04$	$0.47^{a} \pm 0.02$

$^{a, b, c, d}$ Means in the same column followed by different superscripts differ significantly ($P \leq 0.05\%$)

Hardness is a primary quality characteristic of dough and it measures the force (N) necessary to attain a given deformation (the first peak in TPA analysis). Adhesiveness (N) is measured as the work necessary to overcome the attractive forces between the surface of the food and the plate of the instrument. Cohesiveness is the extent to which a material can be deformed before it breaks (Daniel and Viviana, 2006).

It is evident from the results that doughs incubated with combination of hydrogen peroxide (0.05%) and peroxidase (1 mg level) showed maximum hardness value of about 7.16 N (Table 25). Incorporation of 2 mg peroxidase had showed significant increase in dough hardness compared to control, however, it was significantly lower than 0.5 mg and 1 mg peroxidase incorporated doughs. Earlier, Selinheimo et al., (2006) reported similar observations when the dough was treated with higher levels of laccase. They speculated that softening phenomenon of dough might be due to radical catalyzed break down of the cross-linked arabinolxylan net work at high dosage. Incorporation of peroxidase and hydrogen peroxide did not bring about much changes in cohesiveness, while, addition of peroxidase along with hydrogen peroxide decreased the adhesiveness of the dough significantly compared to control. For dough, this property (adhesiveness) is of vital importance, because it is related to handling properties and machinability and low value of adhesiveness is preferred. These results indicated that dough became less sticky after the addition of peroxidase. Decrease in stickiness of dough by the addition of peroxidases was also reported earlier (Hilhorst et al., 2002).

The addition of peroxidase along with hydrogen peroxide may catalyze oxidative gelation of arabinoxylan molecules via the formation of diferulic acid linkages (Schoonveld-Bergmanns et al., 1999). In dough, such cross linking was suggested to be responsible for the improvement of dough physico-chemical properties (Van oort, 1996). Further, hydrogen peroxide and peroxidase also cross-link arabinoxylans to side chains of amino acids of

proteins (Neukom and Markwalder, 1978). Evidence for the occurrence of thiol-ferulic acid linkages has also been observed in wheat dough (Jackson and Hoseney, 1986). Accordingly, peroxidase action in dough has been attributed to the formation of inter-chain bonds between arabinoxylans or by the coupling of arabinoxylans to gluten proteins. Ultimately, peroxidase in dough may modify arabinoxylan and gluten chemical properties which in turn lead to changes in the textural properties (Piber and Koehler, 2005).

3.4.2. Effect of peroxidase on molecular weight of arabinoxylans

Pentosans isolated from the whole wheat dough of varying levels of peroxidase along with hydrogen peroxide (0.05%) were subjected to gel permeation chromatography (Sepharose CL-2B column) analysis. Results indicated that all the samples eluted as single broad peak (Fig. 26). Out of all the chromatographs, T_4 which represents pentosans isolated from dough incorporated with 1 mg of peroxidase eluted earlier indicating that they have larger sized arabinoxylans compared to those from control and other treated doughs. Increase in size of arabinoxylans could be attributed to the cross-linking between the arabinoxylan molecules in presence of peroxidase (Izydorczyk and Biliaderis, 1995; Yeh et al., 1980). Pentosans isolated from dough incorporated with 2 mg peroxidase showed slight increase in size but it is much lesser than that of pentosans isolated from 1 mg peroxidase incorporated dough. As explained earlier, it may be due to radical catalyzed break down of the cross-linked arabinoxylan network at high dosage of peroxidase (Selinheimo et al., 2006; Van Oort, 1996).

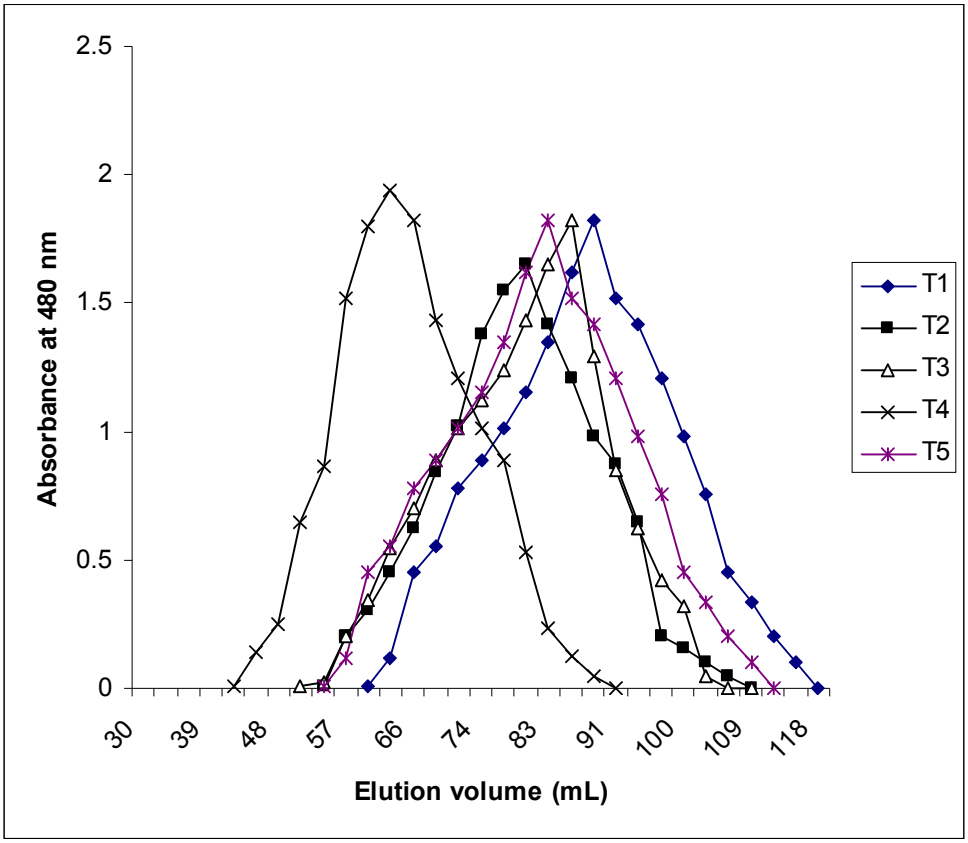


Fig. 26: Elution profile of the pentosans extracted from control and treated dough (DWR-162 variety of wheat): Control (T_1), 0.05% H_2O_2 (T_2), 1 mg peroxidase (T_3), 1mg peroxidase + 0.05% H_2O_2 (T_4) and 2 mg peroxidase+ 0.05% H_2O_2 (T5).

3.4.3. Effect of peroxidase on relative viscosity of pentosans

Purified pentosans from doughs incubated with varying levels of peroxidase along with hydrogen peroxide showed a concentration dependent increase in viscosity (Fig. 27). The pentosans from native samples showed low relative viscosity compared to pentosans isolated from peroxidase treated doughs. Among the peroxidase treated doughs the pentosans isolated from dough incorporated with peroxidase (1 mg) and hydrogen peroxide showed relatively high viscosity. The viscosity of pentosans depends on the size of arabinoxylans, degree of substitution of arabinosyl residues along with the xylan backbone and content of ferulic acid molecules in the arabinoxylan fractions (Courtin and Delcour, 2002). Similar results were also obtained earlier in wheat arabinoxylans treated with peroxidase (Izydorczyk et al., 1990; Schooneveld-Bergmans et al., 1999). Increase in relative viscosity could be attributed to cross-linking of pentosans in the peroxidase treated dough and results in increase in molecular weight of arabinoxylans which in turn leads to increase in relative viscosity of the pentosans. However, further increase in peroxidase (2 mg) concentration leads to decrease in relative viscosity compared to pentosans isolated from dough incorporated with 1 mg of peroxidase. The decrease in viscosity might result from the oxidative degradation of carbohydrate chains taking place competitively with the cross linking reaction. Such degradation of polysaccharides is known to be caused by hydroxyl radicals formed from hydrogen peroxide and other reducing agents (Ciacco and D 'Appolonia, 1982).

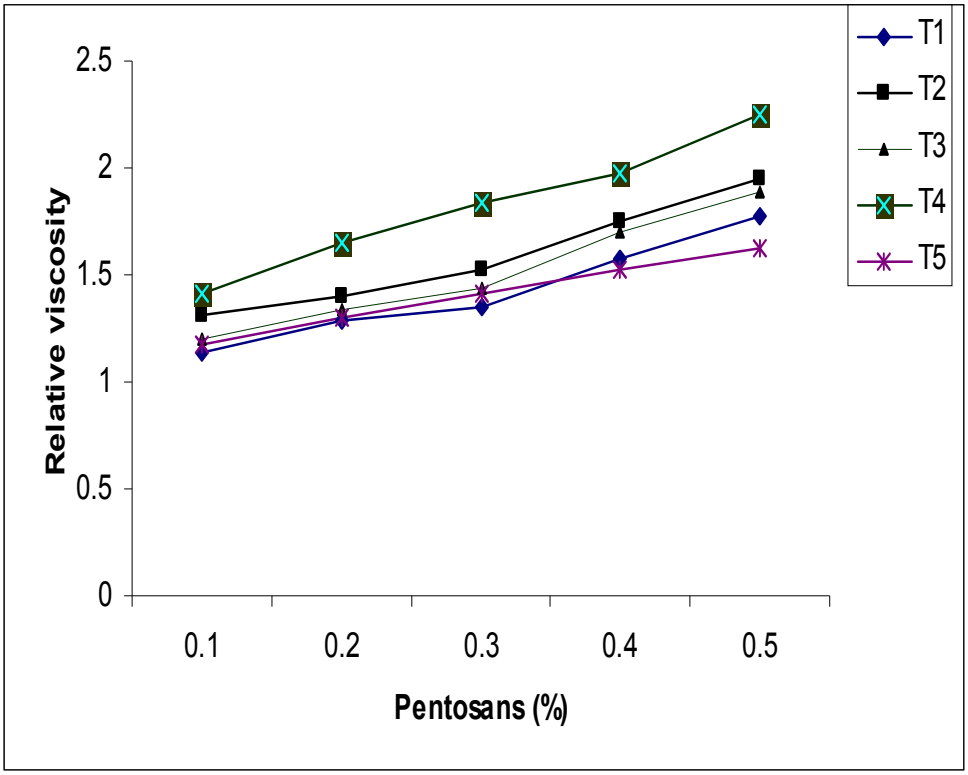


Fig. 27: Relative viscosity of purified pentosans from control and treated dough (DWR-162 variety): Control (T_1), 0.05% H_2O_2 (T_2), 1 mg peroxidase (T_3), 1mg peroxidase + 0.05% H_2O_2 (T_4) and 2 mg peroxidase+ 0.05% H_2O_2 (T5).

3.4.4.

3.4.4.1. Effect of peroxidase on amount and chemical composition of pentosans

Comparison of the amount and composition of purified pentosans from doughs of varying levels of peroxidase gives information on the mechanism of action of peroxidase. Notably, changes in ferulic acid, proteins, and arabinose and xylose contents are of interest, since peroxidase could affect all these components (Hilhorst et al.,1999). Amount of pentosans increased in treated doughs compared to control dough (Table 26). Increase in pentosans could be due to cross-linking of water-soluble arabinoxylans extracted by barium hydroxide and leads to formation of more amounts of water-insoluble arabinoxylans (Hilhorst et al., 2002) in treated dough. Uronic acid content was highest in pentosans extracted from peroxidase (1 mg) along with hydrogen peroxide (0.05%) treated dough (Table 26). Significant changes were observed in protein and ferulic acid contents among the pentosan fractions extracted from dough samples. Pentosans contain higher levels of proteins and ferulic acids in doughs incubated with peroxidase (1 mg) and hydrogen peroxide (0.05%).

Table 26. Yield levels, uronic acid, protein and ferulic acid contents of purified pentosans from different treatments of dough (DWR-162)

Treatments	Yield of pentosans (%)	Uronic acid (%)	Protein (%)	Ferulic acid (µg/g)
Control	1.2	2.4	4.5	424.6
0.05% H_2O_2	1.6	2.0	4.8	470.2
1 mg peroxidase	1.4	2.3	5.2	464.6
1 mg peroxidase + 0.05% H_2O_2	1.8	2.8	7.3	534.5
2 mg peroxidase+ 0.05% H_2O_2	1.7	2.5	6.9	480.4

Table 27. Carbohydrate composition (%) of purified pentosans from enzyme-treated and control dough (DWR-162 variety)

Treatments	Total sugar (%)	Arabinose	Xylose	Glucose	Ara/Xyl ratio
Control	82.5	41.3	52.7	6.0	0.82
0.05 % H_2O_2	87.6	43.4	50.4	6.2	0.86
1 mg peroxidase	83.8	46.2	50.2	3.6	0.92
1 mg peroxidase + 0.05% H_2O_2	86.7	48.5	46.8	4.7	1.03
2 mg peroxidase+ 0.05% H_2O_2	84.3	44.5	52.3	3.2	0.85

Abreviations as in Table 3.

Peroxidase has been shown to cross link arabinoxylans by the formation of ferulic acid dimers (Neukom and Markwalder, 1978; Hilhorst et al., 2002). Proteins have also been suggested as targets for cross-linking to arabinoxylans by peroxidase (Van Oort, 1996). Increase in ferulic acid and proteins in pentosans from dough treated with peroxidase could be due to cross linking between arabinoxylans or between arabinoxylans and proteins and leads to changes in chemical properties of pentosans (Hilhorst et al., 2002). The oxidizing agents might intensify the association between carbohydrates and proteins as indicated by increase in proteins and carbohydrates in treated dough. This suggests conformational change and molecular reorganization of pentosans from dough treated with peroxidase along with hydrogen peroxide (Patil et al., 1975b).

Analysis of sugar composition by GLC revealed changes in the contents of arabinose and xylose between the pentosan fractions isolated from doughs treated with varying levels of peroxidase along with hydrogen peroxide (Table 27). The changes in arabinose to xylose ratio were prominent among the pentosan fractions. Higher arabinose to xylose ratio was observed in the pentosan fractions isolated from dough having peroxidase (1 mg) and hydrogen peroxide (0.05%). Increased arabinose to xylose ratio indicated higher degree of substitution in arabinoxylan fractions due to the action of peroxidase. Degree of substitution of arabinoxylans could influence solubility of arabinoxylans, viscosity and distribution of water in dough and leads to changes in textural and chemical properties of dough (Van Oort., 1996; Izydorczyk et al., 1990).

Hilhorst et al., (2002) reported that peroxidase had no significant effect on the composition of water extractable pentosans, while, it had significant effect on the water unextractable pentosans with respect to arabinose to xylose ratio and yields were higher in wheat dough treated with peroxidase. These results indicated that by the appropriate combination of hydrogen peroxide and peroxidase to dough could leads to the formation of arabinoxylans with higher degree of substitution with higher amount of ferulic acid and protein contents.

3.4.5. Effect of peroxidase on UV-absorption spectra of purified pentosans

The UV absorption spectra of the pentosans from different dough samples are shown in Fig. 28. Pentosans extracted from peroxidase treated doughs had higher absorption intensity at 280 nm than from control dough. The increased absorption could be due to increased amount of protein associated with pentosans which is resulted due to cross-linking of pentosan components with proteins (Yeh et al., 1980). Patil et al., (1975b) also found increased absorption intensity at 280 nm when dough is treated with oxidative agents and the increase was attributed to protein component. The absorption peak at 320 nm is due to the presence of ferulic acid and it was more pronounced in pentosans isolated from peroxidase (1 mg) treated dough compared to control dough. Earlier, It was reported that increase in absorption at 280 nm and 320 nm was due to increased content of associated proteins and ferulic acid with pentosans (Yeh et al., 1980)

Thus, the results indicate that pentosans isolated from peroxidase (1 mg) treated dough had more amounts of protein and ferulic acid, and also showed high absorption at 280 and 320 nm. It has been reported that peroxidase is involved in cross-linking of arabinoxylans (pentosans) through ferulic acid (Van Oort, 1996) and/or cross-linking of gluten proteins or cross-linking of arabinoxyalans and gluten proteins (Ciacco and D 'Appolonia, 1982; Izydorczyk and Biliaderis, 1995). Increase in contents of protein and ferulic acid in pentosan fraction give a possible indication of formation of cross-linking of protein and pentosans through ferulic acid.

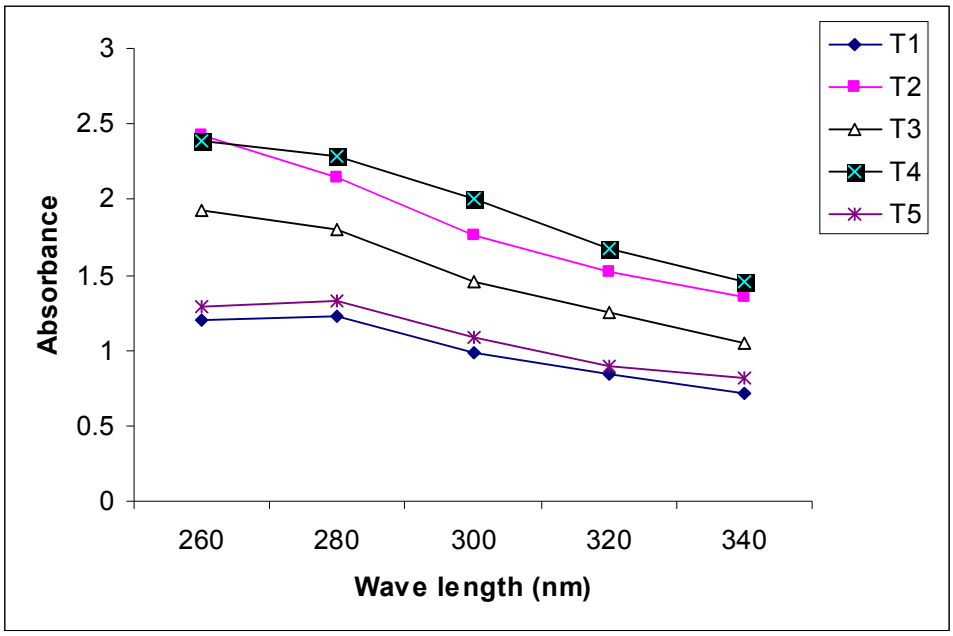


Fig. 28: UV spectra of purified pentosans from control and treated dough (DWR-162 variety): Control (T_1), 0.05% H_2O_2 (T_2), 1 mg peroxidase (T_3), 1 mg peroxidase + 0.05% H_2O_2 (T_4) and 2 mg peroxidase+ 0.05% H_2O_2 (T5).

Peroxidase acts on pentosans by cross-linking of arabinoxylans to each other through ferulic acids. In agreement with this there was an increase in the yield of pentosan fractions in dough treated with hydrogen peroxide (0.05%) and peroxidase (1 mg). This suggests that water extractable arabinoxylans are converted into large molecular weight compounds and higher degree of substitution in water unextractable arabinoxylans as revealed by GPC and sugar profile (Hilhorst et al., 2002).

In the present study amounts of proteins and ferulic acid are also increased due to the action of 1 mg of peroxidase in the presence of hydrogen peroxide. Covalent coupling of proteins to arabinoxylans by peroxidase occurs in smaller amounts. It was reported that ferulic acid was the preferred substrate for peroxidase that was converted 100 times faster than tyrosine and tyrosine derivatives (Jackson and Hoseney, 1986). Therefore, ferulic acid dimers are more readily formed than dityrosine or tyrosine-ferulic acid linkages.

The results presented here indicate that peroxidase (1 mg) along with hydrogen peroxide cross-link arabinoxylans through diferulic acid bridges into larger aggregates and exerts its action by mainly changing the composition of pentosan fractions. The results of gel permeation chromatography, viscosity and UV spectroscopy indicate that mixing flour into dough and treating with peroxidase causes conformational change in pentosan molecules and intensifies the association between carbohydrate and protein components in pentosans (Patil et al., 1975b). In addition, changes shown by treating dough with peroxidase along with hydrogen peroxide suggests that peroxidase plays an important role, with pentosans and proteins, in maintaining the bridge between protein and carbohydrate components during various stages of dough formation.

Hardness and cohesiveness increased with the addition of peroxidase and hydrogen peroxide to dough, while adhesiveness was reduced. Peroxidase prevents degradation of arabinoxylans by endogeneous xylanase (Hilhorst et al.,2002; Selinheimo et al., 2006), and hence peroxidase treated

dough had pentosans of larger size. Peroxidase prevents dough from becoming sticky by preventing extensive degradation of the arabinoxylans and by cross linking them into larger aggregates. This brings about increase in their water binding capacity and changes in the water distribution of dough and consequently affects physico-chemical properties of dough and hence functional properties.

Chapter 4
Summary and Conclusions

The results obtained in the present investigation can be summarized as follows:

1. Wheat varieties DWR-162 and GW-322 yielded highly acceptable *chapati*s. MACS-2496 and HD-2189 yielded poor *chapati*es.
2. Wheat flour and its various polysaccharide fractions were rich in carbohydrates and uronic acid content varied between varieties and in different extracts of the same variety. Arabinose, xylose and glucose were the major sugars present with minor amounts of mannose and galactose. The alkali-insoluble residues (AIR) were mainly cellulosic in nature in poor varieties (MACS-2496 and HD-2189) and appear to be more tightly bound to pentosans in good *chapati* making varieties (DWR-162 and GW-322).
3. Barium hydroxide soluble polysaccharides, hemicelluloses A and B were rich in pentosans (arabinoxylans). Arabinoxylans of barium hydroxide extracted polysaccharides were highly branched in wheat varieties-DWR-162 and GW-322 having good *chapati* making quality as indicated by high A/X ratio, where as those of MACS-2496 and HD-2189 wheat varieties (poor-*chapati* making characteristics) showed lower degree of branching as revealed by low A/X ratio.
4. Whole-wheat flours contain significant amounts of dietary fibres in all the wheat varieties. Variations in the contents of insoluble (IDF) and soluble dietary fibres (SDF) were observed among these wheat varieties. Both soluble and insoluble dietary fibres were rich in carbohydrate contents.
5. The content of dietary fibres was highest in MACS-2496 followed by HD-2189. Analysis of sugar composition by GLC revealed the presence of arabinose and xylose, along with glucose as major sugars in soluble (SDF) and insoluble dietary fibres (IDF) and indicates presence of arabinoxylans along with cellulose as major contributors.
6. Free and bound phenolic acid contents differed significantly among these wheat varieties. Ferulic, p-coumaric and vanillic acids were the major phenolic acids in

free form. Syringic and protocatechuic acid were present in small amounts. Ferulic acid was the predominant phenolic acid in bound form, along with small amount of p-coumaric acid in all the wheat varieties.

7. Wheat varieties such as MACS-2496 and HD-2189 had higher reducing power in bound phenolic extracts. DWR-162 had the lowest reducing power in both free and bound phenolic extracts.

8. Bound phenolic extracts from MACS-2496 had highest free radical scavenging activity followed by HD-2189. Free radical scavenging activities of free and bound phenolic acids was found to be significantly lower than that of synthetic antioxidants such as BHA and BHT.

9. Arabinoxylans purified from barium hydroxide extract (BE) and Hem B revealed differences in branching patterns in all the wheat varieties. Methylation analysis revealed that most of the xyloses in arabinoxylans from BE and Hem B were unsubstituted in MACS-2496 and HD-2189 where as DWR-162 and GW-322 are more branched as revealed by higher number of branches at O-3 of xylose. Main chain arabinosyl residues were also present in all the varieties.

10. ^{13}C NMR spectroscopy of barium hydroxide extracted arabinoxylans from all the wheat varieties showed signals characteristic of arabinose and xylose residues. Signals due to anomeric carbon atoms of xylose residues were observed in the region between 101- 103 ppm. The signals due to anomeric carbon atoms of α-linked arabinofuranose were observed around 108.4- 109.2 ppm. Signal due to ring carbon atoms of singly branched xylose residues were observed between 73.2-81.0 ppm (sugar A). Those of main chain xylose residue gave signals due to ring carbon atoms between 75.4-76.42 ppm (sugar C). Signals due to ring carbon atoms of arabinose residues were observed between 80-84.9 ppm (sugar D).

11. From the relative peak intensities of the anomeric region of arabinose residues for arabinoxylans from the wheat varieties, it could be observed that O-3

substituted xylose residues were present in higher amounts in arabinoxylans of DWR-162 and GW-322 than MACS-2496 and HD-2189 varieties.

12. Proton NMR analysis of arabinoxylans from barium hydroxide extracted polysaccharides and Hem B of wheat varieties indicated signals around 5.4, 5.30, and 5.20 ppm corresponding to anomeric protons of α-L-arabinofuranose substituted at O-3 (monosubstituted) and at both O-3 and O-2 (disubstituted) of xylose residues, respectively.
13. Optical rotation measurements indicated that the xylan backbone of arabinoxylans was made up of β-linkages between xylose residues and α-linkage to arabinosyl residues.
14. Periodate oxidation results indicated higher degree of substitution in DWR-162 and GW-322 varieties having good *chapati* making quality compared to MACS-2496 and HD-2189 (poor *chapati* making quality) and these results are further substantiated by the release of formic acid and Smith degradation products obtained were glycerol, arabinose and xylose.
15. Arabinoxylans from DWR-162 and GW-322 contained high amounts of bound ferulic acid in BE and Hem B.
16. FT-IR spectra of arabinoxylans from wheat varieties showed characteristic bending and stretching vibrations of arabinoxylans.
17. Hardness and cohesiveness of the dough increased with the treatment of peroxidase with hydrogen peroxide, while, stickiness is reduced in treated dough. Significant changes were not observed in cohesiveness. However, significant changes were observed in physicochemical properties of treated pentosans. Pentosans from enzyme treated dough had higher relative viscosity, arabinose to xylose ratio, ferulic acid and protein contents compared to native dough.

The present study clearly indicated that wheat varieties, GW-322 and DWR-162 revealed good *chapati* making characteristics, while, MACS-2496 and HD-2189 had poor *chapati* making quality. Arabinoxylans (AX) from barium hydroxide extract were having higher degree of branching compared to AX from Hem A and Hem B. Pentosans from Hem A were mainly xylan in nature. AX from Hem B had moderate degree of branching. Arabinoxylans from DWR-162 and GW-322 (good *chapati* making quality) are highly branched, compared to MACS-2496 and HD-2189 (poor *chapati* making quality) wheat varieties. Higher branching pattern of arabinoxylans along with ferulic acid substitution (DWR-162 and GW-322) could be the reasons for the good *chapati* making characteristics of these wheat varieties. Wheat varieties such as MACS-2496 and HD-2189 had higher content of phenolic acids and dietary fibre and can be exploited as potential dietary sources of nutraceuticals and functional foods. The studies also showed that peroxidase along with hydrogen peroxide could be used to improve functional properties of wheat and improve *chapati* making quality.

Chapter 5
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List of Publications

Research papers published / communicated

1. **Revanappa, S.B.,** Bhagwat, S.G. and Salimath, P.V. (2007) Studies on pentosans in Indian wheat varieties in relation to *chapati* making quality, ***Food Chemistry*****, 104**, 896–902.
2. **Revanappa**, **S.B.,** Nandini, C.D. and Salimath, P.V. (2009) Structural characterization of pentosans from hemicelluloses B of wheat varieties with varying *chapati* making quality, (***Food Chemistry*** accepted for publication-In press).
3. **Revanappa**, **S.B.** and Salimath, P.V. (2009) Phenolic acid profiles and antioxidant properties of wheat varieties, (Communicated to ***Journal of Food Biochemistry*****).**
4. **Revanappa, S. B.,** Nandini C.D. and Salimath, P.V. Structural features of arabinoxylans from wheat varieties having differences in *chapati*-making quality, (To be Communicated to *Journal of Agricultural and Food Chemistry*)**.**
5. **Revanappa**, **S. B.,** Salimath, P.V. and Prasad Rao, U. J. S. Changes in textural quality and arabinoxylan characteristics of whole-wheat dough by the incorporation of peroxidase, (To be *Communicated to Journal of Cereal Science*).
6. **Revanappa, S. B.** and Salimath, P.V. Isolation and characterization of hemicellulose A from wheat varieties varying in *chapati* making quality, (To be communicated to *Carbohydrate Polymers*).

ABSTRACTS IN SYMPOSIA / CONFERENCES

- **Revanappa**, **S.B.** and. Salimath P.V. Studies on Non-starch polysaccharides in Indian wheat variety DWR-162, ICFOST, CFTRI-DFRL, Mysore. 2005.
- **Revanappa**, **S.B**. Bhagwat**.** S.G. and Salimath P.V. Carbohydrate composition of Indian wheat varieties in relation to *chapati* making quality, Carbohydarte Conference**,** held at University of Lucknow, Lucknow, 2006.
- **Revanappa**, **S.B.** and Salimath, P.V. Antioxidant activities of free and bound phenolic acids of Indian wheat varieties, Presented in International Conference on Emerging trends in Free radical and Antioxidant research, Lonavala, 2007.
- **Revanappa**, **S.B.** and Salimath, P.V. Hemicellulosic polysaccharides: Isolation and structural characterization, Carbohydrate Conference held at PGMIR, Chandigarh, 2007
- **Revanappa**, **S.B.** and Salimath, P.V. Structural features of arabinoxylans from Indian wheat varieties, Society of Biological Chemist (SBC) held at Sri Venkateshwar University, Tirupati . 2007.
- **Revanappa**, **S. B.** and Salimath, P.V. Non-starch polysaccharides from durum wheat (*Triticum durum*) variety (DWR-2006) having good pasta making quality, IFCON, CFTRI, Mysore, 2008.