EXTRACTION, COMPOSITION

AND SOME OF THE

PHYSICAL AND CHEMICAL PROPERTIES

OF

COMPONENTS OF DIETARY FIBRE

by

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"The value of what one knows is doubled if one confesses to not knowing what one does not know. What one knows is then raised beyond the suspicion to which it is exposed when one claims to know what one does not know."

Schopenhauer

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A B S T R A C T

The extraction and some of the chemical and physical properties of components from plant cell walls are described in this thesis. The chemical composition of the extracted polymers and the morphological and physical changes occurring in wheat bran at various stages of an extraction sequence and the metal binding capacities of the extracts were determined.

A sequential extraction procedure using water, amylase, oxalate and alkali (before and after delignification) was used to isolate components of plant cell walls. This enabled water soluble and water insoluble fibres from bean, cabbage, lettuce, tomato, peach, pumpkin, kumera, onion, pear, wheat bran, lucerne, clover and ryegrass to be obtained. The water soluble fibres were shown to be composed predominantly of arabinose, galactose and uronic acid, whereas the water insoluble fibres contained mainly arabinose and xylose.

The viscosities of the alkali soluble fibres extracted from wheat bran, before and after chlorite delignification, and after solubilisation in N-methyl-morpholine-N-oxide were determined. The arabinoxylan extracted before delignification, yield of 7.9 g/100 g, had a limiting viscosity number of 220.6 ml/g, whereas the arabinoxylan extracted after chlorite delignification, yield of 3.8 g/100 g, had a limiting viscosity number of 74.2 ml/g. When the solvent N-methyl-morpholine-N-oxide had been used to dissolve the nondelignified arabinoxylan, a considerable decrease in viscosity, to 6.3 ml/g, was observed. It was concluded that direct extraction (no delignification) of wheat bran, enables a less degraded arabinoxylan to be extracted in adequate yields. The use of

N-methyl-morpholine-N-oxide as a solvent for arabinoxylan resulted in extensive degradation.

The structural changes in wheat bran at each stage of the extraction sequence and when dimethylsulphoxide (DMSO) was substituted for alkali were observed using light and scanning electron microscopy. It was shown that the commercially ground sample of wheat bran contained a high proportion of starch, which was removed after the amylase treatment. Alkali removed cell wall material predominantly from the aleurone layer. DMSO was not an efficient extractor of arabinoxylans from cell walls, a yield of only 0.4% being obtained and the aleurone cell walls remaining intact. The arabinoxylan, extracted with DMSO, had a higher ferulic acid and acetyl content than the arabinoxylan extracted with alkali.

The interactions of fibres with metal ions (copper, iron, zinc, calcium, potassium, magnesium, manganese and sodium) using concentrations that would be expected in the human small bowel after a 'typical' meal were investigated. It was found that the water soluble fibres bound more copper, iron and zinc than the water insoluble fibres. The copper, iron and zinc binding occurred with a displacement of calcium, magnesium and manganese. The water insoluble fibres (hemicelluloses) contained a higher calcium content than the soluble fibres (pectins). After acid treatment, sodium was bound preferentially rather than calcium to hemicellulose. Possibly divalent calcium ions play a role in stabilising the hemicellulose components of plant cell walls.

The binding capacities and mechanisms of zinc binding to wheat bran, its components and to phytate were determined. Zinc binding capacities (μM/g dry weight of plant material) in order of magnitude were; phytate (6582 ± 192), DMSO soluble hemicellulose (5089 ± 921), water soluble fibre (4038 ± 216), cell walls (1012.6 ± 193), lignocellulose (510 ± 41.9), cold water soluble fibre (440.0 ± 15.3), alkali soluble hemicellulose (227.9 ± 61.4), bran (167.7 ± 12.7), bran ex oxalate (148.3 ± 50.0), bran ex ethanol (142.3 ± 4.4) and cellulose (57.4 ± 5.3).

The water soluble fibre, fractionated using ammonium sulphate, composed predominantly of arabinose (24.0%), galactose (20.3%), xylose (18.6%), mannose (16.2%), glucose (10.9%) and rhamnose (6.0%), bound zinc more strongly than phytate or the DMSO hemicellulose. The Scatchard plots of zinc binding to phytate and to the fibres, except for the water soluble fibres, were concave and markedly nonlinear, suggesting that the binding mechanism is by negative cooperativity or site heterogeneity. The Scatchard plots of zinc binding to the water soluble fibres showed well pronounced maximum, indicating the binding mechanism is by positive cooperativity.

Part I of this thesis describes the studies undertaken to isolate and determine the chemical composition of different types of fibres from bean, cabbage, sweet potato, lettuce, onion, peach, pear, pumpkin, tomato, wheat bran, white clover, lucerne and ryegrass. This study has been published in the Journal of the Science of Food and Agriculture 1983, 34: 1236-1240 (see Appendix F).

Part II of this thesis describes studies undertaken to investigate the viscosity of hemicelluloses obtained using the extraction procedure and after solubilisation in N-methyl-morpholine-N-oxide. The work has been published in Carbohydrate Research 1985, 143: 271-274 (see Appendix G).

Part III describes studies undertaken to observe the morphological structure of wheat bran, changes occurring during the extraction sequence and influence of DMSO when substituted for alkali.

Part IV describes studies on binding of metals to water soluble and insoluble fibres from fruits, vegetables, bran and grasses.

Part V describes a more detailed study of zinc binding to wheat bran, its fibre components, and to phytate.

The thesis concludes with a general discussion of the findings and a summary of the conclusions.

I N T R O D U C T I O N

The quality of food is dependent on a high content of nutritionally desirable components and low levels of detrimental substances. The fibre component of plant foods has had its status changed from that of an undesirable component, that reduced the baking quality of flour, affected food texture and did not make a significant contribution to the caloric intake, to a very desirable component that can prevent major health disorders.

In response to the consumers' demand for white flour, processes were developed which lowered the fibre content of the diet of Western populations (Trowell, 1973). It has been observed that over the last one hundred years, the incidence of certain types of diseases have increased (Trowell, 1973). A hypothesis has been advanced relating the increased incidence of certain non-infectious diseases of unknown aeteology, to a reduction in the intake of plant fibre in the diet (Burkitt, 1972, Burkitt et al. 1972). In contrast to the possible advantages of the consumption of high fibre diets, there is the possibility that it may lead to mineral deficiencies of calcium, iron and zinc (Reinhold, et al, 1981).

Despite this apparent conflict, as yet unresolved, fibre in foods has become the basis of new and growing industries in the West. Often with apparent disregard for the type of fibre, from pea hulls (Collins et al. 1982) to linseed meal (Dubois, 1978) being added back to foods to increase their 'fibre' content.

Dietary fibre has so far eluded a simple or single definition. It may be best considered in terms of a concept proposed by the

hypothesis that certain diseases are linked to fibre-depleted diets (Cleave, 1956, Trowell, 1976, Burkitt, 1972b).

Dietary fibre was initially considered to be the remnants of plant cell walls that are resistant to hydrolysis by the alimentary enzymes of man and are therefore present in the ileum, but may be partly hydrolysed by the bacteria in the colon (Trowell, 1974). Trowell (1974), Van Soest and McQueen (1973) considered that the major components of dietary fibre were cellulose, hemicellulose and lignin. However Southgate et al. (1976), Jenkins et al. (1976) and Spiller and Shipley (1977) considered that pectin, mucin, gums, waxes and cell wall bound proteins should also be included. Therefore the definition of dietary fibre was expanded to include the storage polysaccharides. Thus dietary fibre was redefined as the plant polysaccharides and lignin which are resistant to hydrolysis by the digestive enzymes of man (Trowell et al. 1976). Southgate et al. (1976) considered that "this definition is essentially a philosophical one and the term applies to all the indigestible polysaccharides and lignin that may be imagined to reach the large intestine". If this philosophical approach to the definition of dietary fibre is accepted, it then denies the possibility of determining the chemical identity of dietary fibre, for it is not possible to measure compounds imagined to have reached the beginning of the large intestine. However, in order to substantiate the dietary fibre hypothesis, it is necessary to identify the plant cell wall components that reach the start of the large intestine. One of the impediments in achieving this objective has been the lack of established procedures by which plant cell walls and

their constituents may be determined. Earlier studies placed the emphasis on the measurement of cellulose, hemicellulose, pectin and lignin (Van Soest and McQueen, 1973; Southgate, 1969; Holloway, et al 1977). This experimental approach is limited, however, as it has not enabled the composition of the water soluble and water insoluble polysaccharides present in different foods to be determined, nor has it enabled the investigation of the physical and chemical properties of plant cell walls and their constituents to be undertaken.

Purpose of this Study

This study was undertaken in response to the need for:

(1) Further definition of the types of polysaccharides present in a variety of foods and feeds.

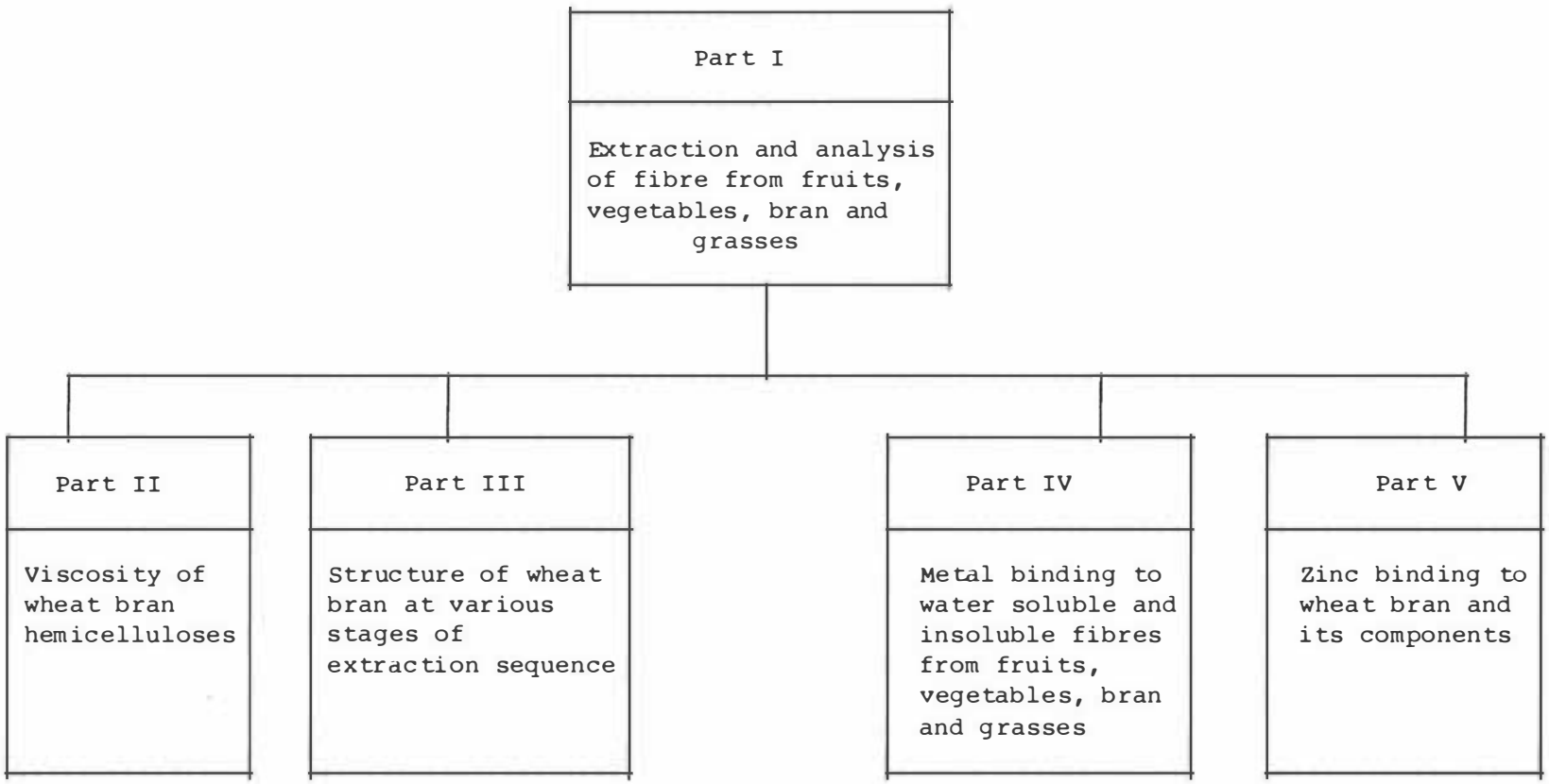
(2) Investigation of different methods of extraction of plant cell wall polymers and preparation of whole cell walls.

(3) To determine the binding of metals to different types of fibres from a variety of foods and feeds.

(4) To quantitate the zinc binding properties of wheat bran, its intact cell walls, cell wall components and phytate.

A flow diagram showing the various stages in the investigation of the extraction, composition and metal binding of components of dietary fibre is shown in figure 1.

Figure I: Flow diagram showing the various stages of the extraction, composition and some of the physical and chemical properties of components of dietary fibre.



Part I

Isolation and determination of the chemical composition of some of the fibre components of fruits, vegetables, pasture grasses and wheat bran.

Introduction

This section of the thesis was initiated in the Applied Biochemistry Division of DSIR in 1980. The experimental procedures for extraction were devised and preliminary experiments were carried out in that establishment. The entire work was completed and submitted for publication at the end of 1982. This part of the thesis was written at that time. Appendix D of this thesis reviews this work in light of subsequent publications as does the discussion in Part I.

Studies of the geographical and chronological incidence of non-infectious diseases of the intestinal tract resulted in the fibre hypothesis being proposed (Burkitt, 1973). Over the last 100 years there have been many changes in Western Societies that may have an influence on the human intestinal tract. These include a decrease in physical activity, increase in cigarette smoking and stress associated with urbanisation. Changes in the diet are associated with an increased calorie intake in the form of fats, simple sugars and proteins and a corresponding decreased consumption of cereals and vegetables (Walker, 1974). The decreased fibre content of cereals used in developed societies can be traced to the changes in milling technology with the introduction of steel roller mills at the end of last century (Trowell, 1973).

Initially the fibre hypothesis laid stress on the decreased wheat bran content of the Western diet (Burkitt, 1973). However, many Societies that have a low incidence of the 'fibre deficiency diseases' have traditionally had a low cereal consumption and high content of fruits and vegetables in their diets (Eastwood et al. 1974).

Plant cell walls have been suggested to be a major component of dietary fibre (Trowell, 1974). The structure and chemistry of plant cell walls has been reviewed by Albersheim (1973), Aspinall (1980), Rogers and Perkins (1968), Preston (1974), Selvendran (1983), Monro et al. (1976), McNeil et al. (1984) and Rees (1977). However few studies have investigated the extraction and composition of components of cell walls from a variety of fruits, vegetables, bran and pasture grasses to enable comparisons between species to be undertaken.

Extraction of the polymeric constituents of cell walls has been subjected to extensive investigations see for example Aspinall (1982). In Aspinall s' (1982) review of cell wall chemistry he stated that the objectives of the isolation procedures, are to obtain polymeric material in as high a yield and as chemically pure and homogeneous form as possible. Sometimes these two objectives may work in opposition to each other. The initial problems are those of isolation without degradation, then purification so that the polysaccharides are obtained free of contaminating materials and fractionation to give substances that are chemically and physically homogenous within acceptable limits (Aspinall, 1982).

In some instances, as with capsular polysaccharides from bacteria and exudate gums, polysaccharides are readily solubilised and may often be simply isolated in homogeneous form (Aspinall, 1982). In other cases, especially cell wall components, the polysaccharides are present in close association forming a supermolecular cell wall structure (Fengel, 1976). Graded extraction can selectively solubilise macromolecular components of the cell wall (Rogers and Perkins, 1968).

The simplest extraction method for polysaccharides is by using water alone at various temperatures (Wise, 1942). Some polysaccharides may be brought into solution by the use of polar nonaqueous solvents such as dimethylsulphoxide, as in the extraction of starch (Leach and Schoch, 1962) and the isolation of O-acetyl-4-O-methylglucuronoxylans from hardwoods and of O-acetyl-galactoglucomannans from some softwoods (Bowering et al. 1961).

Extraction of pectins can be achieved by water extraction or with aqueous solutions of ethylenediaminetetraacetic acid or ammonium oxalate (Rogers and Perkins, 1968).

Extraction of other cell wall polysaccharides such as cellulose and hemicellulose, is generally more difficult. Wilkie (1979) reviewed the chemistry of plant cell walls and their hemicelluloses. He cited the statement by Norman (1937) that he considers still holds true.

"The chemistry of the hemicelluloses remains to be written....their isolation is beset with pitfalls and their separation

and purification with uncertainty. The investigator who wishes to analyse plant material for its content of cellulose or hemicellulose must first of all be clear in his own mind about the questions which the analysis is expected to answer. It can not be too strongly emphasised that 'cellulose' and 'hemicellulose' are normally determined as the resultants of certain sets of operations, rather than as chemically defined species. The term hemicellulose has no unique definition in spite of, and indeed, in part because of, its long usage. For working purposes, hemicelluloses are often implicitly or explicitly defined as the cell wall and intercellular polysaccharides that can be extracted by alkali from higher land-plant tissues that are, or were, lignified, but similar material can be extracted by water from the delignified tissues of grasses".

A review, conducted to determine the methods used by other investigators for the isolation of hemicelluloses showed that the most common method used is extraction with alkali (Timell, 1965; Jermyn and Isherwood, 1956; Furda, 1979; Whistler et al. 1970; Tharanathan et al. 1975; Whistler and Feather, 1965; Blake and Richards, 1970; Brillouet et al. 1982; Mares and Stone, 1973; Keegstra et al. 1973; Siegel, 1962; Norris and Preece, 1930; MacArthur and D'Appolunia, 1980; Monro et al. 1976; Reid and Wilkie, 1969 and Shibuya and Iwasaki, 1978).

However, only minor quantities of hemicelluloses can be removed from soft woods by direct extraction and generally a preliminary delignification is required to obtain adequate yields. Timell (1965) postulated that hemicellulose may be protected by the lignin present in the secondary cell walls.

The most frequently used material for the extraction of hemicelluloses is holocellulose, that is plant material that has been subjected to delignification. A survey of published procedures used to isolate hemicelluloses, from woods and plant foods showed that delignification of the material is commonly carried out before commencing the hemicellulose extraction (Timell, 1965; Jermyn and Isherwood, 1956; Blake and Richards, 1970; Brillouet et al. 1982; Siegel, 1962).

However the severe conditions of oxidation (ozone, chlorine-2-aminoethanol, sodium chlorite and chlorine) and elevated temperatures and/or pressure normally used for delignification are a possible cause of polysaccharide degradation (Timell, 1965).

The yields obtainable by direct extraction from a variety of plant foods, nor the possible degradation caused by delignification of food hemicelluloses, does not appear to have been determined.

This study was designed (see appendix D) to isolate and then to determine the chemical composition of some of the components of dietary fibre in fruits, vegetables, pasture grasses and wheat bran.

Materials and Methods

Sampling procedure and general sample treatment

The samples selected were largely determined by their common usage in New Zealand. Commercially grown and marketable fruits and vegetables (1 kg fresh weight or a minimum of 3 samples of each species) were collected by Horticulture Advisory Officers of the

Ministry of Agriculture. The regions of New Zealand where the foods and grasses were obtained is shown in Table III. Three pastoral grasses (1 kg fresh weight of each species) were obtained from Applied Biochemistry Division of DSIR and wheat bran (5 kg) was obtained from Manawatu Mills Ltd. On arrival at the laboratory the samples were separated from the waste, weighed, then freeze-dried to a constant dry weight. The dried material was ground so that it would pass through a 44 mesh sieve. The homogenised samples were stored, as a dry powdered product, in a deep freeze (-20°C).

Extraction

All the extractions were carried out in duplicate. Samples (5 g) of the dried foods (bean, cabbage, lettuce, tomato, peach, pumpkin, kumera, onion, pear, wheat bran and the three grasses, lucerne, clover and ryegrass) were extracted for 10 hours in a Soxhlet with 200 ml of 95% ethanol and the ethanol insoluble solids were then air dried.

Cold Water Extraction

Water (150 ml) was added to the ethanol insoluble solids and the suspension slowly shaken for 15 hours. The liquid layer was decanted and added to 3 volumes of ethanol. After standing 12 hr the resulting precipitates were separated by centrifugation. The precipitates were washed with ethanol then dialysed against running water for 36 hours against distilled water for a further 24 hours before being freeze-dried. The dried extracts were weighed then stored at -20°C until they were required for chemical analysis.

Hot Water Extraction

Water (150 ml) was added to the cold-water extracted residues. The suspensions were then heated to 95°C for 2 hours. After cooling to room temperature 20 ml of alpha amylase solution (Sigma A 6880, Porcine Pancreatic, 5 g/litre^{-1} in phosphate buffer at p7.0) and a few drops of toluene were added to the suspensions which were then incubated for 17 hours at 37°C. The suspensions were re-heated and re-incubated until the KI-I_2 spot test for starch was negative. The supernatants were decanted and added to 3 volumes of ethanol. The resultant precipitates were treated in the same way as the cold water extracts.

Oxalate Extraction

To the solids remaining after the hot water extraction were added 100 ml of ammonium oxalate (10 g litre^{-1}). The suspension was mixed, and allowed to stand for 90 minutes. The supernatants were decanted and precipitated as for the other extracts.

Alkali Extraction

To the oxalate insoluble material was added 100 ml of sodium hydroxide (100 g litre^{-1}) under nitrogen. The suspensions were mixed and allowed to stand for 6 hours. The aqueous layers were decanted and added to 3 volumes of ethanol. These precipitates were treated in the same way as the previously obtained precipitates.

Delignification

To the alkali insoluble residues were added water (100 ml) and glacial acetic acid (5 ml). The mixtures were heated to 75°C and sodium chlorite (1 g) was added slowly every 25 minutes until a total of 4 g had been added. After a further 90 minutes at 75°C the mixtures were then cooled, the supernatants decanted and discarded. The insoluble residues were then re-extracted with sodium hydroxide using the previous process. All the extracts, water soluble and alkali soluble were dialysed for 48 hours before freeze-drying, followed by drying in a dessicator to a constant dry weight. The sequence used in the extraction of the polysaccharides is summarised in figure 2.

Analysis

The compositions of the dried polymers were determined by measuring the individual sugars, after trifluoroacetic acid hydrolysis, by GLC of the alditol acetates (Jones and Albersheim, 1972). The results of GLC analysis of sugar standards are shown in figure 3. The uronic acids were measured using m-hydroxydiphenol (Blumenkrantz and Asboe-Hansen, 1973), acetyl groups by the hydroxylamine procedure (McComb and McCready, 1957), methoxyl groups by the pentane-2,4-dione method (Wood and Siddiqui, 1971) and protein using the Folin-Ciocalteu reagent (Schacterle and Pollack, 1973). All the extraction procedures were carried out in duplicate, as were all the analyses. Reported mean values were within 10% variation.

The yields were obtained by determining the dry weights (after freeze drying) of all the extracts and expressed relative to the dry

weights (freeze dried) of the starting material. All the freeze dried extracts were stored in weighed air-tight plastic containers immediately after drying, then after weighing they were stored at -20°C.

Results

Figure 3 shows the separation of sugar standards through a column packed with 3% ECNSS-M at 190°C using a Hewlett-Packard 5840A gas chromatograph.

Table I shows the yield in g of freeze-dried polymer per 100 g of freeze-dried starting material.

Table II shows the composition of the isolated components of dietary fibre from fruits, vegetables, grasses and wheat bran.

Table III shows the regions of New Zealand where the foods and grasses, used in this study, were obtained.

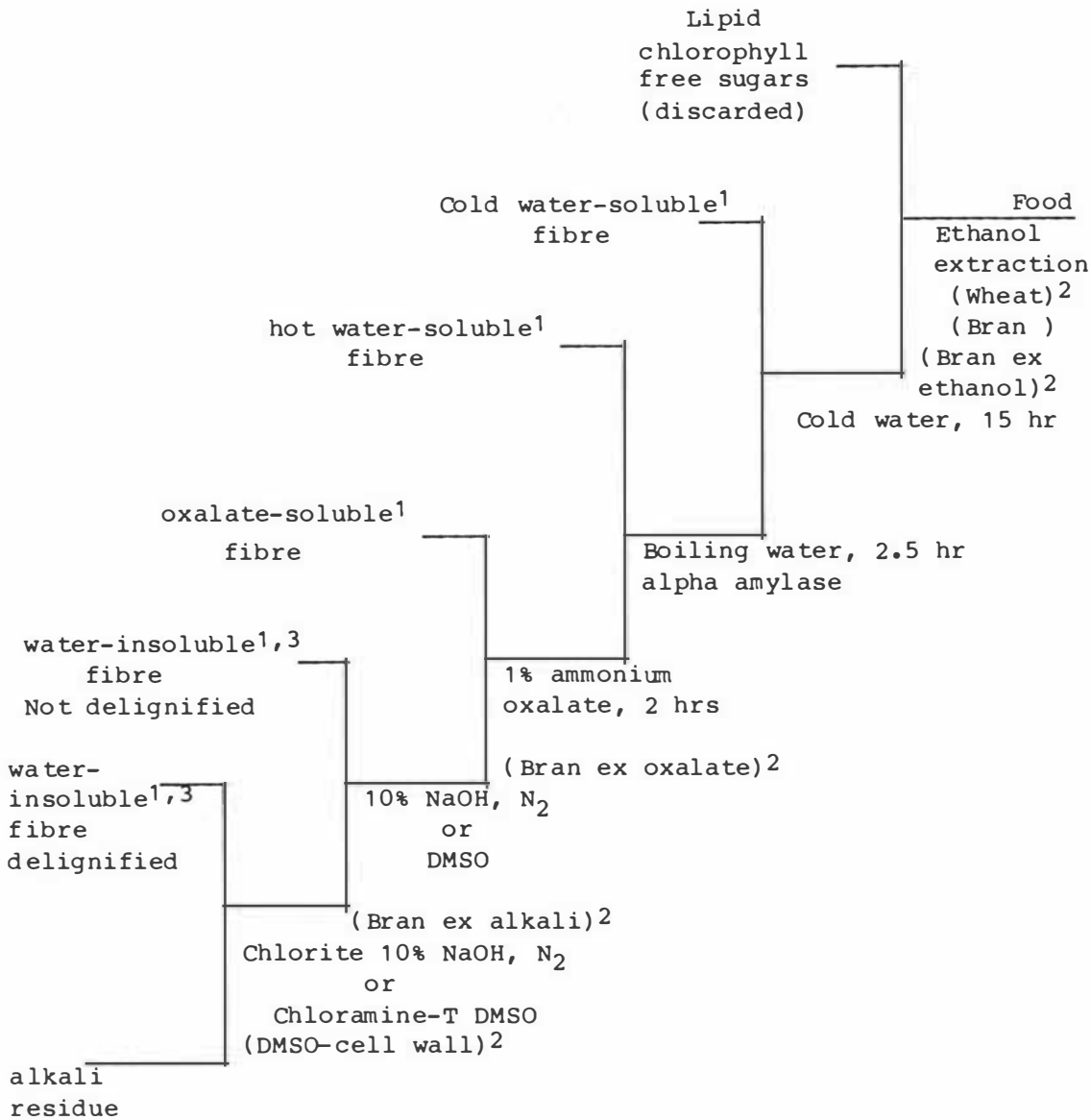


Figure 2: Sequence used in the extraction of the water soluble and water insoluble fibres from fruits, vegetables, wheat bran and grasses.
[1], Precipitated in ethanol, dialysed, freeze dried.
[2], Insoluble material subjected to light and scanning electron microscopy.
[3], Alkali soluble (hemicellulose) and DMSO soluble (DMSO hemicellulose)

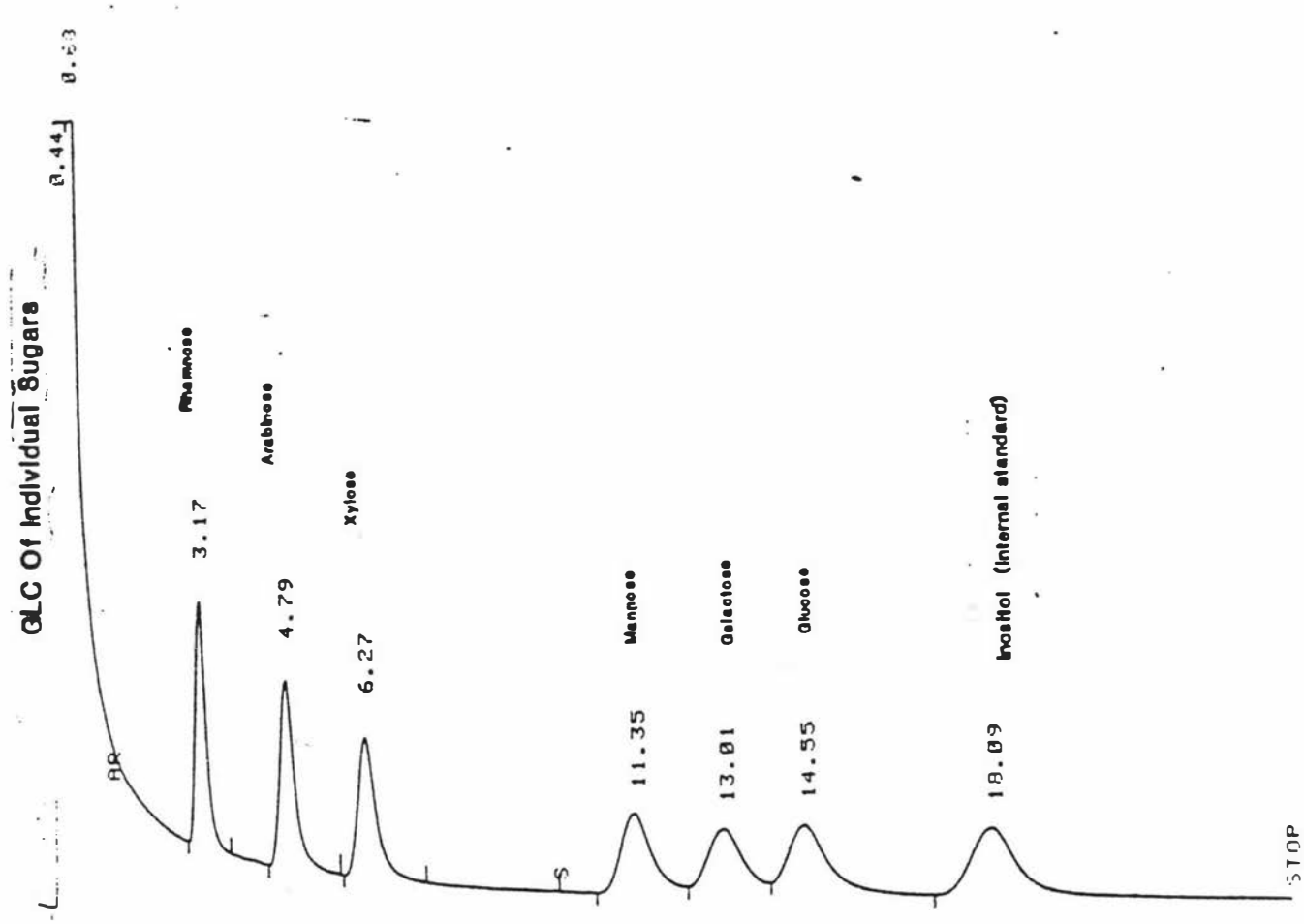


Figure 3: Separation of sugars through a column packed with 3% ECNSS-M at 190°C using a Hewlett Packard 5840A gas chromatograph.

Table I

Mean yields in g of freeze-dried polymer per 100 g of freeze-dried starting material

Food	Cold Water Soluble	Hot Water Soluble	Oxalate Soluble	Hemicellulose before delignification	Hemicellulose after delignification
Beans	1.0	4.0	1.1	3.7	1.3
Cabbage	1.5	1.3	2.4	4.1	0.6
Kumeras	0.5	4.3	4.3	11.0	1.5
Lettuce	2.0	2.1	3.3	3.7	1.0
Onion	1.4	2.8	2.9	2.0	0.7
Peach	2.1	3.3	1.3	3.9	2.5
Pear	0.8	2.4	1.8	2.2	1.9
Pumpkin	0.9	-	3.7	3.6	1.1
Tomato	3.5	0.8	0.7	2.8	0.5
Wheat Bran	0.5	1.1	0.8	7.9	3.8
Feeds					
Clover	0.9	2.0	5.0	4.0	2.4
Lucerne	1.3	2.0	3.2	6.2	2.7
Ryegrass	0.8	0.5	1.4	11.3	2.5

TABLE 11

COMPOSITION OF THE ISOLATED COMPONENTS OF DIETARY FIBRE FROM FRUITS, VEGETABLES, GRASSES AND WHEAT BRAN. (mean values)

(EXPRESSED AS A % OF THE ANALYSED COMPONENTS)

Food	Fraction	Rhamnose	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acid	Methoxyl	Acetyl	Protein
Bean	Cold water	0.6	10.3	1.7	1.3	40.9	5.5	27.6	3.0	0.5	8.6
(*Phaseolus*	Hot water	7.2	6.5	0.7	0.4	23.5	3.4	49.5	1.4	1.1	6.1
vulgaris)	Oxalate	5.4	8.9	0.3	0.0	24.1	13.1	33.0	3.0	1.5	10.7
	NaOH	1.0	2.1	21.9	4.1	12.3	15.7	14.4	1.0	3.3	24.2
	NaOH (after delignification)	4.4	11.7	23.8	0.0	6.8	18.4	5.3	1.9	2.4	25.2
Cabbage	Cold water	0.8	4.2	0.3	0.4	6.1	1.1	71.5	1.0	0.2	14.3
(*Brassica*	Hot water	3.3	16.3	0.7	0.3	12.7	0.0	53.6	2.1	0.6	10.3
oleracea, var.	Oxalate	4.2	22.0	0.9	0.2	17.5	2.2	40.4	1.5	0.4	10.7
Capitata)	NaOH	1.8	11.0	13.8	5.8	24.1	17.6	13.0	1.0	3.0	8.9
	NaOH (after delignification)	0.4	4.2	29.5	2.7	8.0	18.0	7.7	3.0	1.1	25.3
Sweet potato	Cold water	3.9	14.0	0.5	1.9	25.6	6.8	15.9	9.7	0.5	21.3
(*Ipomoea*	Hot water	0.9	0.3	2.0	0.1	2.3	82.4	8.7	1.6	1.1	0.7
batatas)	Oxalate	0.4	3.1	0.1	0.3	3.2	72.6	13.5	0.7	3.1	2.9
	NaOH	0.0	0.8	2.3	0.2	0.7	90.6	2.6	0.3	1.2	1.2
	NaOH (after delignification)	0.0	1.7	24.4	0.0	0.0	55.0	8.8	1.3	0.4	8.4
Lettuce	Cold water	2.4	10.2	1.1	2.8	25.6	5.5	45.3	1.1	0.3	5.6
(*Lactuca*	Hot water	6.9	5.7	0.4	0.4	15.5	0.0	58.3	4.4	0.9	7.5
sativa,	Oxalate	7.5	5.7	0.3	1.3	10.1	5.7	55.7	3.1	0.6	10.1
var. Yateslake)	NaOH	1.6	19.7	23.3	8.2	0.9	0.0	24 9	1.9	1.9	18.3
	NaOH (after delignification)	6.3	2.2	40.8	3.8	8.3	16.2	7.6	1.6	0.3	18.8
Onion	Cold water	0.8	8.9	3.2	7.7	21.0	13.7	9.7	11.3	0.4	23.4
(*Allium*	Hot water	0.3	0.6	0.6	9.3	0.0	40.2	27.4	11.2	0.0	10.3
cepa)	Oxalate	3.3	3.8	0.7	0.0	49.3	0.8	31.5	2.7	0.7	8.0
	NaOH	0.7	9.9	12.4	1.8	24.4	36.5	8.0	1.1	0.0	5.3
	NaOH (after delignification)	0.0	1.3	31.9	2.6	7.5	46.3	5.3	1.0	0.3	4.0
Peach (*Prunus*	Cold water	0.9	10.9	1.3	0.7	8.5	2.4	54.3	8.5	0.7	12.0
persica)	Hot water	5.0	42.6	2.4	0.6	11.9	1.7	23.6	8.7	1.1	2.4
	Oxalate	2.7	31.6	1.2	0.4	7.5	3.3	44.1	2.7	1.6	4.9
	NaOH	1.8	9.4	18.3	4.2	11.9	19.6	9.5	1.3	2.5	21.4
	NaOH (after delignification)	0.1	2.7	75.5	0.0	4.1	3.6	6.7	1.3	0.2	5.7
Pear (*Pyrus*	Cold water	2.5	23.1	1.7	0.6	23.5	5.0	27.6	5.8	0.4	9.7
communis)	Hot water	1.7	45.1	1.3	0.2	12.2	4.2	21.3	9.1	0.8	4.0
	Oxalate	2.3	50.1	1.4	0.4	15.4	3.7	19.9	2.1	0.4	4.3
	NaOH	1.6	17.2	19.8	3.8	13.4	27.7	8.3	0.7	1.7	5.8
	NaOH (after delignification)	0.0	4.2	80.6	0.0	2.6	1.9	4.2	1.5	0.0	5.1
Pumpkin	Cold water	0.0	18.4	0.5	0.7	43.0	0.0	17.6	3.2	0.3	16.3
(*Cucurbita*	Hot water	0.0	10.3	0.5	0.5	17.5	5.3	—	—	—	—
pepolmaximn)	Oxalate	3.3	4.6	0.4	0.0	14.5	5.2	58.9	7.3	0.4	5.2
	NaOH	0.0	0.0	14.2	15.5	32.7	7.2	12.5	2.6	0.8	14.5
	NaOH (after delignification)	0.0	3.9	3.9	0.0	19.8	30.2	17.5	4.0	0.8	19.8
Tomato	Cold water	1.1	5.8	0.7	0.1	12.1	0.0	69.6	1.3	0.7	8.6
(*Lycopersicon*	Hot water	1.7	11.9	2.2	0.4	14.5	2.2	41.7	3.0	6.7	15.8
esculentum)	Oxalate	1.6	15.4	1.6	0.8	14.6	2.4	26.8	5.7	1.6	29.3
	NaOH	0.0	3.7	27.0	12.1	6.9	31.9	9.2	0.5	0.5	8.2
	NaOH (after delignification)	0.0	6.6	35.7	7.7	7.0	16.4	10.8	2.1	0.7	12.9
Wheat bran	Cold water	0.3	23.5	29.9	1.7	7.2	33.1	0.6	0.1	0.4	3.3
(*Triticum*	Hot water	0.0	20.8	39.0	1.1	2.7	33.1	0.6	0.1	0.2	2.5
aestivum)	Oxalate	0.0	12.9	40.1	0.0	0.0	38.7	1.9	0.2	0.6	5.6
	NaOH	0.0	31.9	60.1	0.3	2.1	4.0	0.7	0.1	0.1	0.9
	NaOH (after delignification)	0.0	29.4	61.8	0.0	0.8	5.7	1.2	0.1	0.5	0.5
White clover	Cold water	3.1	9.8	10.9	0.7	15.3	5.4	20.0	3.4	9.5	22.0
(*Trifolium*	Hot water	7.0	10.5	5.1	0.6	15.0	2.5	40.3	2.3	1.9	14.8
repens)	Oxalate	2.1	3.9	0.4	0.0	5.0	6.4	49.5	2.8	1.4	28.5
	NaOH	8.1	28.4	27.2	0.0	3.0	0.0	19.0	1.2	1.6	11.5
	NaOH (after delignification)	0.1	3.3	50.2	5.6	5.4	13.3	6.4	1.5	0.3	13.3
Lucerne	Cold water	1.9	8.9	0.8	0.4	11.1	4.1	29.7	1.9	8.5	32.8
(*Medicago*	Hot water	9.8	11.6	1.9	0.4	14.0	4.3	42.5	1.4	0.9	13.2
sativa)	Oxalate	5.2	9.4	1.0	0.0	7.1	1.0	64.9	1.6	0.0	9.7
	NaOH	2.0	2.8	53.1	4.4	6.1	11.5	10.9	0.8	0.5	7.9
	NaOH (after delignification)	2.4	26.9	39.1	11.9	0.0	2.7	9.5	2.0	0.4	5.1
Ryegrass	Cold water	2.0	9.4	2.5	16.3	12.6	23.0	11.9	3.2	4.9	14.3
(*Lolium*	Hot water	2.0	7.2	2.7	0.7	8.6	7.9	24.3	6.4	5.4	35.1
perenne)	Oxalate	1.3	8.1	2.6	0.9	9.8	7.7	41.5	2.1	2.1	23.9
	NaOH	0.2	14.3	52.8	0.3	5.1	10.5	10.5	0.7	1.2	4.9
	NaOH (after delignification)	0.2	10.1	63.9	0.0	4.8	9.9	4.4	0.4	0.3	5.9

Table III

Regions of New Zealand where the foods and grasses, used for fibre extraction, were grown. All food samples were picked by Ministry of Agriculture and Fisheries, Horticultural Advisory Officer's in a fresh state, and at the stage of ripeness normally used for human consumption.

Name	Area grown and collected
Bean	Horticultural Research Centre, Levin.
Cabbage	Horticultural Research Centre, Levin.
Sweet potato	Horticultural Research Centre, Pukekohe.
Lettuce	Horticultural Research Centre, Levin.
Onion	Horticultural Research Centre, Pukekohe.
Peach	Horticultural Research Centre, Hastings.
Pear	Horticultural Research Centre, Hastings.
Pumpkin	Horticultural Research Centre, Pukekohe.
Tomato	Horticultural Research Centre, Levin.
Wheat bran	Manawatu Mills, Palmerston North.
White clover	Grasslands, DSIR, Palmerston North.
Lucerne	Grasslands, DSIR, Palmerston North.
Ryegrass	Grasslands, DSIR, Palmerston North.

Discussion

The yields of the polymers soluble in cold water, hot water, oxalate and sodium hydroxide (before and after delignification) are shown in Table I. The highest yields of the water soluble polysaccharides were obtained after treatment of the plant material with amylase and extractions into hot water. The greatest yields of hemicelluloses (non-cellulosic polysaccharides soluble in sodium hydroxide) for all the foods and feeds were obtained before delignification. This is in contrast to studies on the extraction of hemicellulose from wood. Generally wood requires delignification before any quantities of hemicellulose can be extracted (Timell, 1964).

The water soluble polymers are composed predominantly of the sugars arabinose, galactose and uronic acid. The amount of protein present in the water soluble polymers varied from 3% (bran) to 35% (ryegrass) as shown in Table II. The high levels of uronic acid in the water soluble as well as the oxalate soluble fractions confirms that methods of analysis for the uronic acid content of foods should include the water and oxalate soluble fractions (Holloway et al. 1983). The methoxyl and acetyl groups present also showed considerable variation. This large variation in content makes it difficult to substantiate the findings of Selvendran et al. (1979) that oxalate soluble polysaccharides have a lower methoxyl content. Possibly this is due to the diverse monocot and dicotyledonous plant food groups analysed. Onion had the highest level of methoxyl groups at 22.5 percent; in contrast wheat bran had the lowest at 0.4 percent. It has been suggested that high methoxyl

pectins are important in reducing serum cholesterol levels (Mokady , 1973) so that onions could possibly be an interesting source of high methoxyl pectins for dietary studies.

The water insoluble fibre components were composed predominantly of the pentose sugars arabinose and xylose. The xylose content of the polymers, in many cases, increased markedly after delignification, possibly due to loss of arabinose side chains. The protein content of the water insoluble polymers varied from 0.5 percent in wheat bran to 25 percent in beans. The protein is likely to be the hydroxyproline rich protein of the cell walls (McNeil et al. 1984).

Other investigators have also endeavoured to isolate and/or quantitate plant cell wall components (Southgate et al. 1978 and James and Theander, 1981). Sequential extraction for dietary fibre analysis has been reported, by among others, James and Theander (1981), Stevens and Selvendran (1984 A,B), Asp et al. (1983). Isolation of cell wall components for compositional studies include the study by Aspinall and Fanous (1984). Using stepwise extraction of cell wall materials from apples using water, ammonium oxalate and dilute alkali, three polysaccharides were obtained. They were found to be a highly branched α-L-arabinofuranan, a linear 4-linked α-D-galacturonan with α-L-arabinofuranan subunits and a fucogalactoxyloglucan. The structural features of apple and citrus pectic substances were studied by De Vries et al. (1984). Using alcohol insoluble solids (De Vries et al. (1984) they fractionated the polysaccharides by ion-exchange chromatography and gel filtration. They found that the pectin contained rhamnose, arabinose, galactose, glucose and galacturonic

acid residues, xylose was present only at low levels. Enzymatic degradation of the pectin showed that neutral sugar side chains were present in 'hairy' regions i.e. blocks of neutral sugar side chains (De Vries et al. 1984). The cell wall material from parenchymatous tissue of the pods of French and Runner beans were extracted from wet ball-milled tissues with 1% aqueous dimethyl sulphoxide (O'Neill and Selvendran, 1980). The results of that study are similar to other sequential methods of extraction. However the more mild treatment used by O'Neill and Selvendran (1980) resulted in predominantly galacturonic acid fractions being obtained. Presumably the less soluble hemicelluloses were not able to be extensively extracted using dimethyl sulphoxide.

The hemicelluloses present in both dicot and monocot cell walls are composed of xyloglucans and xylans (McNeil et al. 1984). In dicots xyloglucans predominate, whereas xylans constitute the major hemicellulose of monocots (McNeil et al. 1984). But both types of polysaccharides have been shown to be present in monocots and dicots. Xyloglucans being found in many different dicots including mung and runner beans, potato and in the cell walls of the monocots of rice, oats, barley and bamboo (McNeil et al. 1984). The structure of cell wall xyloglucan has been reviewed extensively (McNeil et al. 1979). The basic structure of this cell wall polymer consists of a backbone of β-4-linked-D-glucosyl residues with D-xylosyl side chains α-linked to O-6 of some of the glucosyl residues, O-acetyl groups and galactosyl-containing side chains also have been shown to occur (McNeil et al. 1979). The basic structure of the xylans has been reviewed by

Wilkie (1979). All known xylans consist of a backbone of β-4-linked xylosyl residues with various side chains attached through O-2 or O-3 of the xylosyl residues (Wilkie, 1979). Both xyloglucans and xylans probably have a structural role in plant cell walls, some or all being hydrogen bonded to cellulose, as evidenced by the requirement for strong alkali for their extraction (McNeil *et al.* 1984).

The integration of the accumulated knowledge of the chemical structure of cell walls has been reviewed by including McNeil *et al.* (1979), Aspinall (1980), Knee and Bartley (1981) and McNeil *et al.* (1984). The complexity and diversity of plant cell wall components has resulted in difficulty in postulating comprehensive plant cell wall models. With the development of new analytical tools such as NMR and HPLC/mass spectrometry the possibility of further developments in this field, particularly the study of whole cell walls, would appear to be advantageous. This is discussed further in Appendix D.

The high glucose content in the water, oxalate and sodium hydroxide extracts from the sweet potato, kumera (*Ipomoea betatas*) may be due to amylase resistant starch. Englyst *et al.* (1983) reported that the content of amylase resistant starch was between 0 and 3% in a wide variety of foods. However this amylase resistant starch has been proposed to be a component of dietary fibre (Englyst, 1983). A further fractionation of the hemicellulose from wheat bran was undertaken. The results of this study are discussed in the general discussion and results shown in appendix A and B.

The extraction and composition of the fibre components from fruits, grasses and vegetables in this study indicate that they are

composed of a high proportion of water soluble polymers composed predominantly of arabinose, galactose and uronic acids, whereas the fibre composition of wheat bran is mainly water insoluble polymers of arabinose and xylose. These results are similar to that found by Theander and Åman (1979), however the fractions in their study were generally higher in glucose and lower in uronic acid. This is presumably due to dissimilar starting materials and the different extraction procedure. However the results of both these studies show there is considerable difference in the solubility and chemical composition of the fibre components. This would support the hypothesis that the greater water affinity of the fibre components of fruits and vegetables may be associated with gel formation within the small bowel thereby affecting the absorption of other nutrients such as glucose (Jenkins et al. 1976). This would account for the slowed glucose absorption hence reduced insulin requirements in diabetic patients on high fruit and vegetable diets (Douglass, 1975). The fibre of wheat bran being less water soluble appears to be also less easily degraded in the upper intestinal tract (Holloway et al. 1978). Wheat bran fibre may exert its major influence in the colonic environment rather than that of the small bowel.

The results of this study and those of Theander and Åman (1979) and Selvendran et al. (1979) suggest that one type of extraction scheme should not be applied to all types of plant foods. The variability in cell types and chemical composition, particularly the high starch containing foods, makes quantitation of dietary fibre difficult because each step removes an indefinable amount of

polysaccharide (Selvendran et al. 1979). There is considerable appeal in using one extraction scheme for the quantitative analysis of fibre in foods to obtain support for the epidemiological studies, such as applying the Southgate (1969) procedure of sequential extraction or the removal of nonfibre components by detergents as in the Van Soest method (Van Soest and Wine, 1976). However these procedures can only give a general measure of the fibre content of foods. Possibly what is required is a more detailed fractionation of different plant foods to isolate and determine components of fibre that are 'active' in influencing disease prevention and do not adversely affect other food components.

The next section, Part II, was undertaken to investigate the influence of the delignification procedure on the viscosity of hemicelluloses extracted from one of the foods used in this study.

PART II

Study of the Viscosity of Wheat Bran Hemicelluloses

Introduction

Methods of extraction of polymeric constituents of plant cell walls have been subjected to extensive investigations, see for example the work of Whistler et al, 1965; Whistler and Richards, 1970; Ring and Selvendran, 1980) and the review by Aspinall (1982). One of the principal difficulties that still remains is the selective solubilisation of the hemicelluloses. Their isolation from woody tissues is usually preceded by delignification to obtain an appreciable yield (Whistler and BeMiller, 1963). However, even in woody tissue there is the possibility that delignification may cause depolymerisation of the polysaccharides. (Glaudemans and Timell, 1957). This procedure, however, is still widely used in the isolation of hemicelluloses from feeds (Aspinall and McGrath, 1966) and foods (Brillonet et al. 1982). As shown in Part I of this thesis hemicelluloses from foods and feeds can be obtained, using alkali, in adequate yields, without prior delignification.

Alkali, while being the most commonly used reagent for obtaining hemicelluloses, does have disadvantages. They have been reviewed by Whistler and BeMiller (1958) and Aspinall (1959). Aspinall (1959) considered that there are four types of reaction, resulting from the use of alkali, which may cause modification of the hemicelluloses as they occur in the plant; (1) de-esterification of partially acylated polysaccharides, (2) chemical degradation initiated at reducing

groups, (3) alkaline hydrolysis of glycosidic linkages, and (4) the breaking of chemical bonds between hemicelluloses and other cell wall components.

The influence these reactions have in degrading hemicellulose during extraction was studied by Aspinall et al. (1961). The degradation of xylans by dilute alkali at room temperature was followed by the analysis of the low molecular weight acids formed and the determination of changes in xylan molecular weights. The conclusion from this study was that under the conditions normally employed for their extraction from plant tissues, alkaline degradation of xylans is slow and proceeds only from the reducing end-groups. Prolonged alkali extraction (25 days) led to a 20 per cent diminution of the molecular weight of the xylan. They also reported that alkaline degradation can be minimised by reducing latent aldehydic end-groups with potassium borohydride.

However, possibly one of the great disadvantages in alkaline extraction of hemicelluloses, is that the naturally occurring ester groups are saponified (Whistler, 1960). Other solvents for extraction have also been used. Dimethylsulfoxide (DMSO) has been used to extract glucuronoxylan with a 17 per cent acetyl content (Bouveng et al. 1959). DMSO has also been used in studies on the distribution of the O-acetyl groups in pine glucomannan (Kerne et al. 1975). However, DMSO generally enables cell wall polysaccharides to be extracted only in a low yield (Timell, 1960), possibly because the lignin-hemicellulose linkages are stable in DMSO (Morrison, 1973).

The differences in composition, yields and physical properties of hemicelluloses extracted from foods, by alkali and DMSO do not appear to have been determined.

A new solvent N-methylmorpholine-N-oxide (NMMNO) was reported by Joseleau et al. 1981, as a 'remarkable solvent' for certain polysaccharides (Aspinall, 1982). This solvent has been reported to have been used to dissolve cellulose nondegradatively (Chanzy et al. 1979) and whole cell walls (Joseleau, et al. 1981). Its use as a solvent for hemicelluloses has not been reported in the scientific literature.

The isolation of hemicelluloses, obtained in the least degraded form, would enable appropriate samples for further studies of the properties of dietary fibre to be undertaken.

Possible degradation of wheat bran hemicelluloses isolated before and after delignification and after solubilisation in NMMNO. Can be determined by measuring the limiting viscosity number. The limiting viscosity number(η)(intrinsic viscosity) has been shown to be related to the molecular weight (m) by the general equation

$$[\eta] = K\ m^{\alpha}$$

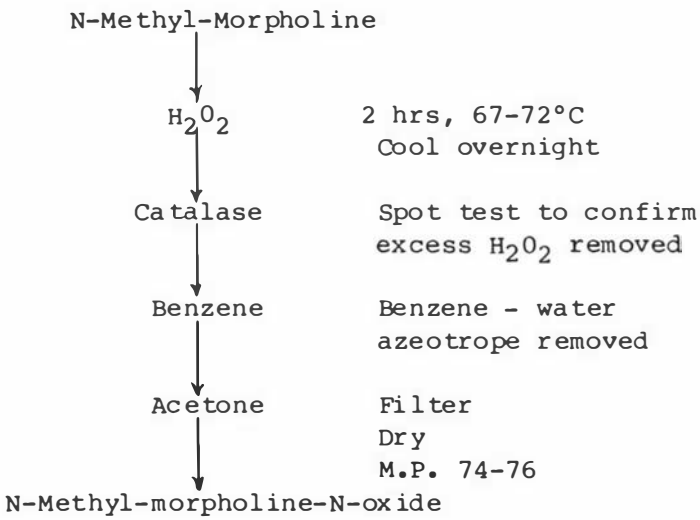
where K and α are empirical constants for a given polymer-solvent system (Greenwood, 1964).

Materials and Methods

Synthesis of N-Methyl-Morpholine-N-Oxide

The solvent NMMNO was not available in New Zealand at the commencement of this study, it was therefore synthesised using the procedure described in the patent application (Johnson, 1969). N-methyl-morpholine was oxided using hydrogen peroxide, excess peroxide being removed by the use of catalase. The N-methyl-morpholine-N-oxide was then obtained from a benzene-water azeotrope by precipitation in acetone, dried in a dessicator, and the melting point established to be between 74 and 76°C. The synthesis sequence is shown in figure 4.

Figure 4: Sequence of synthesis of N-methyl-morpholine-n-oxide. Johnson D.L. 1969, British Patent No 1,144,048.



Preparation of solution of hemicellulose in N-methyl-morpholine-N-oxide

The suitability of NMMNO as a nondegradative solvent for hemicelluloses was investigated by adding NMMNO (12.5 g) to

nondelignified hemicellulose (0.5 g). As a control, a similar sample of hemicellulose was added to water (12.5 g). Both samples were then heated (120°C) under nitrogen for 30 minutes. Dimethyl sulphoxide (50 ml) was then added to each sample. After cooling to room temperature both solutions were dialysed separately against water for 48 hours. The nondialysable contents were then freeze-dried. The dialysis tubing remained intact throughout the dialysis procedure, thus indicating that the tubing was not degraded by the solvents.

Measurement of viscosity of hemicellulose solutions

Samples of wheat bran hemicellulose used for viscosity determinations were extracted in alkali before and after delignification, and after solubilisation in NMMNO.

The viscosity measurements were made at 25° ± 0.05°C using Cannon-Fenske size 50 viscometers in a Tamson TCV 40 water bath. Solutions of the hemicelluloses (250 mg in M NaOH, 25 ml) were stirred under nitrogen for one hour, filtered through number two sintered glass filters. The small amounts of undissolved material obtained on filtration were washed twice with ethanol and dried at 105°C. These weights were subtracted from the starting weights in order that the final concentrations could be calculated. The filtered solutions were then diluted in filtered M NaOH to make a series of solutions of 5.0, 3.0, 1.25 and 0.625 mg/ml.

The individual solutions (5.0 ml) were placed into five viscometers that had previously been cleaned and calibrated according to the procedures described by Kragh (1961). The limiting viscosity

numbers were determined by measuring the flow times of all the solutions to within 0.1 of a second. The viscosity ratio for each solution (V/Vo) was calculated by dividing the flow time of the hemicellulose solutions by that for the pure solvent (M NaOH). The results were plotted as:

$\frac{V/Vo - 1}{C}$ against concentration (g/ml) and extrapolated to zero concentration.

Effect of exposure to alkali on hemicellulose viscosity

The influence of time of exposure in alkali on the viscosity of hemicellulose was determined. Hemicellulose was extracted from wheat bran ex oxalate as described in Part I. The filtered solution of hemicellulose was introduced to the viscometer 20 minutes after the addition of alkali to the bran. The relative viscosity (V/Vo) was determined until 165 minutes had elapsed.

Results

The viscosity of the wheat bran hemicelluloses extracted before and after delignification are shown in figure 5.

The viscosity of bran hemicellulose after solubilisation and recovery from NMMNO and the control sample is shown in figure 6.

The limiting viscosity numbers of the hemicelluloses extracted before and after delignification and after recovery from NMMNO are shown in Table IV.

The relative viscosity of alkali nondelignified hemicellulose in alkali with time is shown in Table V.

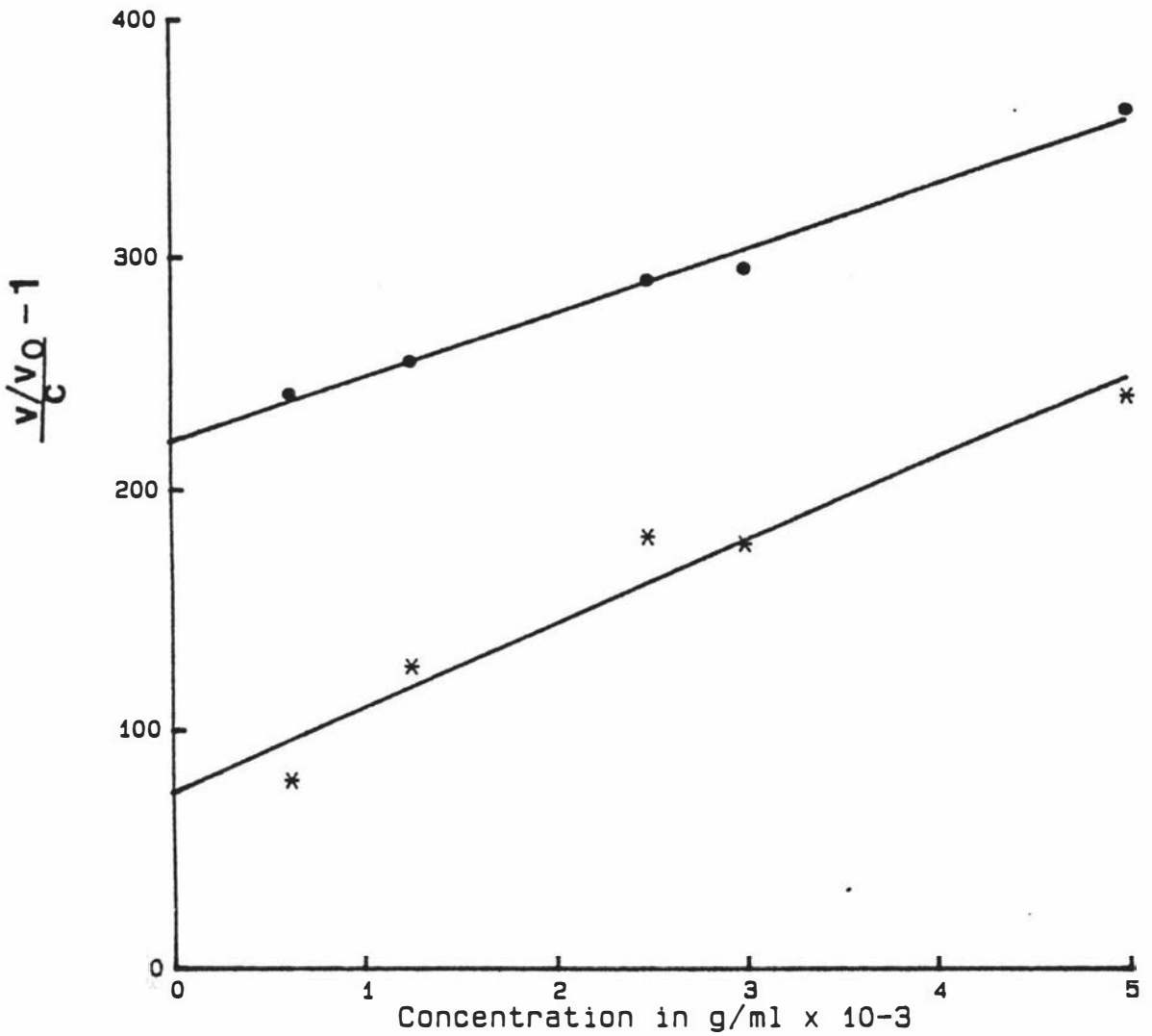


Figure 5:The Viscosity of Wheat Bran Hemicellulose Extracted in Alkali Before (●) and after Delignification (*).

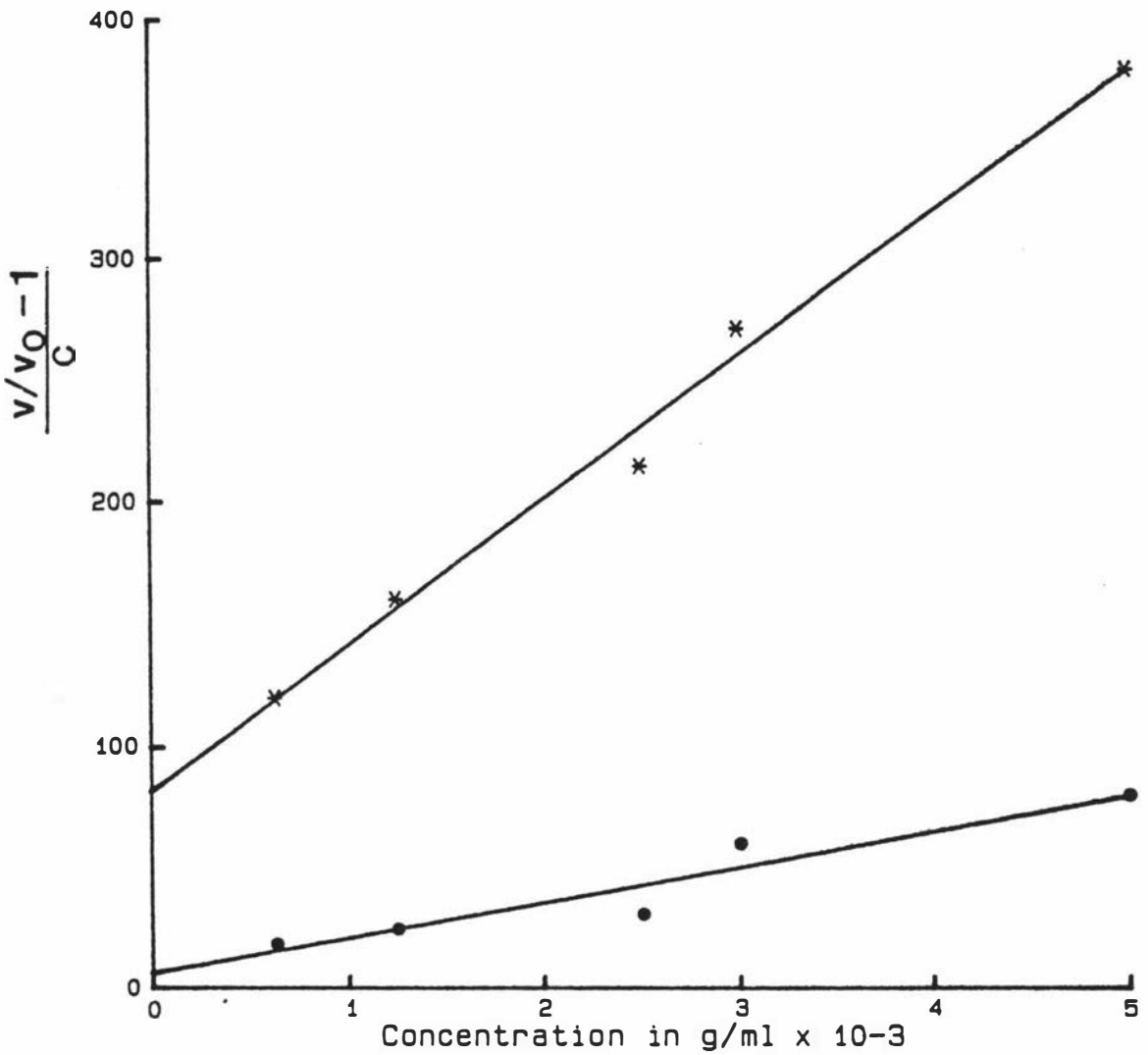


Figure 6: The Viscosity of Wheat Bran Hemicellulose After Solubilisation and Recovery from NMMNO (●) and Control Sample (*)

Table IV

Limiting viscosity number of hemicelluloses before and after delignification and after recovery from solubilisation in N-methyl-morpholine-N-oxide (NMMNO)

Treatment of Hemicellulose	Viscosity Number ml/g
Alkali extraction	220.6
Alkali extraction then delignified	68.3
Prior delignification, alkali extraction	74.2
Alkali extraction, water control	81.9
Alkali extraction, NMMNO solution	6.3

Table V

Relative viscosity of nondelignified hemicellulose in alkali as a function of time

Relative viscosity	Time (minutes)
1.824	25
1.823	35
1.821	45
1.818	55
1.817	85
1.815	95
1.815	120
1.814	165

Discussion

The chemical composition of the polysaccharides isolated from wheat bran using alkali, before and after delignification, showed only small differences in composition as seen in Part I, Table II. The pre and post-delignification hemicelluloses were composed predominantly of xylose 60.1 and 61.8%, and arabinose 31.9 and 29.4% respectively. Table I in Part I shows that the greatest yield of 7.9% was obtained in the extraction prior to delignification and that in the subsequent extraction the yield was 3.8%. Despite the similarity of composition the arabinoxylans differed considerably in their viscosity, as seen in figure 5. The viscosity numbers of the arabinoxylans extracted prior to and after delignification were 220.6 and 74.2 ml/g respectively as shown in Table IV. As the two polymers are of similar composition, it is possible that the arabinoxylan extracted after chlorite delignification may have been degraded as indicated by its lower viscosity. The delignification treatment of the hemicellulose, extracted from wheat bran prior to delignification, showed a similar decrease in viscosity.

The cell walls of wheat bran contain two main types of polysaccharides, arabinoxylans and cellulosic polymers and there is evidence that phenolic ester linkages play a role in holding them together (Ring and Selvendran, 1980). The lignin-hemicellulose ester linkage has been shown to be alkali labile (Wilkie, 1979). Therefore the alkali treatment prior to chlorite delignification would be expected to have cleaved the accessible alkali sensitive linkages between the lignin and arabinoxylan. Extraction of more hemicellulose

requires further delignification. The method generally used for delignification (Wilkie, 1979) is the aqueous acetic acid and sodium chlorite procedure (Whistler and BeMiller, 1963). The requirement for further delignification could indicate that the accessibility of the lignin-hemicellulose linkage to the reagents is prevented by the close packing of the polymers, and that partial degradation of the lignin and/or the hemicellulose is required to open up access to the solubilising reagent. A more likely possibility is that linkages between lignin and hemicellulose contain bonds other than alkali sensitive ester linkages (Smith and Hartley, 1983). Such as isodityrosine, which may form an inter-polypeptide cross-link, possibly being responsible for plant cell wall glycoprotein insolubility (Fry, 1982A). Phenolic components of such as ferulic and P-coumaric acid appear to be associated with water insoluble polymers (Fry, 1982B). The mechanism is probably by forming cross-links to the cell wall protein (Fry, 1982A). Cross-linking of cellulose and other cell wall polysaccharides by 5-keto-D-mannuronic acid in Sphagnum Moss has been reported (Painter, 1983) as have feruloylated pectins in primary cell walls (Fry, 1983). It is possible that the influence of the various means of cross-linking reduce the alkali solubility of the cell wall polysaccharides.

The partial degradation of hemicellulose, as indicated by the reduced viscosity after delignification, suggests that an appropriate starting material for the isolation of hemicelluloses from foods is not the chlorite delignified holocellulose. As hemicellulose can be extracted without prior delignification and in a reasonably high yield

from wheat bran, which has one of the highest lignin contents of human foods (Holloway et al. 1977) delignification appears to be an unnecessary as well as undesirable step in preparation of polysaccharides representative of those present in the parent material. If the chlorite delignification step is used prior to extraction of the arabinoxylan, appropriate material for investigations of molecular properties may not be obtained.

The possible degradation of arabinoxylans extracted using alkali (Aspinall et al. 1961) has not been overcome. Other solvents such as dimethylsulphoxide have been used, however only low yields of hemicellulose have been obtained (Goring and Timell, 1960, Part III of this thesis). The reported use of the new solvent NMMNO (Joseleau et al. 1981) in which cellulose is soluble and stable at elevated temperatures appeared to offer a new method of nondegradative solubilisation of hemicelluloses. However as seen in Table IV the limiting viscosity number of hemicellulose that had been solubilised in NMMNO was only 6.3 ml/g compared with the control sample of 81.9 ml/g. The lower limiting viscosity number of the control sample compared to the hemicellulose that had not been heated (figure 5) suggests that some degradation had occurred, possibly due to the elevated temperature (120°C for 30 minutes), that the procedure recommended by Joseleau et al. (1981) required. However the degradation was not as extensive as for that observed for the sample that had been dissolved in NMMNO. The application of NMMNO as a nondegradative solvent for wheat bran arabinoxylan is not supported by this study. The alkali extraction procedure used to extract

nondelignified wheat bran was found to result in minimal decrease in relative viscosity as seen in Table V, thus supporting the conclusion reached by Aspinall *et al.* (1961) that under the conditions normally employed for the extraction of xylans from plant tissues, alkaline degradation is slow.

It was concluded from the results of the studies described in this part of the thesis, that the least degraded alkali soluble hemicellulose isolated from bran, is that obtained prior to delignification, and that the use of the new solvent N-methyl-morpholine-N-oxide results in extensive hemicellulose degradation. Therefore the hemicelluloses extracted before delignification, obtained in Part I, were used as samples of water insoluble fibres for the studies on metal binding described in Part IV and Part V of this thesis.

PART III

Morphological Changes observed in Wheat Bran after Sequential Extraction with Ethanol, Water, Oxalate and Alkali or Dimethylsulphoxide.

Introduction

Studies using whole foods have been undertaken in attempts to relate advantageous or detrimental properties to their fibre components (Kelsay, 1978). This approach, however, is limited by the complexity of the components and by the plant structures. Some of the components, which may normally be digested, could mask the properties of the fibres in *in vitro* studies.

There have been two approaches to prepare material that would enable scientific studies to be undertaken to substantiate or refute the fibre hypothesis. The first approach, as used in Part I, is to isolate polymeric components from the cell walls of foods and to examine their individual properties. The other approach is to prepare plant material with intact morphological features, such as whole cell walls, and to investigate their *in vitro* and *in vivo* properties and to compare with whole foods and isolated cell wall components.

A method of preparing whole cell walls from wheat endosperm using a sequence of grinding, amylase and ethanol extraction was described by Mares and Stone (1973). The isolation and ultrastructure of aleurone cell walls has also been described by Bacic and Stone (1981A). The material isolated in these studies were, however, not used to

investigate the properties of fibre. The isolation of cell wall material from ground cabbage by sequential extraction with ethanol, treatment with pronase, followed by extraction with phenolic-acetic acid-water and DMSO was described by Stevens and Selvendran (1980). The same method was also used to prepare cell wall material from wheat bran (Ring and Selvendran, 1980). The cell wall material produced from these studies were used for clinical trials (Stephen and Cummings, 1980).

The importance of wheat bran because of its major contribution to the dietary fibre intake of most Western nations (Trowell, 1973) suggests that studies using this material may be the most appropriate for investigating the fibre hypothesis.

The composition of wheat bran has been reviewed by Saunders (1978) and its anatomy by Esau (1965). The main morphological features of wheat bran are shown in Figure 7. The fibrous properties of plant material have been reviewed by Setterfield and Bayley (1961). However a clear distinction between botanical fibre and dietary fibre has, as yet, not been established. Studies of cell walls, their structure and changes that occur when extracted, may assist in this distinction being made.

The studies described in Part III were undertaken to observe:

(1) The morphological structure of commercially ground wheat bran.
(2) To observe changes in morphological structure after wheat bran has been subjected to ethanol, oxalate and alkali extractions.
(3) To observe the influence on wheat bran, of DMSO when substituted

for alkali extraction.

(4) To determine the chemical composition of alkali and DMSO extracted polysaccharides.

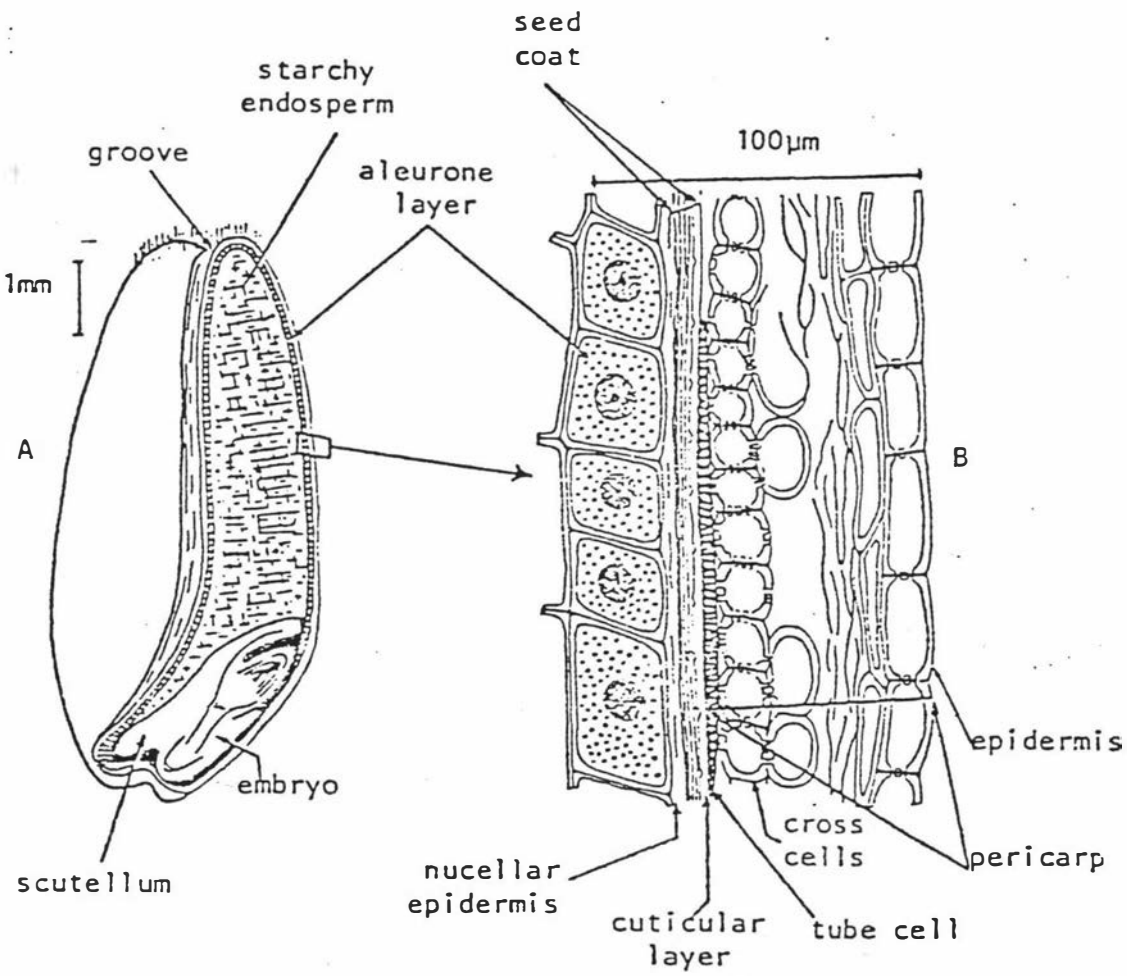


Figure 7: Caryopsis (A) of wheat (Triticum aestivum) and parts of its pericarp in longitudinal section (B) from Esau, 1965.

Materials and Methods

Samples[1] of wheat bran before and after extraction in ethanol (reflux) hot water, amylase, ammonium oxalate and alkali, before and after chlorite delignification, as indicated in figure 2, were dried in a dessicator over phosphorus pentoxide to constant weight.

Dimethylsulphoxide extraction procedure: To bran ex-oxalate[2] insoluble material (138 g) was added DMSO (400 ml). The suspension was mixed for five hours on a mechanical shaker, allowed to stand for 17 hours at room temperature, decanted and filtered through a number two sintered glass filter. The insoluble material was washed three times with DMSO (100 ml). The solutions were pooled, then added to four volumes of 95% ethanol. The resultant precipitate was collected, dialysed for 12 hours against running water, then freeze-dried. The DMSO insoluble material was then delignified using chloramine-T (8 g) and glacial acetic acid (5 ml) added (Gaillard, 1958). After mixing for two hours it was decanted, and the delignification procedure repeated on the insoluble material. The decanted liquor was discarded. DMSO (500 ml) was added to the insoluble material, the suspension was mixed for five hours on a mechanical shaker, then allowed to stand for 48 hours. The supernatant was separated and added to four volumes of 95% ethanol. The resultant precipitate was dialysed for 12 hours against water and freeze-dried and weighed. The DMSO extraction procedure is shown in figure 8.

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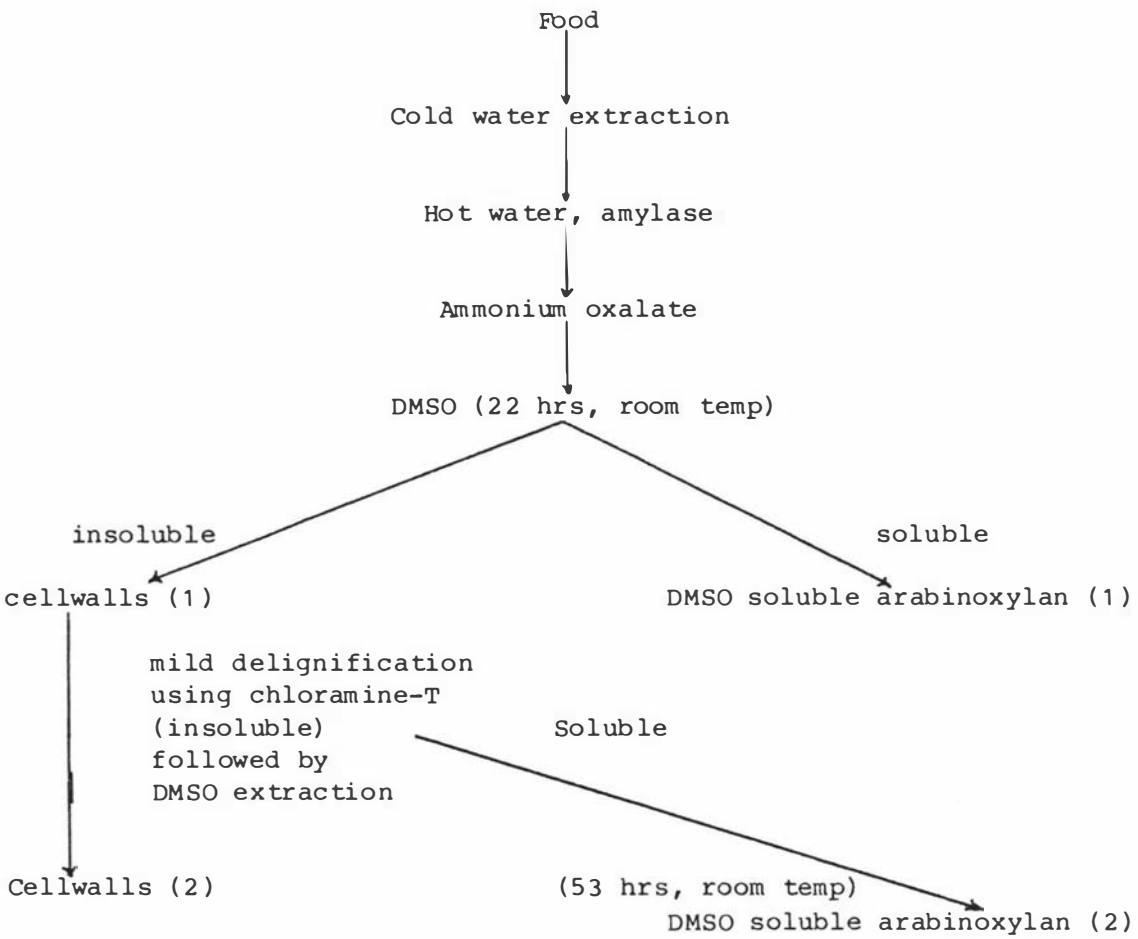
[1]All experiments were carried out in duplicate

[2]Insoluble residue after extraction with ammonium oxalate

Figure 8: DMSO cell wall preparation procedure



The chemical composition of the extract was determined by GLC and the colorimetric procedures as previously used in Part I of this thesis.

Ferulic acid determination

The ferulic acid content of the extracted polymers was determined by a modification of the procedure of Hartley (1971). Samples (0.2 g) were oxidised with nitrobenzene (1.0 ml) and 2m sodium hydroxide (10 ml) in screw-capped stainless steel tubes (25 ml capacity in 304 stainless steel). The tubes were heated (160 ± 2°C) for three hours

then the contents filtered through number one sintered glass filters, washed with water (10 ml) then with peroxide-free ether (20 ml). The filtrate and washings were then extracted with ether (2 x 50 ml) and the aqueous layer was then acidified with concentrated hydrochloric acid to pH2.5. Solid sodium chloride was added to saturate the solution before extraction with ether (2 x 60 ml). The combined ether solutions were dried (anhydrous sodium sulphate) and the solvent evaporated. The residue was dissolved in acetone (1.0 ml).

The phenolics were separated by the thin layer chromatography on Silicagel-G plates using toluene:methanol:acetic acid (79:14:7) as the solvent (Brand, 1966). Ferulic acid standards (2 mg/ml) and samples were quantitated using a Shimaduz Dual-Wavelength Chromatograph Scanner, with reference at 556 nm and the sample at 366 nm using the procedure as described by Yamamoto et al. (1976).

Light microscopy

Dried samples of all the insoluble materials were embedded in resin before sectioning for light microscopy. All the light micrographs were at 380 magnification.

Scanning electron microscopy: The freeze-dried samples at various stages of the extraction sequence (figure 2) were separately glued on to an electron microscope stub by sprinkling on to a thin layer of conductive silver paint or doubled sided tape. The loose particles were blown away, then the stub was sputter coated with 100-200Å of gold in a Polaron E5100 sputter coater.

Scanning electron microscopy of the samples was carried out on a CWIKSCAN 100 field emission scanning electron microscope operated at 16 KV. Micrographs were obtained at 40, 200, 400, 800 and 12,000 magnification using Ilford PF4 film at 16 seconds exposure time.

Results

Light microscopy of wheat bran enabled the starch granules, aleurone layer, cuticular layer, intact pericarp with tube cells, cross cells and epidermis to be observed as seen in figure 9A.

Figure 9B shows that after refluxing in ethanol the starch granules are considerably reduced in amount. The contents of the aleurone cells are also reduced in volume and the nuclei of the aleurone cells are more easily observed. The pericarp is now more compressed.

Figure 9C shows the bran after sequential extraction with cold water, hot water, amylase and hot ammonium oxalate. The starchy endosperm is no longer evident. Some of the contents within the aleurone layer still remain. The cuticular layer and some of the cellular elements of the pericarp are also still intact. The epidermis however, is no longer attached.

Figure 9D shows bran which has been treated with alkali. The cellular structure of the aleurone layer is no longer identifiable, the cellular structure of the pericarp does however remain.

Figure 10A shows scanning electron microscopy of ground wheat bran with starch granules.

Figure 10B shows a more 'open' structure, possibly the epidermis removed, after ethanol reflux and a preponderance of starch granules.

Figure 10C shows the structure of bran after water, amylase and oxalate treatment. At this stage of the extraction sequence most of the cell contents appear to have been removed.

Figure 10D shows that after treatment with sodium hydroxide no cell walls remained, but only a nondescript striated material.

Figure 11A shows the material remaining when DMSO was substituted for alkali. The cell walls of the aleurone layer, the cellular elements of the pericarp and the cutical layer remain.

Figure 11B shows that after delignification using chloramine-T and repeated DMSO extraction that there was minimal detectable change in the structure observed.

Figure 12A shows the micrograph of the bran after DMSO extraction. There appears to be only cell wall material present.

Figures 12B and C show that after mild delignification and repeated DMSO extraction minimal structural change has occurred except that the height of the cell walls appear to be diminished.

Figure 12D shows the cell walls of wheat bran at 4,000 magnification.

The yields in percent dry weight of wheat bran of the DMSO soluble extract and for comparison the alkali extracts, are shown in Table VI.

The composition of the nondelignified alkali and DMSO extracted polymers are shown in Table VII. The standard procedure when undertaking a microscopy study is to take a large number of photomicrographs. From these a representative selection is made. This procedure was adopted in this present study and therefore the results presented are typical of the tissues examined.

Light Microscopy

Figure 9A: Commercially ground wheat bran at 380 magnification

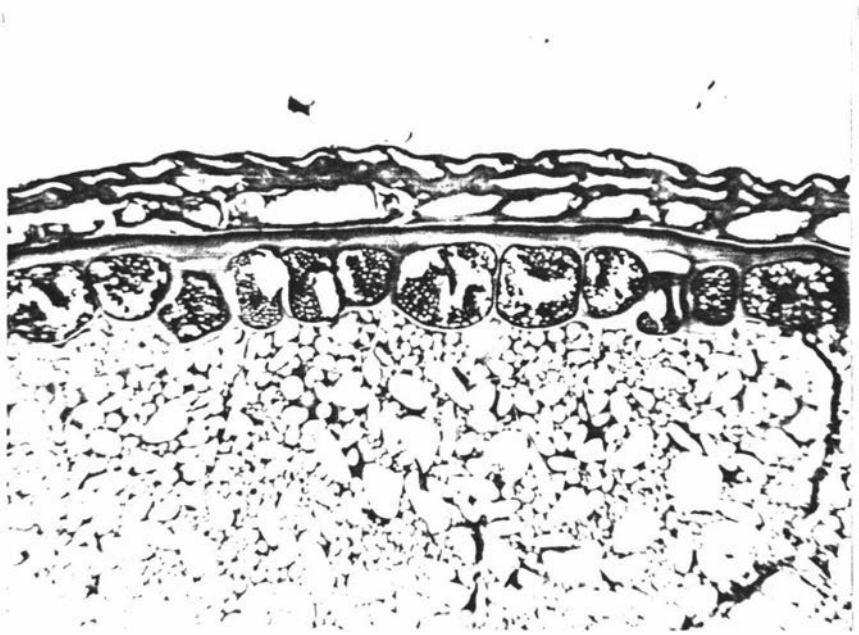
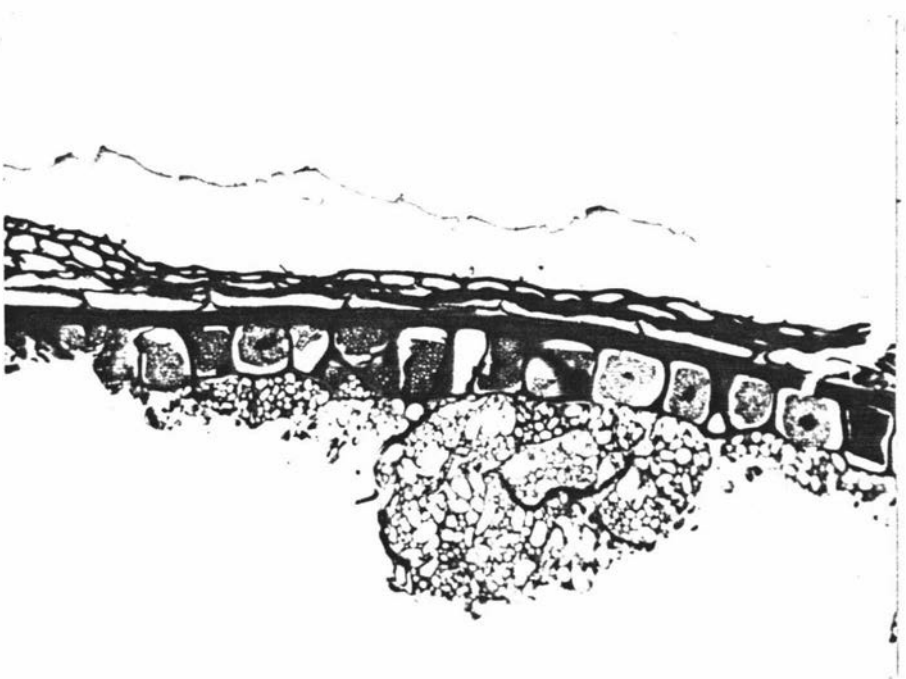


Figure 9B: Wheat bran ex ethanol at 380 magnification



Light Microscopy

Figure 9C: Wheat bran ex oxalate at 380 magnification

Figure 9D: Wheat bran ex sodium hydroxide at 380 magnification

Scanning Electron Microscopy

Figure 10A: Commercially ground wheat bran at 2000 magnification

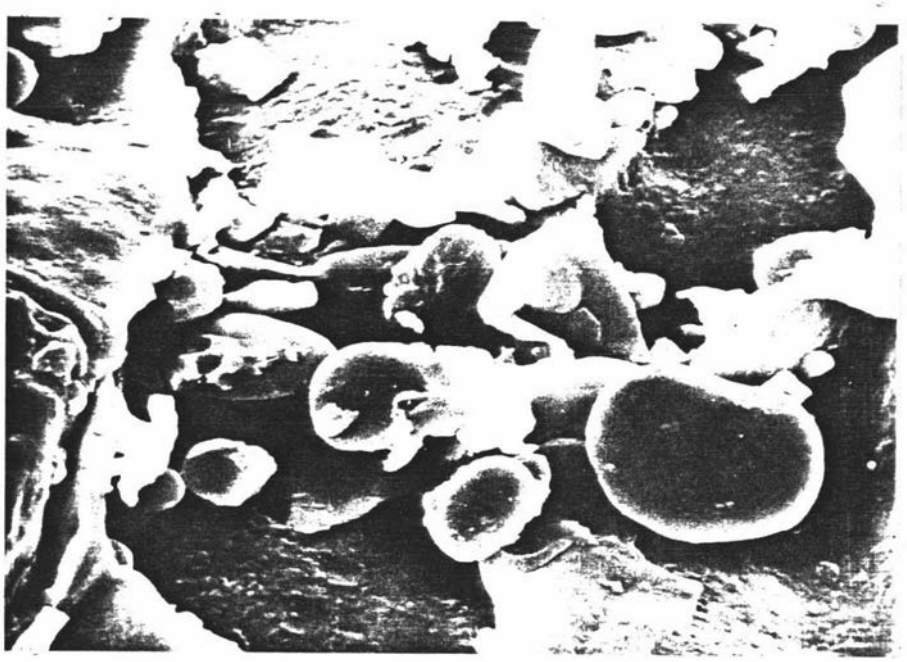
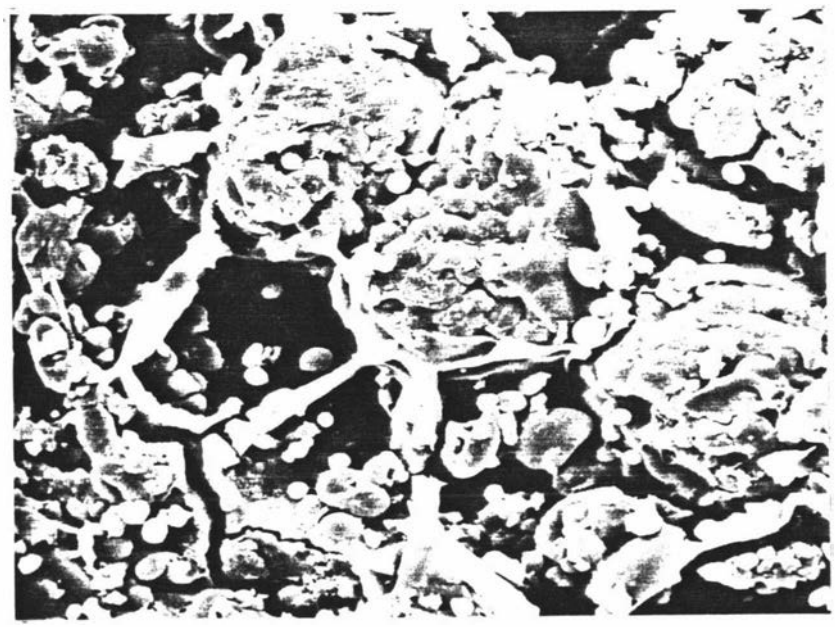


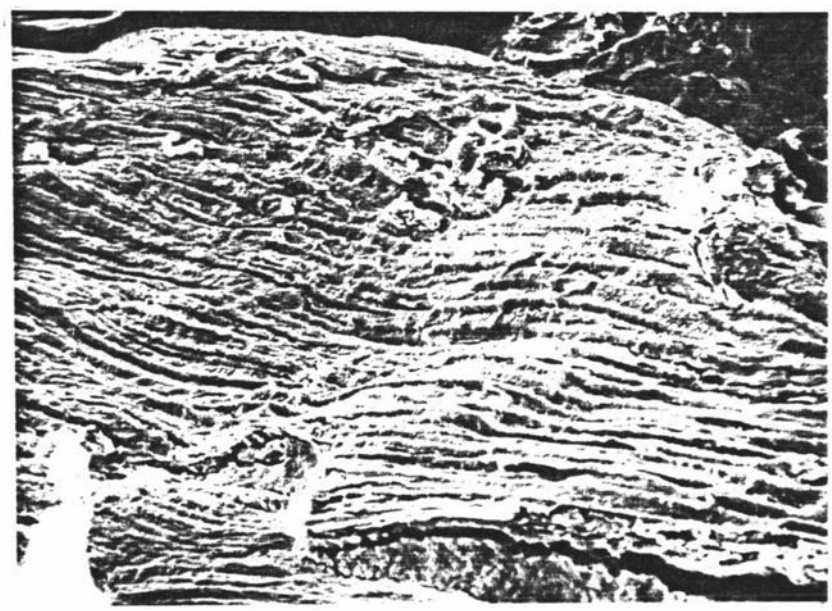
Figure 10B: Wheat bran ex ethanol at 800 magnification



Scanning Electron Microscopy

Figure 10C: Wheat bran ex oxalate at 400 magnification

Figure 10D: Wheat bran ex alkali at 400 magnification



Light Microscopy

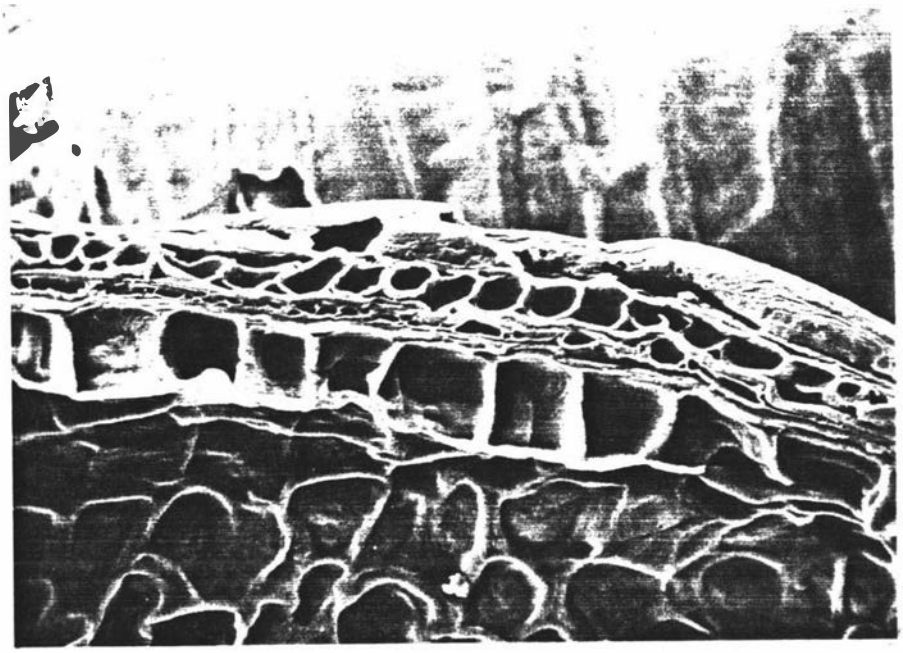
Figure 11A: Wheat bran ex DMSO at 380 magnification

Figure 11B: Wheat bran ex chloramine-T, DMSO at 380 magnification

Scanning Electron Microscopy

Figure 12A: Wheat bran ex DMSO at 400 magnification

Figure 12B: Wheat bran ex DMSO at 400 magnification



Scanning Electron Microscopy

Figure 12C: Wheat bran ex chloramine-T, DMSO at 400 magnification

Figure 12D: Wheat bran ex chloramine-T, DMSO at 4,000 magnification

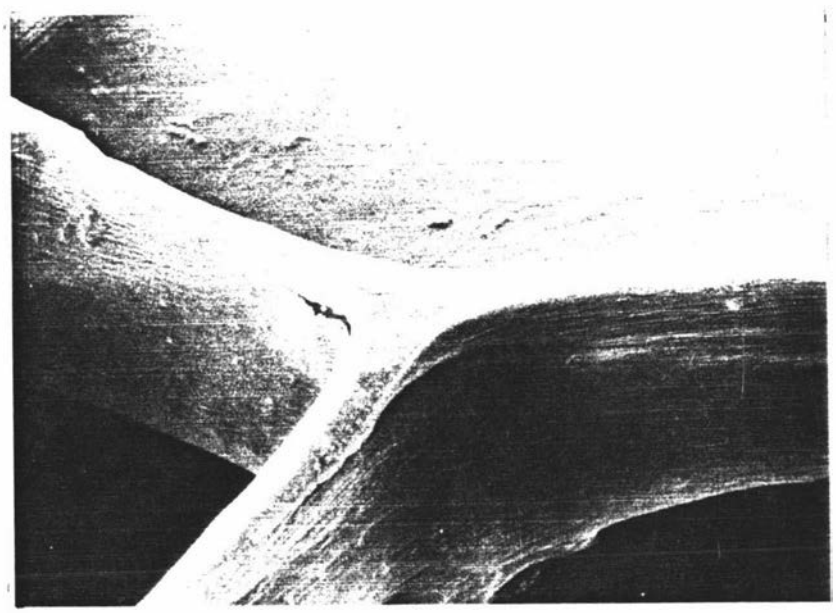


Table VI.

Yields of hemicellulose obtained by alkali and DMSO extraction from wheat bran

Extraction Reagent	Yield % Dry Weight
Sodium hydroxide before delignification	7.4
Sodium hydroxide after delignification	3.8
DMSO before delignification	0.4
DMSO after delignification	0.8

Table VII

Composition of nondelignified hemicellulose extracted from wheat bran with alkali and DMSO (expressed as % of the analysed components)

Extraction Reagent	Rhamnose	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acid	Methoxyl	Acetyl	Protein	Ferulic acid
Alkali	0	31.9	60.1	0.3	2.1	4.0	0.7	0.1	0.1	0.5	12.0
DMSO	0	26.3	51.9	0.4	0.8	16.1	1.1	0.2	1.6	1.6	14.0

Discussion

This study of the insoluble portions of bran has enabled the influence of the major steps of the extraction sequence used in Part I to be observed.

In the commercial sample of wheat bran the predominant features observed were the cellular components and the starchy endosperm. The morphological structures can be identified as described in figure 7. Refluxing in ethanol appeared to compress the pericarp, possibly by removing tightly bound water, and reduction of the amount of starchy endosperm granules was observed. After treatment with water, amylase and ammonium oxalate, the starch had been removed, confirming the negative results of the KI-I_2 spot test. Some of the cell contents of the aleurone layer and the cellular elements of the pericarp remain at this stage of the extraction sequence. The epidermis, is however, no longer attached. After alkali extraction it was not possible to identify any cell structure of the aleurone layer. Thus the alkali treatment appeared to have removed cell wall material predominantly from the aleurone layer. This observation suggests that the alkali soluble cell wall polysaccharides of the aleurone are less strongly bound, possibly due to a lower lignin/phenolic content.

The substitution of alkali by DMSO resulted in the cell wall structures being retained. The cellular contents, however, appear to be reduced. After mild delignification using chloramine-T and re-extraction in DMSO the cell walls of the aleurone, tube and cross cells appear to be the only structural elements remaining.

The procedure using DMSO to prepare cell walls as reported in this thesis, and the procedures used by Mares and Stone (1973), Bacic and Stone (1981A) and by Selvandran *et al.* (1980) are difficult to compare, as the results of the different methods have not been subjected to the same evaluation procedures by scanning electron microscopy of the cell walls. However Bacic and Stone's (1981A) study did show a high magnification electromicrograph of striated cell walls of similar appearance to those shown in Figure 12D. The chemical composition of the cell walls has however been reported (Mares and Stone, 1973; Ring and Selvendran, 1980; Bacic and Stone 1981B). There is general agreement that the cell walls of bran are composed predominantly of arabinoxylans and cellulose.

The low yields of the DMSO extracted arabinoxylans (0.4 and 0.8% of the dry weight of bran) support the observation that cell walls of wheat bran are predominantly retained in DMSO extraction. This is in contrast to the disintegration of the cell walls in alkali, when 7.4% of the bran dry weight is removed, prior to delignification.

The two polymers, extracted by alkali and DMSO, respectively, are similar, with respect to monosaccharide, protein and ferulic acid content, both polymers being composed predominantly of arabinose and xylose (Table VII). There was however small differences, in that the DMSO extract had lower levels of arabinose, xylose and galactose and higher levels of glucose, acetyl, ferulic acid and protein than the alkali extract. The relative acetyl contents of the DMSO and alkali extracts are consistent with the findings of Timell (1960) that alkali extraction reduces the acetyl content of polysaccharides. The lower

yield of the DMSO soluble polysaccharide is probably the result of noncleavage of the alkali sensitive bonds.

Aspinall (1982) stated that it is extremely unlikely that different methods of isolation result in the same polymer being extracted from the complex of cell walls. The results obtained in this study would confirm that observation.

The significance of the small differences in composition in the different preparations of fibres is not known. Samples of the various fibre preparations and the cell walls obtained in these studies were used to investigate relationships between the morphological and chemical structures and the binding of zinc is described in Part V of this thesis.

PART IV

Metal adsorption to the water soluble and water insoluble fibres[3] from fruits, vegetables, bran and grasses

Introduction

The presence of a component or of a nutrient in a particular food or diet does not necessarily indicate its bioavailability (O'Dell, 1969). The traditional analysis of food contents tabulated in Food Tables gives no indication of the availability of nutrients present.

There is some evidence that humans on marginal mineral intakes may become deficient in some essential elements (Kelsay et al. 1979), particularly after prolonged consumption of unrefined foods (Ismail-Beigi et al. 1977). The most consistent finding on the effects of high intakes of unrefined plant foods on mineral bioavailability has been the reduction in calcium (McCance et al. 1942; Ismail-Beigi et al. 1977), iron (Jenkins et al. 1975) and zinc absorption (Davies and Nightingale, 1975; Reinhold et al. 1974).

The metal binding capacity of dietary fibre has been reviewed by Rasper (1982), Staub, et al. (1983), Royal College of Physicians (1980) and Kelsay (1978). However, the relative importance of different types of fibres, the mechanisms and sites of metal binding has not been completely elucidated.

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[3] noncellulosic, non starch polysaccharides of edible plants isolated by the procedures described in Part I.

In mixed diets, minerals are obtained from both plant and animal foods. As undigested plant material passes through the small intestine, bound minerals may be unavailable for absorption into the small intestine. If the binding capacity of the plant material is unsaturated, then the excess binding capacity would appear to increase the likelihood of minerals from other food sources being complexed, thereby rendering them unavailable for absorption.

The location of minerals in edible matter from plants is often different than in meat. Minerals in plants are often closely associated with the plant cell wall and may be concerned with the structural integrity of the polysaccharides, as in the stabilisation of the pectin structure by calcium (Rees, 1977). Weak binding of calcium ions has been observed to occur to monomeric uronates, the calcium binding properties of the polyuronates are therefore assumed to be due to their polymeric nature, and the differences between them to be caused by differences in their sterochemistry (Kohn et al., 1968). It has been suggested that the steric arrangement of the fixed groups on the polymer should be more important with multivalent cations than with monovalent cations (Haug and Smidsrød 1970).

Bound metals may also occur on other cell wall constituents. The carboxyl groups of lignin have been suggested to be the sites of magnesium binding (Jones, 1978). Morris and Ellis (1976) reported that iron is bound to phytate in wheat bran. Cereals also contain high concentrations of other elements (Steele, 1981). The relative importance of metal binding by different classes of polysaccharides extracted from different foods does not appear to have been established.

This study was undertaken to determine the metal content of water soluble and insoluble fibres from fruits, vegetables, bran and grasses and to determine their interaction with a range of metals that may be present in the region of the intestinal tract where competition between fibres and intestinal absorption would occur.

Materials and Methods

Cold water soluble and water insoluble fibres extracted as described in Part I, of known composition (Table II), were used for the binding studies reported in this part of the thesis.

Standard Zinc Solution

A series of zinc chloride solutions (0.25 g $ZnCl_2$/l) were made up in sodium acetate - and sodium barbital buffer (3.886 g sodium acetate and 5.886 g sodium barbital/l) with pH's of 8.30, 6.92, 6.08, 4.05, 3.15 and 2.12 obtained by the addition of 0.1 M hydrochloric acid. Also a series of zinc chloride solutions containing 8.0 µg/ml of zinc in sodium acetate/sodium barbital buffer at pH 5.5 with varying amounts of glucose (1, 2.5, 5.0 g/100 ml) and sodium chloride (0.45, 0.9 g/100 ml) were prepared. The osmolality of these solutions was determined using an Advanced Instruments Osmometer (USA).

Stock Mineral Solution

A stock solution containing (per litre): sodium chloride (8.168 g), potassium chloride (0.38 g), calcium chloride (0.783 g), magnesium chloride (0.242 g), ferrous ammonium sulphate (0.358 g), zinc chloride (0.025 g), cupric chloride (0.002 g) and manganese

chloride (0.001 g) in sodium acetate/sodium barbital/hydrochloric acid (352.0 ml of 0.1 m HCl) buffer at pH 5.5 was made. The concentration of the metals used for this solution were calculated to be approximately the same as would be expected to occur in the region of the intestinal tract (small bowel, proximal jejunum) where competition between fibre adsorption and intestinal absorption for metals from a 'typical' meal would occur. The concentrations were based on the measurements by Fordtran et al. (1966) of the metals present in a 'typical' meal and at various regions of the intestinal tract. The magnesium levels were calculated from Guthrie (1975). This metal solution was used as a 'standard' solution for the multiple metal binding studies.

Binding Procedure

The procedure used for the binding studies to separate the bound and free metal species made use of six Amicon MPS-1 micropartition systems (Amicon, 1980). The apparatus is shown in Figure 13. It consists of a reservoir cap, sample reservoir, o-ring, YMT membranes, clips, membrane support base and filtrate cup.

The water soluble and insoluble fibres (0.01 g) were individually weighed into an assembled MPS-1 filtration apparatus, and the metal containing solution (1.0 ml) was then added. The apparatus was capped and initially mixed by hand for approximately half a minute, and then mixed on a vertical rotating plate (60 RPM) for one hour (in the timed mixing experiment, mixing times were varied from 0.5 to 180 minutes). After mixing, the MPS-1 systems were centrifuged using a

fixed angle (34°) SS-34 rotor at 1000 RCF (4,000 RPM) on a Sorvall centrifuge. The filtrates (0.5 to 0.1 ml) were transferred to volumetric flasks (5.0 to 100.0 ml), hydrochloric acid (2M, 4.5 ml) added, and then made up to volume with distilled deionised water. Water only was used for the zinc solutions.

The percentage of added zinc bound to wheat bran hemicellulose at pH's of 2.0, 3.15, 4.05, 5.05, 6.05, 6.90 and 8.45; times of mixing of 0.5, 1, 2, 3, 4, 5, 15, 25, 60, 120 and 180 minutes; and the influence of osmalality changes with glucose and sodium chloride from 0 to 298 mOsm/kg, were determined using the various zinc solutions and the MPS-1 filtration apparatus.

The percent retention of the 'stock' metal solution by YMT membranes was determined in duplicate and the reliability of the analysis of the 'stock' solution determined on ten samples.

The binding of metals from the 'stock' solution to the fibres were determined in duplicate with individual samples of 0.1 g fibre and 1.0 ml of metal solution. The water insoluble fibre from ryegrass was also washed with hydrochloric acid (0.1 M) for five minutes, then washed with distilled deionised water until neutral, before binding with the 'stock' solution.

The nondialysable metal content of the fibres were determined in the same way as the metal binding, except the 'stock' solution was replaced by distilled deionised water.

Analyses

Zinc Content

The zinc content of the solutions were determined by atomic absorption spectrometry (Steele, 1981). The sodium, potassium, calcium, magnesium, iron, zinc, copper and manganese levels of the 'stock' solution used for each run of the binding studies and their filtrates were determined by Inductively Coupled Argon Plasma (ICP) emission spectroscopy (Lee, 1981).

Metal Content

The metal contents of the fibres were determined by adding concentrated (A.R. Grade) nitric acid (5.0 ml) to the individual fibres (0.1 to 0.05 g). After standing for 18 hours the nitric acid was removed by evaporation. The nearly dry residues were then dissolved in hydrochloric acid (2.0 M) and quantitatively transferred to 5.0 ml volumetric flasks. The metal contents were determined by ICP.

Metal Binding Capacity

The metal binding capacities of the fibres were calculated as the sum of the metal content of the fibres and the amount bound from the 'stock' solution. The total binding capacities were calculated as the sum of copper, iron, zinc, calcium, potassium, magnesium, manganese and sodium binding capacity.

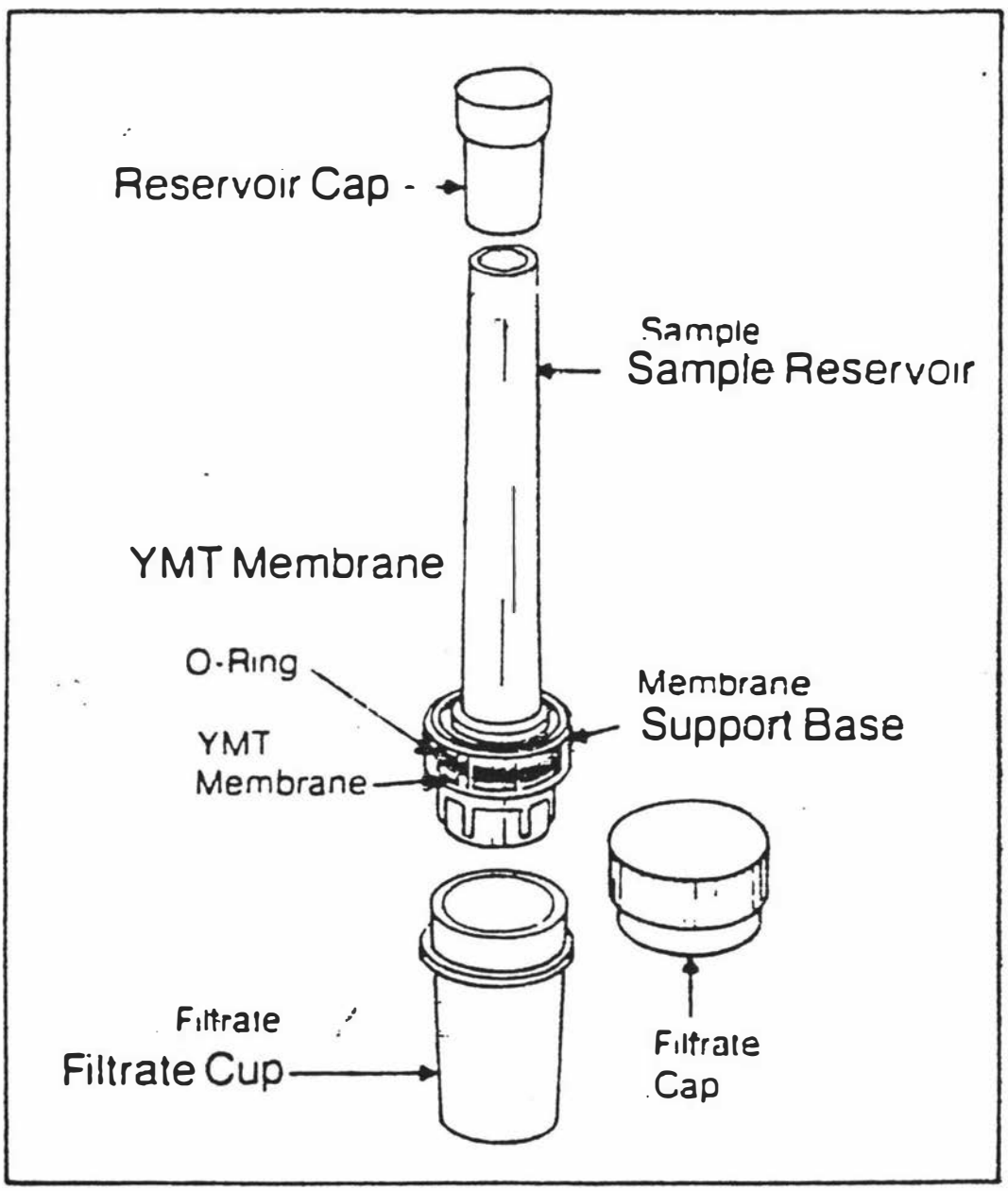


Figure 13: Amicon MPS-1 micropartition unit

Results

Figure 14 shows the percentage of zinc bound to wheat bran hemicellulose over a pH range from 2.12 to 8.30, the maximum zinc binding occurring between pH 5 and 6.

Figure 15 shows the percentage of zinc bound to wheat bran hemicellulose with different mixing times, the maximum zinc binding occurring at less than 0.5 of a minute.

Table VIII compares the influence of osmolality on the binding of zinc to wheat bran hemicellulose.

Table IX shows results of the reliability study of 10 analyses of the metals in the solution used before and after binding to wheat bran.

Table X compares the percentage retention of metals by the Amicon MPS-1 micropartition system by YMT membranes.

Table XI shows the metal content (µg/mg dry weight) of water soluble and water insoluble fibres from fruits, vegetables, bran and grasses.

Table XII shows the mean metal content (µg/mg dry weight) of water soluble and water insoluble fibres from fruits, vegetables, bran and grasses.

Table XIII shows the changes in metal content (µg/mg dry weight) of the water soluble and insoluble fibres from fruits, vegetables, bran and grasses after mixing with 'stock' metal solution.

Table XIV shows the bound metal content (µg/mg dry weight) of water soluble and insoluble fibres from fruit, vegetables, wheat bran and grasses after mixing with a 'stock' metal solution.

Table XV shows the mean bound metal content (µg/mg dry weight) of water soluble and insoluble fibres from fruits, vegetables, bran and grasses.

Table XVI shows the mean, standard deviation (µg/mg) and T values of bound metals from matched paired fibres, before and after mixing with 'stock' metal solution.

Table XVII shows the percentage nondialysable metal content of the water soluble fibres and insoluble fibres.

Table XVIII shows the total ion-binding capacity (mM/g dry weight) of water soluble and insoluble fibres from fruits, vegetables, wheat bran and grasses.

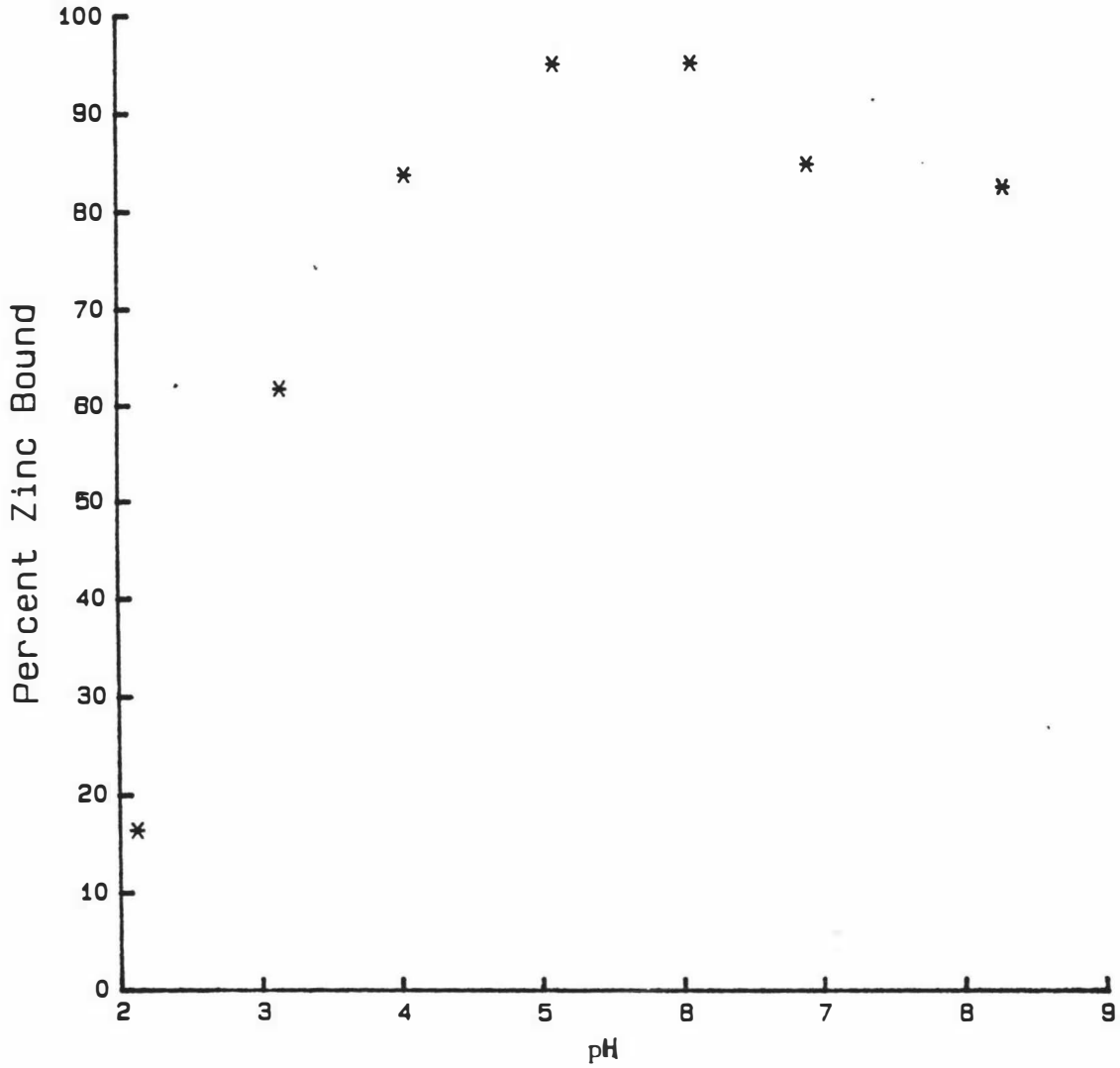


Figure 14: Percent Zinc Bound to Wheat Bran Hemicellulose Over a ph Range from 2.12 to 8.3.

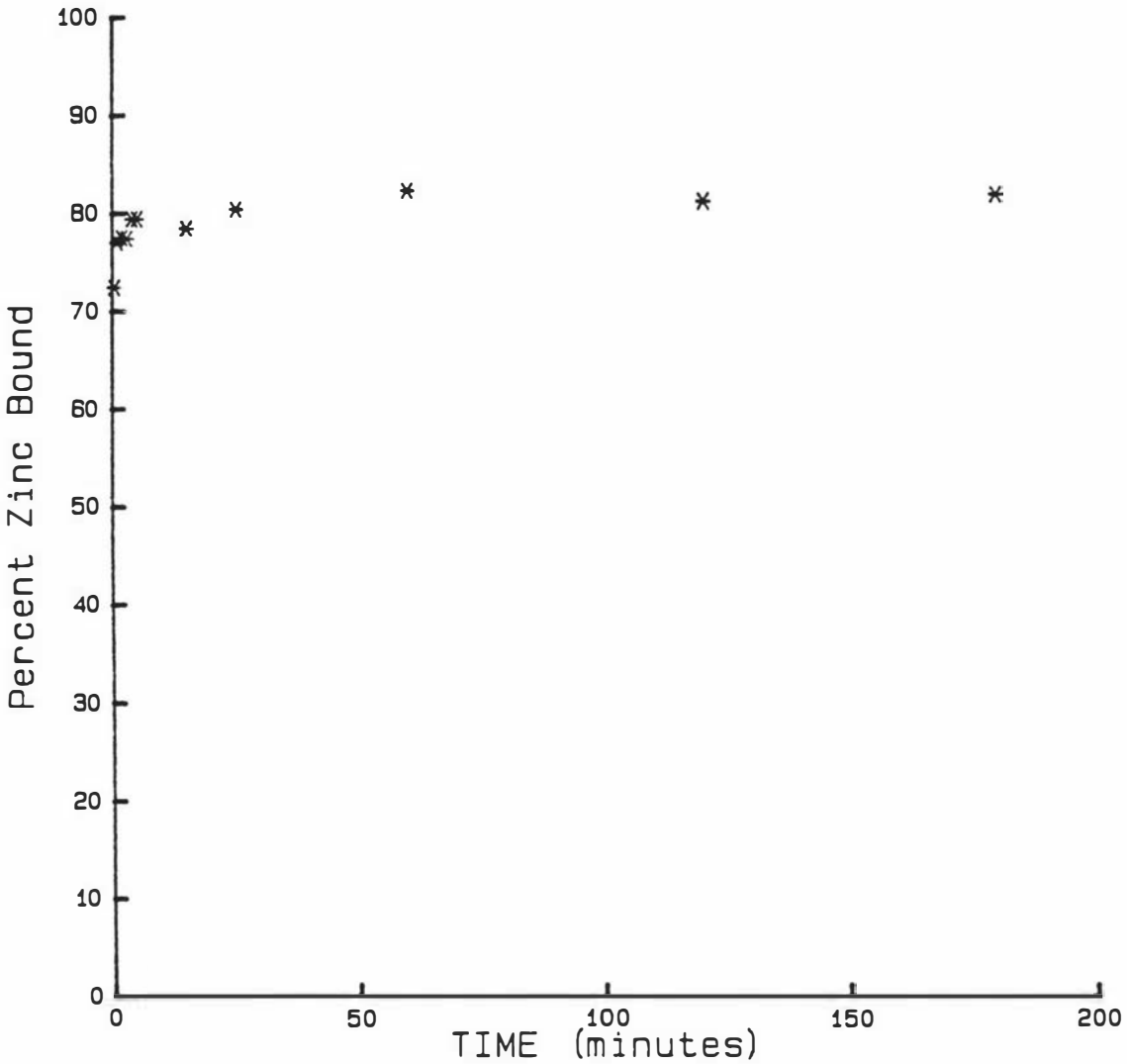


Figure 15: Percent Zinc Bound to Wheat Bran Hemicellulose With Different Times of Mixing.

Table VIII

Influence of osmolality of glucose and sodium chloride on the adsorption of zinc (µg/ml) to wheat bran hemicellulose at pH 5.5.

Solution	Osmolality mOsm/kg	Total zinc	Free zinc	Bound zinc	% zinc bound
glucose %					
0	10	7.8	1.5	6.3	80.3
1%	60	7.7	1.5	6.2	80.1
2.5	149	7.8	1.5	6.3	81.4
5.0	298	7.0	1.3	5.7	81.0
% NaCl					
0	10	7.8	1.5	6.3	80.3
0.45	155	8.4	0.4	8.0	95.2
0.9	289	8.6	0.4	8.3	95.7

Table IX

Reliability of metal binding analysis using standard solution and after mixing with wheat bran

µg/ml		Mean (n=10) metal content (µg/ml)							
		Calcium	Copper	Iron	Potassium	Magnesium	Manganese	Sodium	Zinc
Standard solution	Mean	196.1	1.7	52.0	215.9	29.8	1.5	4838.0	12.7
	S.D.	6.5	0.1	1.2	5.6	1.2	0.2	56.3	0.5
	C.V.	3.3	6.4	2.3	2.6	4.1	15.5	1.2	4.1
Filtrate(mean of ten determinations) after mixing with wheat bran: (1 ml:0.05 g):	Mean	172.5	2.0	19.7	325.4	64.5	3.6	4841.3	3.7
	S.D.	9.4	0.3	1.5	11.3	2.8	0.6	67.8	0.5
	C.V.	5.4	15.5	7.4	3.5	4.3	16.3	1.4	12.3

S.D. = Standard deviation
C.V. = Coefficient of variation

Table X

Total metal content of test solution and amount as percentage retention of metals by Amicon MPS - 1 micropartition system with YMT membranes

	Calcium	Copper	Iron	Potassium	Magnesium	Manganese	Sodium	Zinc
Total metal content µg/ml	196.1	1.7	52.0	215.9	29.8	1.5	4838.0	12.7
Bound metal content µg/ml	2.9	0.3	1.5	3.0	0.3	0.2	83.7	1.1
% Bound	1.5	15.9	2.9	1.4	0.9	11.1	1.7	8.4

Table XI

Metal content (µg/mg dry weight) of water soluble (SF) and insoluble (IF) fibres from fruits, vegetables, bran and grasses.

Fibre Source		Copper	Iron	Zinc	Calcium	Potassium	Magnesium	Manganese	Sodium
Bean	SF	0.3	5.7	1.2	34.6	5.2	4.2	0.4	12.7
	IF	0.1	9.2	0.7	132.2	0.3	0.3	0.1	3.0
Cabbage	SF	0.6	23.1	4.5	106.5	1.2	5.0	1.5	6.7
	IF	0.2	8.3	0.5	126.4	0.3	0.5	0.1	3.4
Kumera	SF	0.6	15.6	2.8	57.6	7.6	5.2	0.2	21.4
	IF	0.2	3.3	0.5	26.6	1.8	2.4	0.1	29.0
Lettuce	SF	0.2	3.3	0.7	44.9	6.8	5.8	0.3	12.2
	IF	0.3	12.0	1.5	137.3	0.3	2.4	0.3	2.1
Onion	SF	0.4	2.3	1.8	72.4	5.4	6.7	0.6	11.3
	IF	0.2	22.3	1.2	119.5	0.3	0.4	0.1	4.3
Peach	SF	0.5	12.5	3.3	14.9	4.8	1.8	0.4	31.1
	IF	0.4	17.4	1.5	203.2	0.6	0.9	0.2	4.5
Pear	SF	0.7	11.4	4.0	24.4	12.1	3.0	0.2	15.2
	IF	0.2	23.8	1.5	96.3	0.5	0.5	0.1	4.4
Pumpkin	SF	0.3	5.3	1.3	30.8	6.6	4.0	0.2	17.5
	IF	0.2	33.2	1.1	118.1	0.3	0.5	0.2	3.8
Tomato	SF	0.2	3.3	0.7	44.1	2.2	3.5	0.1	6.6
	IF	0.2	15.5	2.0	142.8	0.4	0.5	0.2	2.9
Wheat bran	SF	0.1	1.2	0.2	1.0	1.0	1.0	0.1	3.6
	IF	0.2	15.8	0.1	7.8	2.8	0.1	0.0	4.8
White clover	SF	0.5	7.9	1.3	84.0	5.1	6.6	0.3	13.8
	IF	0.2	3.4	0.7	46.3	3.2	4.2	0.1	31.7
Lucerne	SF	0.4	6.2	1.0	79.5	4.8	5.2	0.3	12.1
	IF	0.1	2.8	0.5	52.5	1.6	2.3	0.1	6.6
Ryegrass	SF	0.8	7.0	1.2	34.0	6.3	4.0	0.4	12.8
	IF	0.1	2.2	0.3	36.0	1.2	1.7	0.1	5.3

Table XII

Mean metal content (µg/mg dry weight) of fruit, vegetable, wheat bran and grass fibres

Fibre type	Copper	Iron	Zinc	Calcium	Potassium	Magnesium	Manganese	Sodium
Water soluble fibre	0.4±0.2	8.1±6.2	1.9±1.8	48.4±30.2	5.3±2.9	4.3±1.7	0.4±0.3	13.6±7.0
Insoluble fibre	0.2±0.1	13.0±9.5	0.9±0.6	95.8±57.2	1.0±1.0	1.3±1.2	0.1±0.1	8.1±9.9

Table XIII

Changes in metal content (µg/mg dry weight) of water soluble (SF) and insoluble fibre (IF) after mixing with 'standard' metal solution.

Fibre Source		Copper	Iron	Zinc	Calcium	Potassium	Magnesium	Manganese	Sodium
Bean	SF	0.2	2.9	0.3	-21.3	1.6	0.7	-0.1	12.7
	IF	0.1	1.3	0.2	1.7	1.4	0.4	-0.0	11.4
Cabbage	SF	0.1	3.0	0.2	-16.3	1.2	-2.0	-0.1	13.3
	IF	0.1	-0.4	-0.1	-8.5	2.1	-0.1	0.0	28.2
Kumera	SF	0.1	3.0	0.2	-3.4	-0.3	-0.1	-0.1	-20.9
	IF	0.0	2.9	0.5	-6.2	0.5	-0.3	-0.0	-5.1
Lettuce	SF	0.2	3.8	0.6	-13.0	-2.8	-1.8	-0.2	5.4
	IF	0.1	3.4	0.7	-22.6	0.9	-0.7	-0.1	7.5
Onion	SF	0.1	3.5	0.3	-6.8	-0.2	-0.4	-0.1	-3.0
	IF	0.0	0.7	0.0	-5.8	1.1	-0.0	-0.0	7.4
Peach	SF	0.1	4.0	0.5	-2.5	1.3	0.2	-0.1	3.5
	IF	0.2	2.5	0.5	2.0	1.4	0.5	-0.1	16.5
Pear	SF	0.1	-7.8	0.2	-6.0	-0.0	-0.5	-0.1	-14.7
	IF	0.1	1.2	0.2	-3.3	1.3	0.3	-0.1	7.5
Pumpkin	SF	0.0	3.6	0.2	-11.9	-3.9	-1.4	-0.1	-13.8
	IF	-0.1	1.7	-0.3	-2.2	1.3	-0.4	-0.1	13.9
Tomato	SF	0.1	4.1	0.5	-5.2	1.3	-0.4	-0.1	21.1
	IF	0.1	3.0	0.8	-2.3	1.4	0.3	-0.1	9.9
Wheat Bran	SF	0.0	4.2	1.2	1.0	1.5	1.2	-0.0	12.3
	IF	0.1	3.4	0.9	-0.2	1.5	1.5	-0.0	8.0
White Clover	SF	0.1	4.5	0.5	-27.0	-1.8	-2.8	-0.1	-1.3
	IF	-0.0	3.5	0.8	-19.2	-1.4	2.3	-0.1	-7.8
Lucerne	SF	0.1	4.4	1.0	-25.7	-0.7	-1.4	-0.1	1.9
	IF	0.0	1.8	0.3	-12.3	0.6	-1.3	0.0	16.0
Ryegrass	SF	0.1	4.6	0.5	-8.6	-1.1	-0.5	-0.2	5.3
	IF	0.1	1.8	0.4	-7.1	0.7	-0.6	0.0	11.2

Table XIV

Bound metal content (µg/mg dry weight) of water soluble (SF) and insoluble (IF) fibres from fruits, vegetables, wheat bran and grasses after mixing with a 'standard' metal solution.

Fibre Source		Copper	Iron	Zinc	Calcium	Potassium	Magnesium	Manganese	Sodium
Bean	SF	0.5	8.5	1.5	13.3	6.8	4.9	0.3	25.4
	IF	0.2	10.6	0.9	133.9	1.7	0.7	0.1	14.5
Cabbage	SF	0.7	26.1	4.7	90.2	2.4	3.0	1.4	20.0
	IF	0.2	7.9	0.4	117.9	2.4	0.4	1.1	32.2
Kumera	SF	0.7	18.6	3.1	54.3	7.3	5.1	0.1	0.6
	IF	0.2	6.2	1.0	20.4	2.3	2.1	0.0	23.9
Lettuce	SF	0.3	7.1	1.3	31.0	4.0	4.0	0.1	17.6
	IF	0.4	15.4	2.2	114.7	1.2	1.7	0.2	9.6
Onion	SF	0.5	5.8	2.1	65.6	5.2	6.3	0.5	8.3
	IF	0.3	23.0	1.3	113.7	1.4	0.3	0.1	11.8
Peach	SF	0.6	16.5	3.7	12.4	6.1	1.9	0.3	34.5
	IF	0.5	19.9	1.9	205.2	2.0	1.4	0.1	21.0
Pear	SF	0.8	3.6	4.2	18.4	12.1	2.6	0.1	0.5
	IF	0.3	24.9	1.6	93.0	1.8	0.8	0.1	11.9
Pumpkin	SF	0.3	8.9	1.5	18.9	2.7	2.8	0.1	3.7
	IF	0.1	34.9	0.8	116.0	1.6	0.1	0.0	17.7
Tomato	SF	0.3	7.4	1.3	38.9	3.5	3.1	0.0	27.8
	IF	0.3	18.5	2.8	140.0	1.8	0.8	0.1	12.7
Wheat Bran	SF	0.1	5.4	1.4	2.1	2.5	2.2	0.1	15.9
	IF	0.3	19.3	1.0	7.6	4.4	1.6	0.0	12.8
White Clover	SF	0.6	12.4	1.8	57.0	3.4	3.8	0.2	12.5
	IF	0.2	6.9	1.5	27.2	1.8	6.5	0.0	23.9
Lucerne	SF	0.6	10.6	2.0	53.8	4.1	3.8	0.3	14.0
	IF	0.1	4.6	0.8	40.2	2.1	1.0	0.1	22.6
Ryegrass	SF	0.9	11.6	1.7	25.4	5.2	3.4	0.2	18.1
	IF	0.2	4.1	0.7	28.8	2.0	1.1	0.1	16.5
(after 0.1M) (HCl wash.)	IF	0.1	2.4	0.5	3.0	3.0	0.8	0.1	40.8

Table XV

Mean bound metal content (µg/mg dry weight) of fruit, vegetable, wheat bran and grass fibres after mixing with 'standard' metal solution

Fibre type	Copper	Iron	Zinc	Calcium	Potassium	Magnesium	Manganese	Sodium
Foods and grasses								
Water soluble	0.5±0.2	11.0±6.3	2.3±1.2	37.0±25.6	5.0±2.7	3.6±1.3	0.3±0.4	15.3±10.4
Insoluble	0.3±0.1	15.1±9.4	1.3±0.7	89.2±59.3	2.0±0.8	1.4±1.6	0.1±0.1	17.4± 6.4
Foods								
Water soluble	0.5±0.2	10.8±7.2	2.5±1.3	34.5±27.9	5.3±3.0	3.6±1.5	0.3±0.4	15.4±11.9
Insoluble	0.3±0.1	18.1±8.6	1.4±0.7	106.3±57.1	2.1±0.9	1.0±0.7	0.2±0.3	16.8± 7.0
Grasses								
Water soluble	0.7±0.2	11.5±0.9	1.8±0.2	45.4±17.4	4.2±1.0	3.7±0.2	0.2±0.0	14.9± 2.9
Insoluble	0.2±0.1	5.2±1.5	1.0±0.5	32.1± 7.1	1.9±0.1	2.9±3.2	0.1±0.0	21.0± 4.0

Table XVI

Mean, standard deviation (µg/mg) and T values of bound metals from matched paired fibres, before and after mixing with 'standard' metal solution

Fibre	Type	Copper	Iron	Zinc	Calcium	Potassium	Magnesium	Manganese	Sodium
Soluble fibre	x	0.1	2.9	0.5	-11.3	-0.3	-0.7	-0.1	1.7
	S	0.1	3.3	0.3	9.0	1.7	1.1	0.0	12.3
	T	7.2	3.2	5.5	-4.5	0.6*	-2.3*	-9.0	0.4*
Insoluble fibre	x	0.1	2.1	0.4	-6.6	1.0	0.2	-0.0	9.6
	S	0.1	1.2	0.4	7.6	0.8	1.0	0.1	9.1
	T	3.6	6.3	3.6	-3.2	4.3	0.6*	-2.9	3.8

x = mean
S = Standard deviation
T = T statistic for matched pairs (Walker and Lev, 1969)
* = not significant for 12 degrees of freedom, at $0.01 < 2.68$

Table XVII

Percentage nondialysable metal content against distilled-deionised water of the water soluble and insoluble fibres

Fibre source		Copper	Iron	Zinc	Calcium	Potassium	Magnesium	Manganese	Sodium
Bean	SF	91.6	100.0	95.5	85.7	89.1	87.5	100.0	53.2
	IF	81.1	96.9	87.5	99.3	100.0	91.2	90.0	84.2
Cabbage	SF	85.8	90.9	96.6	60.1	83.1	33.1	97.5	93.2
	IF	83.1	83.6	48.9	94.4	87.7	61.8	96.7	74.2
Peach	SF	88.3	98.8	93.5	91.4	100.0	96.2	100.0	83.9
	IF	98.3	97.5	95.6	99.7	100.0	100.0	98.4	92.8
Ryegrass	SF	91.6	97.5	89.2	87.8	75.2	87.9	99.8	57.0
	IF	62.5	82.7	59.4	90.6	61.3	70.0	96.0	61.8

Table XVIII

Total bound ion content (mM/g dry weight) of water soluble (SF) and insoluble fibres (IF) from fruits, vegetables, wheat bran and grasses.

Fibre Source		Total ion content
Bean	SF	2.0
	IF	4.4
Cabbage	SF	3.9
	IF	4.6
Kumera	SF	2.2
	IF	1.8
Lettuce	SF	2.0
	IF	3.7
Onion	SF	2.5
	IF	3.8
Peach	SF	2.4
	IF	6.5
Pear	SF	1.0
	IF	3.4
Pumpkin	SF	1.0
	IF	4.4
Tomato	SF	2.6
	IF	4.5
Wheat Bran	SF	1.0
	IF	1.3
White Clover	SF	2.5
	IF	2.2
Lucerne	SF	2.5
	IF	2.2
Ryegrass	SF	1.5
	IF	1.6
(after 0.1M HCl)	IF	2.0

Discussion

Preliminary studies were undertaken to establish some of the parameters of the metal binding to fibres.

For this study it was assumed that wheat bran hemicellulose was representative of the water insoluble fibres and that zinc was representative of all the metals used in the test solution. This assumption is probably valid for the fibres, although wheat bran hemicellulose did have an initial zinc content slightly lower than that from other foods. However, for the metals the effect of hydrolysis and precipitation of ferric irons at higher pH, suggest that it may not be entirely valid. In this study, oxidation of ferrous ions were diminished by using fresh solutions. Therefore hydrolysis and precipitation probably do not seriously invalidate this *in vitro* model system.

Effect of pH

As shown in Figure 14, the maximum amount of zinc binding to wheat bran hemicellulose occurred between pH 5 and 6, which is within the range of pH 4 to 7, that Fordtran and Lockler (1966) found in the proximal jejunum. Thus the pH of the absorption region of the intestinal tract and that at which maximum zinc binding to this fibre occurs are in the same pH range.

Effect of Time

The investigation of the time required to obtain maximum zinc binding, shown in Figure 15, indicates that equilibrium is reached

within a short time. The change in binding from 20 to 150 minutes was less than one per cent. It had been suggested by Phillips and Fernandez (1981), that appropriate studies of metal binding to fibres should be undertaken with the same time (greater than 120 minutes) as that occurring in the intestinal tract. It was also pointed out by Scheinberg (1982) that all binding studies should endeavour to reach equilibrium between the binding polymer and the ligand, so that the law of mass action can be applied. The results obtained in this study suggest that equilibrium between the binding polymer and the ligand will be reached before the time Phillips and Fernandez (1981) suggest. However, to incorporate a safety margin, all the binding studies were carried out with a mixing time of 60 minutes.

Effect of Osmolality

The influence of osmolality on the percentage of zinc bound to wheat bran hemicellulose is shown in Table IX. Increases in osmolality of glucose from 10 to 298 mOsmol/kg had minimal affect on the binding of zinc (80.3 to 81.0%). However, the increase of osmolality using sodium chloride, over the same range of osmolality increased the binding of zinc from 80.3 to 95.7 per cent.

Phillips and Fernadez (1981) promoted the concept that investigations of metal binding to fibre should mimic the conditions of the small bowel, the osmolality of which is from 250 to 300 mOsmol/kg (Fordtran and Locklear, 1966). The results of this study suggest that it is not the osmolality *per se* that influences metal binding to fibre but the ionic strength of the solution. This result

is consistent with the findings of Smidsrød and Haug (1968, 1972). They found that the presence of neutral substances such as sucrose did not influence the calcium binding properties of pectic acid. The increase in zinc binding at higher ionic strength may be the result of an increased hydrodynamic volume as has been observed by Simdsrød (1970) in alginate solutions. Therefore metal binding to this polysaccharide is conditional upon the ionic strength of the solution.

Filtration System

The validity of the MPS-1 filtration systems for use in separating bound and free metal species was investigated. This was done by determining the retention of metals to the apparatus. It can be seen from Table X that only small percentages of the metals were retained by the system. Manganese (11.1%) and copper (15.9%) are exceptions and their higher retention is probably related to their low concentration in the standard solution (Mn, 1.4 mg/ml and Cu 1.7 mg/ml). The binding of all the metals to the filtration membrane and apparatus could possibly have been reduced by prior saturation. However a coefficient of variation of less than 10% for most of the metals would appear to be within reasonable experimental error.

The results from this study have been taken to confirm that the MPS-1 microfiltration system does enable the separation of bound and free ligands (Hammond *et al.* 1980; Shah *et al.* 1974). This method has, however, not previously been used to determine metal binding to food components. It has the advantage of ease of experimental procedure compared with the equivalent but slower method of equilibrium dialysis (Sophianopoulos *et al.* 1978).

The initial metal contents of the two groups of fibres varied considerably as seen in Table XI. While the origin of these metals has not been established it is possible that they were of plant origin or derived by a Donnan equilibrium from the dialysis water (which had a content of calcium, 33.8 µg/ml, magnesium, 6.3 µg/ml, potassium, 5.5 µg/ml, copper, 0.84 µg/ml, iron, 1.16 µg/ml, manganese, 0.08 µg/ml and zinc, 2.81 µg/ml) that occurred during the polysaccharide preparations as described in Part I. However, the metals were not readily dialysable against distilled-deionised water as seen in Table XVII, indicating that the metals were firmly bound to the water soluble and insoluble fibres from bean, cabbage, peach and ryegrass. Thus, while the origin of the metals is not established it does indicate a capacity of these plant materials to firmly bind minerals.

Calcium was the predominant metal species present in both classes of fibre.* Generally the water insoluble fibres contained more calcium than the water soluble fibres. The mechanisms of calcium association within plant material have not been clearly elucidated. Pectin, binding calcium by the proposed 'egg-box' mechanism (Rees, 1977) has received prominence as the metal complexing cell wall constituent (Davis et al. 1980). The modes of interchain association for pectins resulting in gels have been investigated using a variety of techniques. Morris et al. (1980) investigated non-ionic interactions using circular dichroism and found that reduced gel strengths at low levels of esterification to be due not only to increased charge density but also from a significant loss that the contribution of ester groups make to the stability of the pectin interchain junctions.

* The highest calcium content (> 88% of the total metal content) was observed in peach water insoluble fibre.

Interchain associations being stabilised by non-covalent forces analogous to those in protein tertiary structure. This was confirmed by Oakenfull and Scott (1984) that the network of polysaccharide molecules in gels of high methoxyl pectins are stabilised by a combination of hydrophobic interactions and hydrogen bonds. The contribution from hydrophobic interactions to the free energy of formation of junction zones being about half that from hydrogen bonding but an essential requirement since hydrogen bonding alone is insufficient to overcome the entropic barrier to gelation, sucrose acts by stabilising the hydrophobic interactions (Oakenfull and Scott, 1984). The elution behaviour of aqueous pectin solutions is also influenced by reversable aggregations as well as the chain length of individual molecules (Davis et al. 1980). Gidley et al. (1980) suggest that there are two modes of pectin interchange association. The first mechanism by interchain chelation of calcium 'egg-box binding' (Powell et al. 1982) and the second by non-ionic interchain associations analogous to those in low water activity gels (Morris et al. 1980). The metal binding characteristics of the high uronic acid containing polysaccharides have been shown to have considerable complexity (Gidley et al. 1980). However, the results from this study suggest that the insoluble fibres, such as the hemicelluloses, are quantitatively more significant binders of calcium. The lower uronic acid content of the water insoluble fibres but greater calcium content than the water soluble fibres, suggests that different binding mechanisms may be operative in the two different groups of polysaccharides.

The site and function of bound calcium in hemicelluloses has not been determined. However, the results of the determination of metal binding to ryegrass hemicellulose with and without acid treatment, showed that there was a marked decrease in bound calcium after acid treatment (Table XIV). It is possible that divalent calcium plays a stabilising role within the insoluble fibres by being a biological crosslinking agent which contributes to the architecture of the cell wall.

The application of the MPS-1 microfiltration system to determine the interaction of metals with the water soluble and insoluble fibres from fruits, vegetables, bran and grasses showed that there was considerable variation in the amounts of the metal species bound, as seen in Tables XIII, XIV, XV and XVI.

Table XV shows that the mean bound metal content of the foods and the grasses do not substantially differ except for the bound calcium content. The foods had a higher calcium content (mean 106.3 ± 57.1 µg/mg) associated with the insoluble fibre compared with the grasses (32.1 ± 7.1 µg/mg). This can not be explained in terms of uronic acid content, for the foods had a mean percentage uronic acid content of 10.3 ± 6.7 compared with the grasses of 13.4 ± 4.8 per cent of the hemicellulose content. This is suggestive that a mechanism other than uronic acid association with calcium is the binding mechanism. However, the results reported in this section of the thesis were not designed to investigate the basic binding mechanisms .

It can be seen from Table XVI that there was significantly more copper, iron and zinc bound to both the water soluble and insoluble

fibres for the matched paired extracts before and after mixing with the 'stock' metal solution. After mixing with the 'stock' metal solution there was significantly less calcium and manganese bound to both classes of fibre, while there was significantly more potassium and sodium bound to the insoluble fibres. At the 0.01% level there were no significant changes in the magnesium levels from both classes of fibre, however, at the 0.025% level there was significant displacement of magnesium from the water soluble polysaccharides.

Haug and Smidsrød (1970) investigated the selectivity of polysaccharides for copper compared to calcium. They concluded that selectivity for copper compared to calcium is a characteristic feature of all carboxylate containing polymers and of polyphosphate, while no such selectivity is observed for sulphated polysaccharides.

They suggested that the binding mechanism responsible for the higher affinity of copper relative to calcium ions in carboxylate containing polyanions is independent of the presence of free hydroxyl groups in the polymer and of the steric arrangements of the carboxylate groups. This exchange reaction is associated with an unfavourable enthalpy change and the driving force of the copper-calcium exchange reaction must therefore be a relatively large positive entropic change connected with the binding of copper ions and liberation of calcium ions from the polyanion. It seems possible that this entropic change is caused by the copper ions having a larger ordering effect upon the water molecules than calcium ions and that hydration of the ions is destroyed when they are bound to the polymer. The difference between carboxylate and sulphate groups may be due to

different effect of the two types of fixed groups in destroying the hydration of the copper ions.

The fact that copper-calcium selectivity seems to be caused by a mechanism different from that governing the selectivities of alkaline earth ions (Haug and Smidsrød, 1970) does not mean that copper ions cannot be bound to polyuronates by the same mechanisms as are the calcium ions. On the contrary, the constancy of copper-calcium selectivity coefficients indicates that, when the structure of the polyuronide allows a preferential binding of calcium compared to magnesium, copper is also bound preferentially, probably by the same mechanism. In addition, the mechanism making the binding of copper to carboxylate groups energetically more favourable than the binding of calcium is operative.

The affinity of copper, iron and zinc binding to the water soluble and insoluble fibres is in general agreement with Mod et al. (1981) who found an affinity order of copper, iron and zinc to water and alkali soluble hemicelluloses from rice. Gregor et al. (1955) who studied the binding of divalent metal ions to polyacrylate, found the complex formation constant for copper was much higher than for magnesium, zinc, cobalt, calcium and manganese which decreased in that order. A study of the formation constants with polymethacrylate was reported to decrease in the order copper, cadmium, zinc, cobalt and manganese (Mandel and Leyte, 1964).

Studies of cell wall fractions from a grass, Holcus lanatus, have shown that pectin and lignin complex a large proportion of the

calcium, whereas magnesium is complexed only by lignin (Molloy and Richards, 1971). The results of this present study showed that there was a significant decrease in calcium and magnesium in the water soluble polysaccharides when mixed with the 'stock' metal solution, but no significant decrease in magnesium in the water insoluble hemicelluloses. As shown in Part V the hemicellulose of wheat bran contains some lignin, and a hemicellulose-lignin bond has been demonstrated (Hartley 1978) and feruloylated pectins (Fry, 1983). Possibly the difference in magnesium binding between these two classes may be due to the lignin content.

The range of total metal binding capacities of the fibres was between 0.2mM/g dry weight for the water soluble fibre from beans to 6.5 mM/g dry weight for insoluble fibre from peach (Table XVIII). The mean value of the total bound metal content of the fibres, from the plant species examined, was 2.0 ± 1.0 mM/g dry weight of water soluble fibre and 3.5 ± 1.4 mM/g dry weight of insoluble fibre. The higher value of bound metal content of the water insoluble fibres is probably a reflection of the high initial calcium content.

The metal binding capacity of the fibres compares favourably with the binding capacity of commercial ion-exchange resins, which range from 0.6 to 5.0 mM/g dry weight (British Drug Houses Bulletin, 1977).

The cation-exchange capacities of a variety of 'whole' foods and of neutral detergent fibres (McBurney et al. 1983) have been determined (McConnell et al. 1974). They ranged from 0.6 mM/g dry weight of pear to 2.0 mM/g dry weight of carrot. While it may not be

valid to compare the ion binding capacity of such diverse samples as manufactured resins, fibres and 'whole' foods, determined by a variety of different methods, the similarity of the quantitative values suggests that the total number of binding sites are similar.

The content of copper and zinc was generally higher in the water soluble fibres (Table XI and XII). This result is in agreement with the findings obtained in investigations of metal tolerant grasses (Turner, 1970), where it was found that pectate extract, contained more zinc and copper than other cell wall constituents. Results of investigations of the mechanism of zinc tolerance suggest that there are different binding sites for copper and zinc (Peterson, 1969).

The binding sites of these metals is not known. It has been suggested that the metal binding properties of dietary fibre are directly related to the uronic acid content (James et al. 1978). Although they obtained a very highly significant correlation ($P < 0.001$) for calcium binding to uronic acid content an R^2 value of only 0.4356 was obtained, indicating that over 50% of the variation in calcium binding was not attributable to a relationship with uronic acid content. Within the complex of plant cell walls and their constituents there are a number of possible metal binding sites, such as the carboxyl, phosphate, phenolic, hydroxyl and carboxyl groups of lignin and ferulic acid (Jones, 1978, and Fry, 1983), binding to silica present in pectins (Schwarz, 1973) and in secondary cell wall polysaccharides (Jones, 1978), and also mechanisms involving structural interactions between polymers can not be excluded.

The quantitation and mechanisms of metal interactions with plant material, particularly food polysaccharides with a low uronic acid content, do not appear to have been extensively investigated.

The next part of this thesis describes experiments undertaken to determine the binding capacities and mechanisms of zinc interaction with various fibres from wheat bran and phytate.

PART V

Zinc adsorption to wheat bran, its fibre components and to phytate

Introduction

Efficient milling technology, resulting in bran removal and fibre depletion, has been suggested to be a contributing factor in the increased human death rate from a variety of degenerative diseases (Burkitt, 1972, 1973; Walker, 1974; Trowell, 1976).

Bran however, has been shown to be not an entirely benign dietary component. A relationship between poorly or unrefined cereals in the aetiology of mineral depletion was shown in an investigation of the role cereals played in the aetiology of nutritional rickets, that occurred in Dublin in 1942, when the extraction rate of flour was increased from 70-100 per cent (Robertson et al. 1981). It has been suggested that phytate is the major factor in reducing mineral bioavailability (Reinhold et al. 1973). Phytate (phytic acid, inositolhexaphosphoric acid), 87 percent of which is associated with the aleurone layer of bran (O'Dell et al. 1972) appears to reduce the bioavailability of metals (McCance and Widdowson, 1942).

Ingestion of phytate containing foods has been shown to interfere with calcium (McCance and Widdowson, 1942) and zinc absorption (Reinhold et al. 1973). The chemistry, occurrence and metal binding of phytate has been recently reviewed by Maga (1982), Graf (1983) and Oberleas (1983).

In studies on the availability of calcium and iron with respect to

refining, McCance and co-workers (1942, 1948) ascribed the deleterious effect of unrefined cereals to the phytate content. However studies by Reinhold and co-workers (1975) investigating the binding of calcium, zinc and iron to various wheat breads and wheat bran, found that even white bread and dephytinised bran, bound zinc. Moreover, phytate consumed in a purified form is less effective in decreasing the retention of zinc and calcium than when equivalent amounts are consumed in the form of wholemeal bread (Reinhold et al., 1973). White bread containing negligible amounts of phytate, also interferes with mineral absorption (Callender and Malpas, 1968). Frølich and Asp (1980) investigated the relationship between the fibre content of wheat flours obtained from milling of different extraction rates and the association of minerals. They concluded that the minerals were mainly associated with a water soluble fraction containing polysaccharides and phytate. The results obtained in Part IV of this thesis suggests that there was significant binding of the three metals, copper, iron and zinc. Preliminary studies on iron binding were undertaken. However, facilities were not available to eliminate the possibility of insoluble metal complexes of copper and iron being formed by oxidation. Therefore a more detailed study of only zinc binding was undertaken.

In Cummings' (1978) opinion there are many questions relating to zinc absorption and fibre that remain unanswered, further information is needed particularly on the mineral-binding properties of dietary fibre as present in commonly eaten fruits and vegetables. Fisher and Berry (1980) on reviewing the present state of research on dietary

fibre, concluded that binding of metals by fibre undoubtedly occurs, but that the relative importance of binding by fibre and of phytate remains controversial. As the role of fibre and of phytate in reducing mineral bioavailability is not clear (Fisher and Berry, 1980), the debate on the importance of wheat refining on food quality (McCance and Widdowson, 1955) has as yet to establish quantitatively the importance of fibre or phytate as the metal binding substance.

Numerous studies have investigated the binding of small molecules such as metals, to macromolecules. A number of methods for graphical and computer assisted analysis of the binding data have been employed. The most commonly used graphical methods include; Scatchard plots (Scatchard, 1949), Hill plots (Dahlquist, 1978) and Klotz plots (Klotz, 1982). It is clear that a given set of binding data contains the same information, independent of the particular method used to interpret it. However, certain graphical methods show some aspects of this information more clearly than others.

Scatchard (1949) described a procedure for determining the way in which small molecules may combine with macromolecules. This procedure can enable the determination of the amount, the tightness and the sites of binding to be determined. The method has been summarised by Scheinberg (1982) as follows: if the initial probability of binding a molecule of A is the same at each of n sites or groups on a

macromolecule, P, the change in free energy $(\Delta G)_v$ for the reaction

$$P + {}_vA = PA_v$$

is zero, if all the components and species of the reaction are in equilibrium when a measurement is made (Scatchard, 1976). If binding the first molecule of A to P has a negligible effect on the tightness of binding of the second molecule of A and so on, then

$$kC_A = \bar{v}/(n-\bar{v})$$

where k is the intrinsic association constant for the reaction at a single site, C_A is the concentration of free ligand molecules and $\bar{v}$ is the average number of A molecules bound to each macromolecule.

A literal interpretation of Scatchard analysis (Scheinberg, 1982) applies only to the binding of small molecules and ions to macromolecules in solution, expressed in molar terms. A more general application has also been applied to more complex systems, involving the suspension of cells or membranes, with the concentration expressed in terms of milligram of polymer (Weisiger et al. 1982). The graphical extrapolation (Rosenthal, 1967) of the binding parameters, in either form, from nonlinear Scatchard Plots can result in difficulties in interpretation (Nørby et al. 1980; Peters and Pingoud, 1982) such as the over estimation of the number of sites and an under-estimation of the binding affinity (Nørby et al. 1980). Weisiger et al. (1982) employed Scatchard Plots for the display of the data, to indicate the model, i.e. the occurrence of positive

cooperativity, negative cooperativity, or the presence of both positive and negativity, and/or the presence of more than one class of sites.

A means of investigating positive and negative cooperativity by Hill Plots was suggested by Dahlquist (1978). All forms of ligand binding can be represented by a single-step interaction between macromolecules and ligands:

$$M + nX \rightleftarrows MXn$$

where M is the macromolecule and X is the ligand.

This equilibrium can be defined by a phenomenological constant, k, such that $[MXn]/[M][X]^n = k^n$.

The apparent value of k is the inverse of the half-saturating ligand concentration.

The Hill binding equation can be linearised by taking logarithms:

$$\log [MX_n]/[M] = n \log k + n \log X.$$

The binding data may then be plotted as log (sites bound)/(sites free) versus log (free ligands). The slope of the region of half saturation gives n , the Hill coefficient, which is a measure of the extent of cooperative-interactions among the potential sites (i.e. the mean square deviation of the number of ligands bound at half saturation). When positive cooperativity is present, the Hill coefficient is greater than unity, and less than unity when negative cooperativity is present (Koshland *et al.* 1966; Dahlquist, 1978).

The experiments described in this section were undertaken in an attempt to quantitate zinc binding to various types of wheat bran fibres and to phytate. The strength of binding, and the mechanisms of interaction between zinc, fibres and phytates were also investigated.

Materials and Methods

Sodium phytate obtained from Sigma (type V), sodium salt from corn, and cellulose from Macherey, Nagel and Co. were used.

(i) Fractions from Part I

The soluble wheat bran fractions obtained in Part I, cold water soluble polysaccharide and the alkali soluble (non-delignified) hemicellulose, were used in this study. Before using the hemicellulose it was suspended in water and neutralised with 0.1 M hydrochloric acid, dialysed for eight hours against deionised water then freeze-dried.

(ii) Fractions from Part III

The dry insoluble wheat bran fractions prepared in Part III were also used; bran, bran ex ethanol, bran ex oxalate, bran cell walls (DMSO insoluble residue after chloramine - T) and DMSO soluble hemicellulose (nondelignified).

(iii) Water soluble polysaccharide from bran

A water soluble polysaccharide from wheat bran was also extracted using the procedure of Neukom and Markwalder (1975). Wheat bran (100 g) was blended in water (500 ml) for 10 minutes in an

homogeniser. The contents were centrifuged (20 minutes, 760 RCF*) and filtered. The solution obtained was then saturated with ammonium sulphate, mixed and allowed to stand overnight at 4°C. The solution was then centrifuged for 20 minutes at 760 RCF. To the supernatant was added alpha amylase (2 g) then dialysed for 120 hours against distilled water. The solution was again saturated with ammonium sulphate, allowed to stand overnight at 4°C, centrifuged, then redialysed for 48 hours. The volume of the solution was reduced from 968 ml to 350 ml by rotary evaporation (32°C), then freeze-dried. The composition of the dry material was determined by the same procedures as used in Part I.

(iv) Lignocellulose fraction

A lignocellulose fraction, from wheat bran, was prepared using mild extraction procedures to remove non-lignin components. Bran ex oxalate (150 g), as prepared in Part III, was used as the starting material. It was washed in deionized water, pepsin (1.5 g) added in 250 ml of 0.1M hydrochloric acid, the mixture was shaken for 120 hours at 30°C, then dialysed and the pepsin procedure repeated until the spot test for amino acids (Feigel, 1956) was negative. The liquid was decanted and the residue washed in water (3 x 500 ml). To the residue was added sodium hydroxide (10%) under nitrogen, mixed and allowed to stand for 2 hours. After decanting the liquid, the residue was washed with water (3 x 500 ml) then neutralised with 0.1 M hydrochloric acid.

****

* (RCF = Relative centrifugal force, $11.17 \times (\text{radius in cm}) \times \left(\frac{\text{RPM}}{1000}\right)^2$

Cellulase/hemicellulase (cellulase 30,000, PFIZER Chemicals) in phosphate buffer (pH 2.5) was then added. The mixture was shaken for 120 hours then dialysed and the enzyme procedure repeated until the spot test for carbohydrates (Vogel, 1958) in the supernatant was negative. The brown insoluble residue obtained on decanting, was washed twice in water and finally in 95% ethanol and dried. The procedure used is summarised in Figure 16.

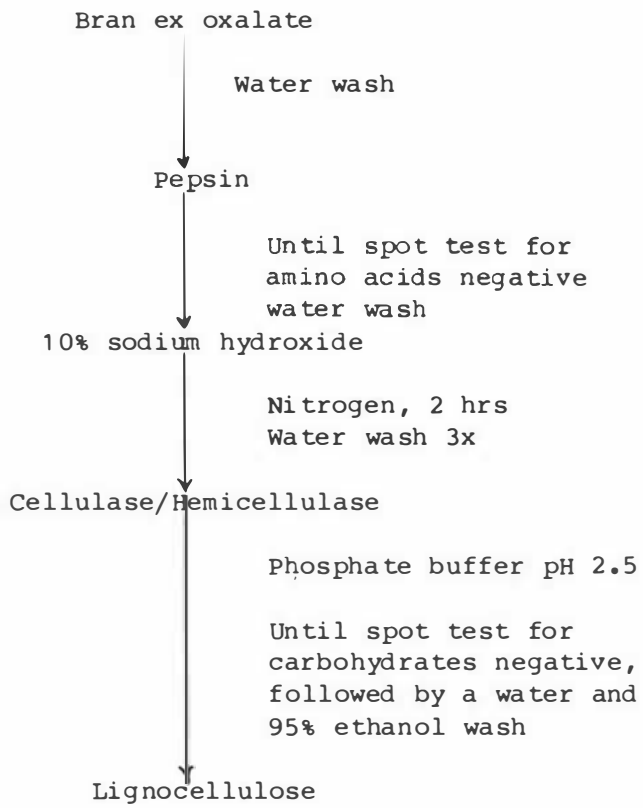


Figure 16: Extraction sequence for the preparation of lignin.

Lignin determination

The lignin content of bran, lignocellulose fraction and the hemicelluloses were determined using the 72% sulphuric acid procedure as described by Holloway et al. (1977). To the dry weighed material

was added 72% sulphuric acid, mixed for four hours, then filtered through No. 2 sintered glass crucibles, washed in water and acetone, oven dried, weighed, then ashed at 550°C.

Determination of zinc binding

The method used for determining the zinc binding to wheat bran and its various fibre fractions and to phytate was as described in Part IV. The membranes used for the phytate studies were UMO5 membranes with a molecular weight cut off 500 (Amicon, 1980), cut to size to fit the MPS-1 filtration systems. The phytate content of the filtrate was determined using the method of Davies and Reid (1979).

A series of zinc solutions from 1.0 µg/ml to 6,000 µg/ml in sodium acetate/sodium barbital buffer at pH 5.5 was made. The fibre free MPS-1 filtrates containing unbound zinc were diluted, when required, with distilled deionised water to obtain solutions with a concentration of zinc between 1.0 and 50.0 µg/ml before measuring by atomic absorption.

Analysis of binding results

The results of the binding studies were analysed using graphical display and Hardman's (1983) adaption of Wood's version (Daniel and Wood, 1980) of the Marquardt maximum neighbourhood method of nonlinear fitting program (Marquart, 1963). This Fortran program, run on a Cromenco CS-2 microcomputer with 64K RAM, is a combination of the Gauss-Newton and steepest-descent methods (Daniel and Wood, 1980). Data, expressed as µmoles zinc bound/g of fibre, were fitted to the following equations:

For one site interacting model;

$$Y = \frac{Ymax * x^n}{L_{0.5}{}^n * x^n}$$

For a two site interacting model;

$$Y = \frac{(Ymax_1 * x^{n1})}{(L_{0.5_1}{}^{n1} * x^n)} \quad \frac{(Ymax_2 * x^{n2})}{(L_{0.5_1}{}^{n2}\ x^{n2})}$$

where Y = bound zinc, x = free zinc, n = Hill coefficient, $L_{0.5}$ = ligand 50% bound.

The minimum least squares values for zinc bound, maximum zinc binding, ligand 50% bound (ligand concentration required for half saturation) and the Hill coefficients for the fibres and phytate were determined using Daniel and Woods (1980) curve fitting procedure (see Appendix E).

Results

The composition of the cold water soluble polysaccharides and the polysaccharide obtained using the ammonium sulphate procedure are shown in Table XIX.

Table XX shows the yields of the two water soluble fibre extracts and of the lignocellulose fraction from wheat bran.

Table XXI shows the lignin content of wheat bran, lignocellulose fraction and the hemicelluloses.

Reproducibility

The reproducibility study was undertaken to assess the variability in the binding and the analysis of zinc. In the determination of the free zinc levels from ten binding studies to wheat bran, with a zinc solution of 2.0 µg/ml, a coefficient of variation of 4.0% was obtained.

Binding

The binding isotherms of zinc to wheat bran, its various fibres and to phytate are shown in Figures 17A to K. The results of the binding experiments, expressed in µM/g of fibre, were plotted against the total amounts of zinc added to the experimental systems. It was observed that the shapes of the binding isotherms varied considerably.

Scatchard plots

Figures 18A to K show the Scatchard plots of zinc binding to wheat bran, its fibres and to phytate.

Table XXII shows the binding capacities, ligand 50% bound and Hill coefficients for zinc binding to wheat bran, its fibres and to phytate.

Table XIX

The percent composition of the cold water soluble fibre and the purified water soluble fibre from wheat bran

Extraction procedure	Rhamnose	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acid	Methoxyl	Acetyl	Protein
Cold water	0.3	23.5	29.9	1.7	7.2	33.1	0.6	0.1	0.4	3.3
Ammonium sulphate	6.0	24.0	18.6	16.2	20.3	10.9	0.9	0.1	0.1	2.9

Table XX

Yields of the water soluble fibres and lignocellulose from wheat bran

	gm/100 g of wheat bran
Cold water soluble extract	0.50
Purified water soluble extract*	0.13
Lignocellulose	7.44

* Extracted using the ammonium sulphate procedure of Neukom and Markwalder (1975).

Table XXI

Lignin content of wheat bran, lignin and the hemicelluloses extracted before and after delignification

Fibre type	Lignin g/100 g dry weight
Bran	3.6
Lignocellulose	30.4
Hemicellulose (not delignified)	1.8
Hemicellulose (delignified)	0

Figure 17: A to C: Zinc Binding to Wheat Bran and its Components in µM/ g. ●Bound, ○Fitted
(1, a one site interacting model: 2, a two site interacting model)

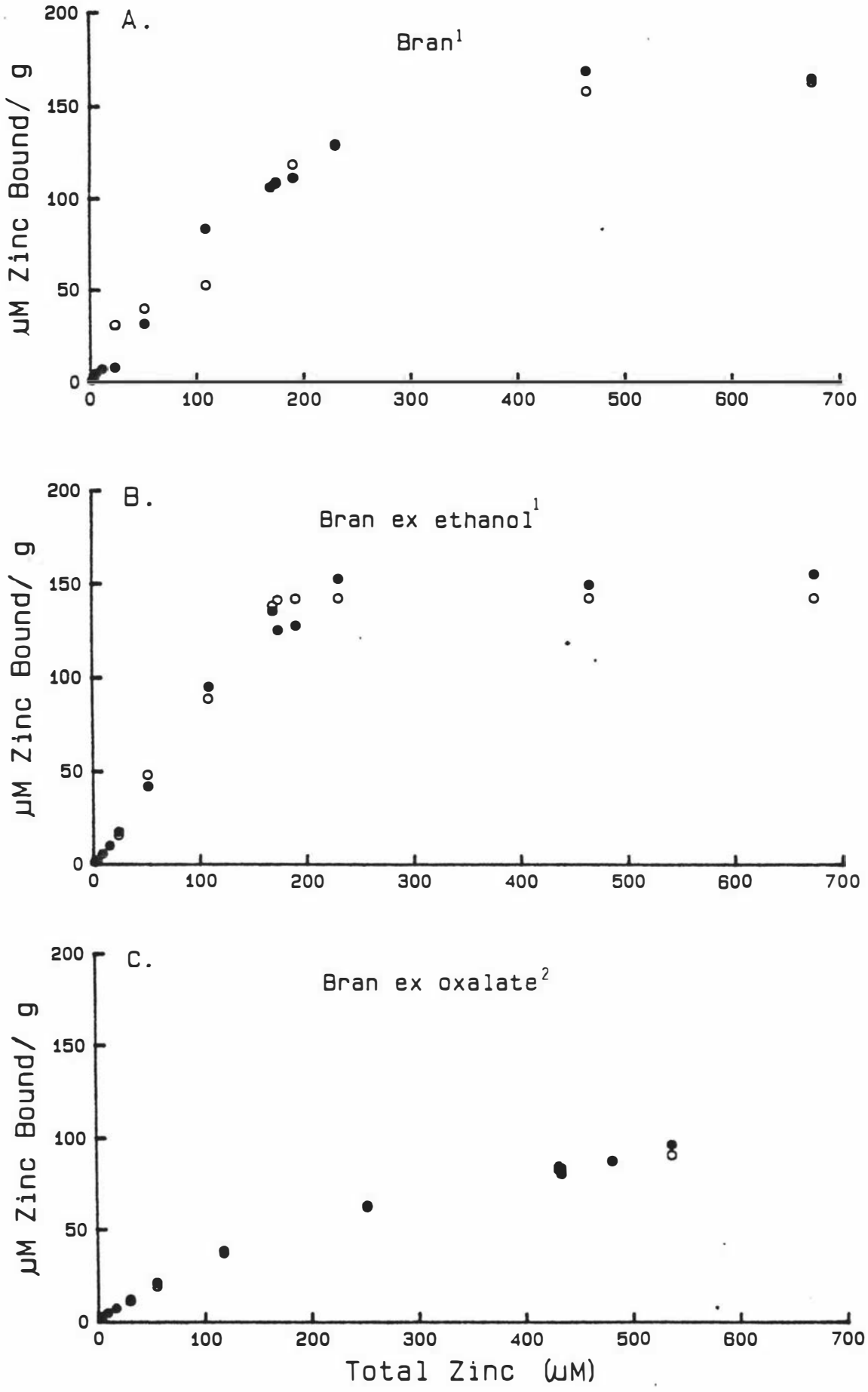


Figure 17: D to F: Zinc Adsorption to Wheat Bran Components in µM/ g. ● Bound, ○ Fitted

(1, a one site interacting model: 2, a two site interacting model)

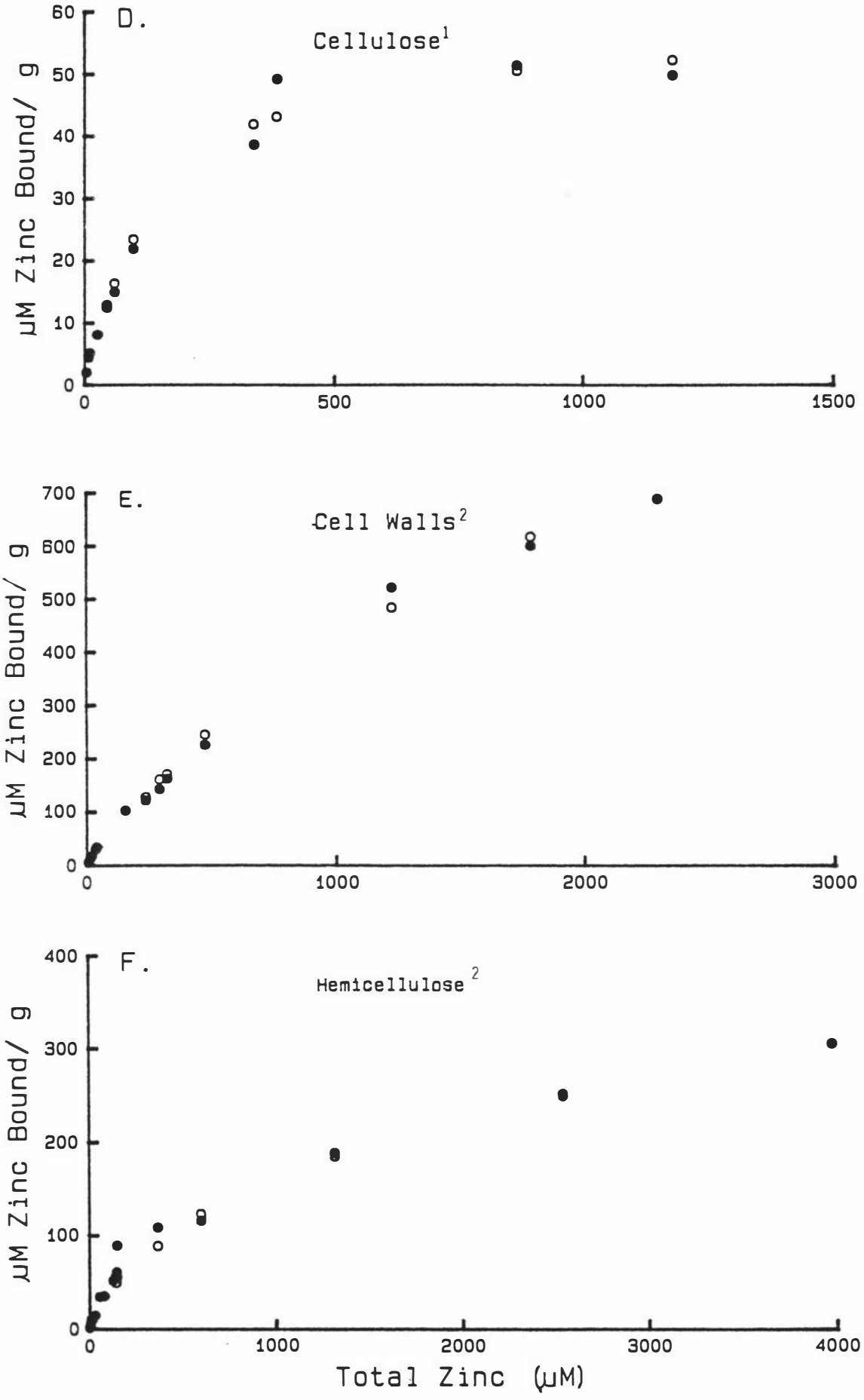


Figure 17: G to I: Zinc Adsorption to Wheat Bran Components in µM/ g.* ●Bound, ○Fitted
(1, a one site interacting model: 2, a two site interacting model)

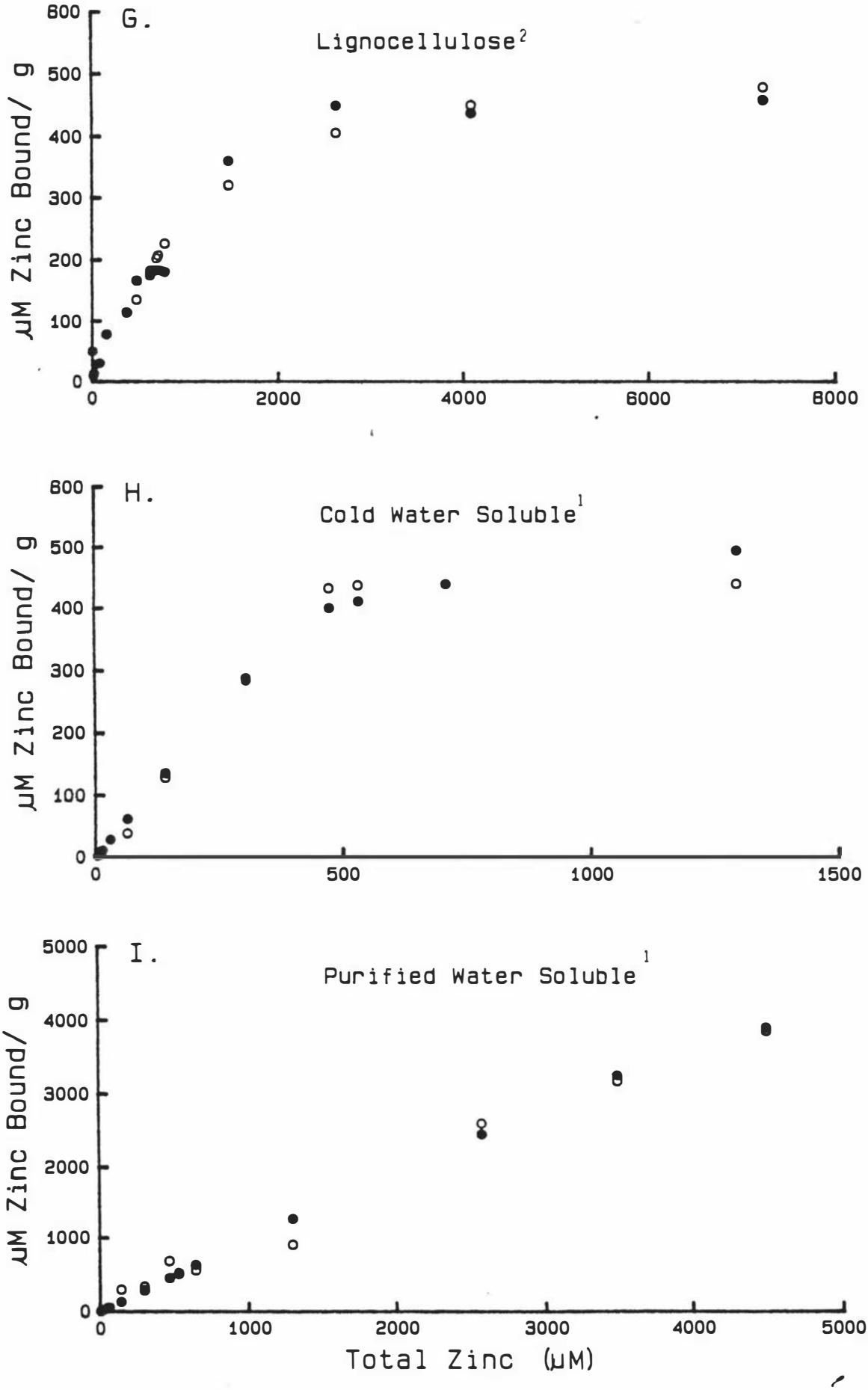
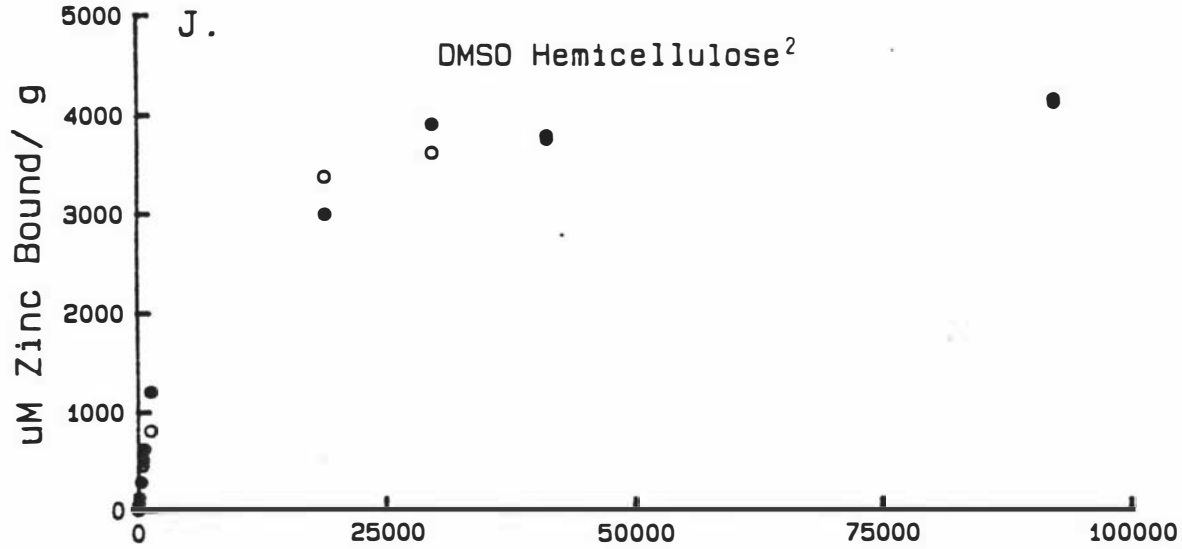
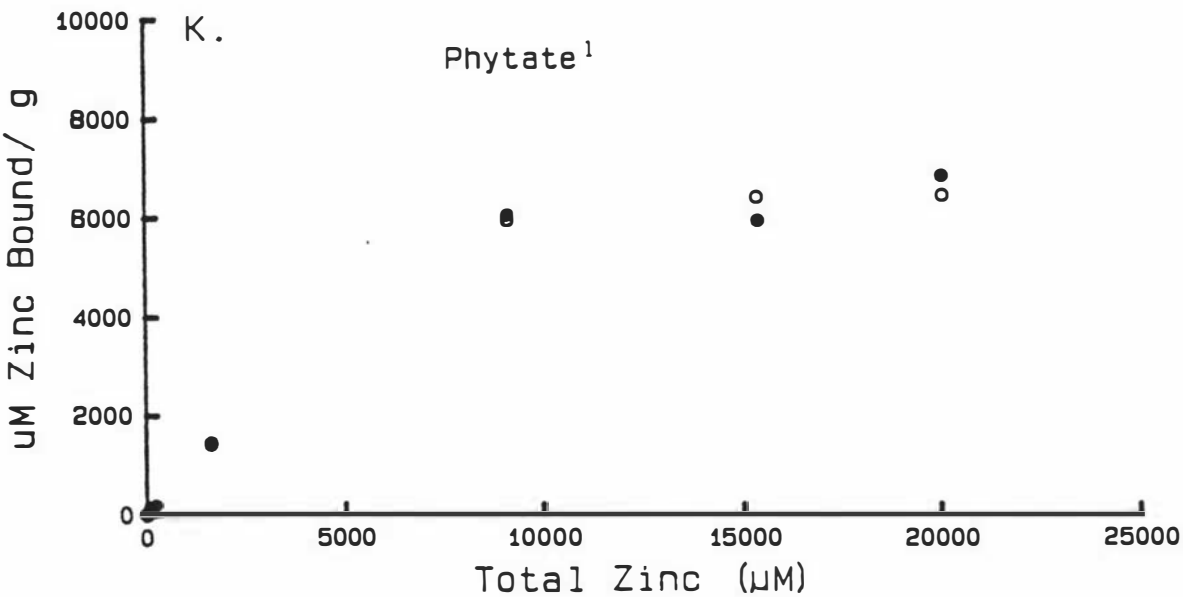


Figure 17: J to K: Zinc Adsorption to Wheat Bran Components in µM/ g.* ●Bound, ○Fitted

(1, a one site interacting model: 2, a two site interacting model)





* Foot Note:
Bound zinc content = total zinc minus free zinc in µM/g dry weight of fibre

Figure 18: A to C: Scatchard Plots of Zinc Adsorption to Wheat Bran Components *

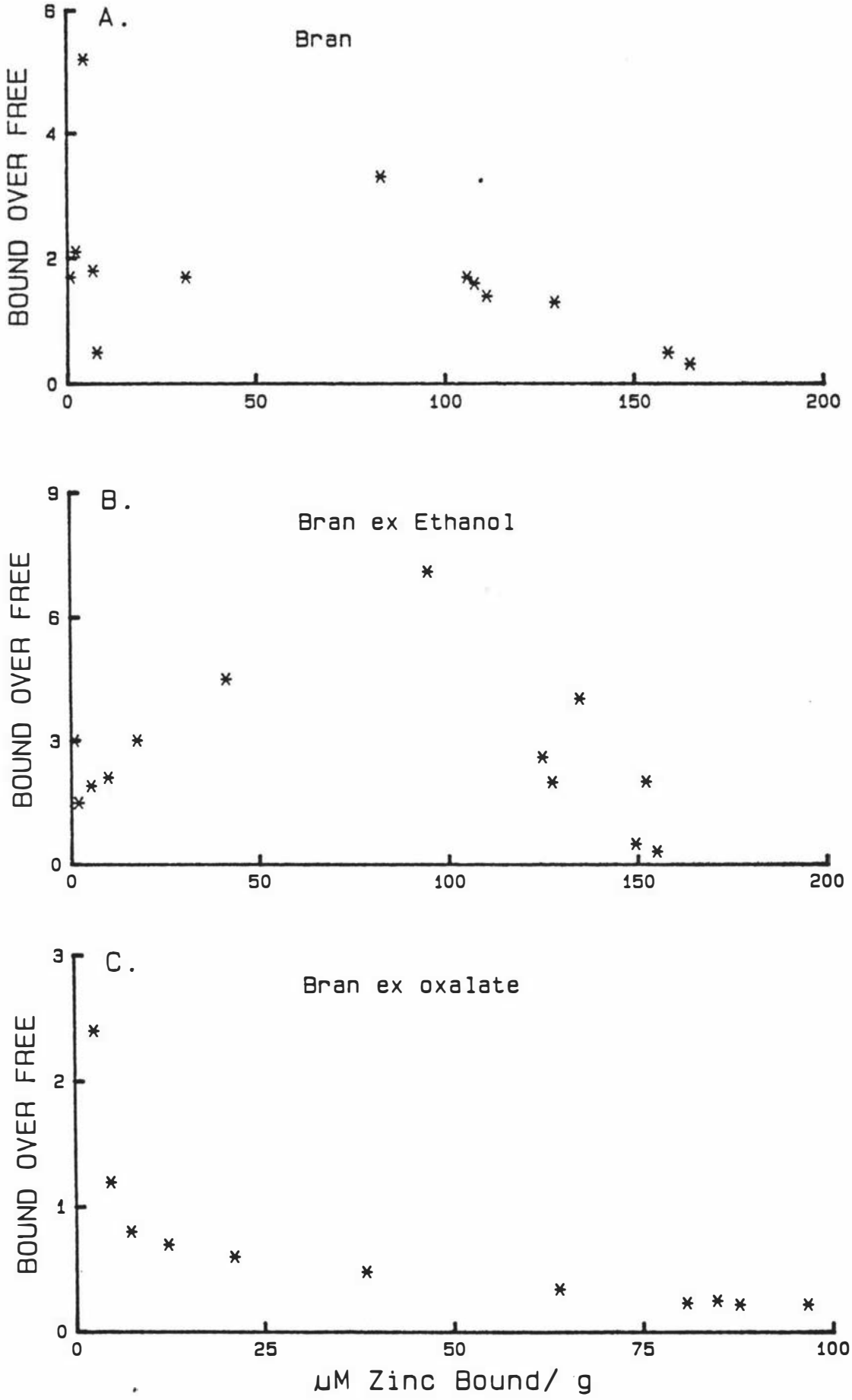


Figure 18: D to F: Scatchard Plots of Zinc Adsorption to Wheat Bran Components *

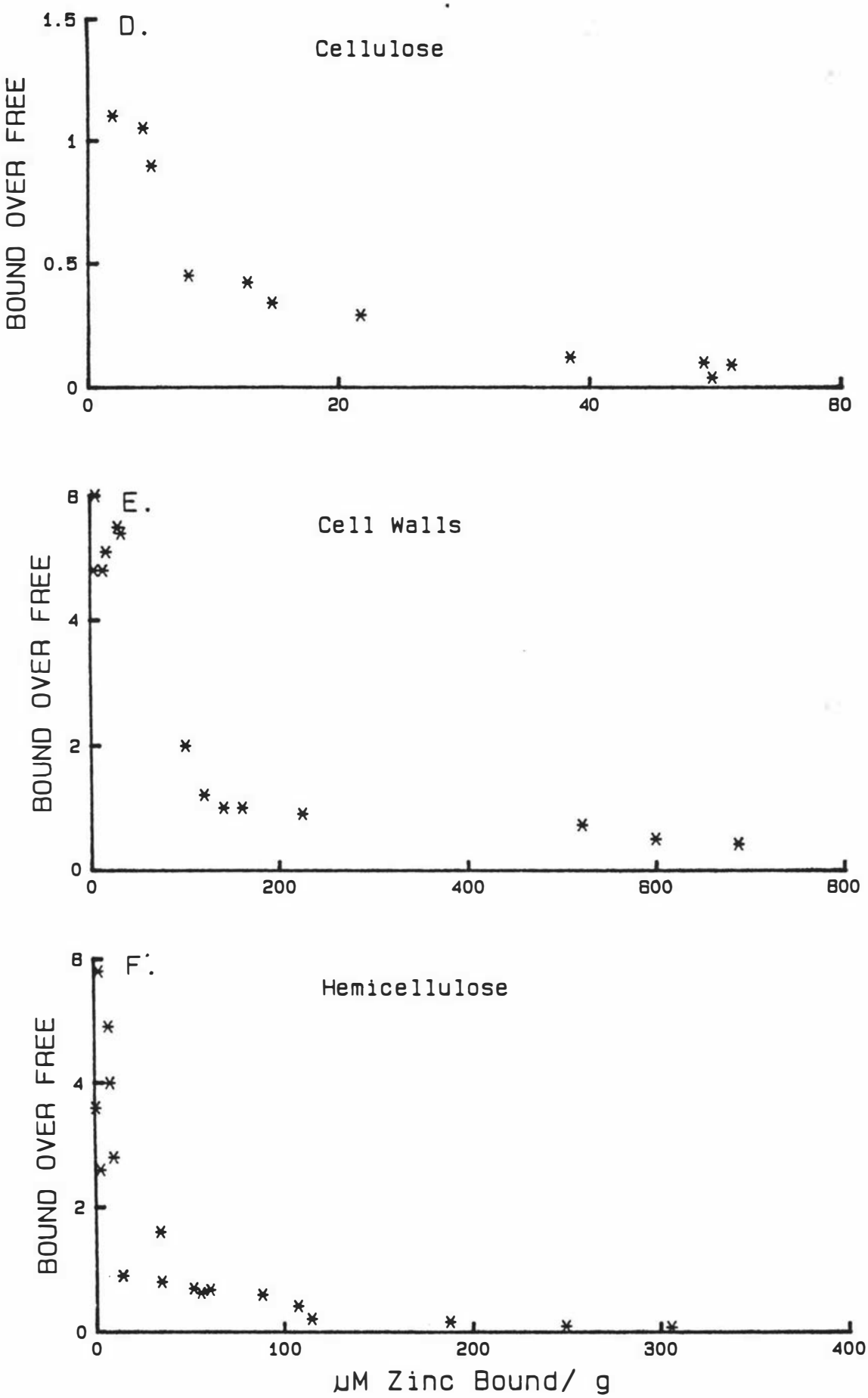


Figure 18: G to I: Scatchard Plot of Zinc Adsorption to Wheat Bran Components *

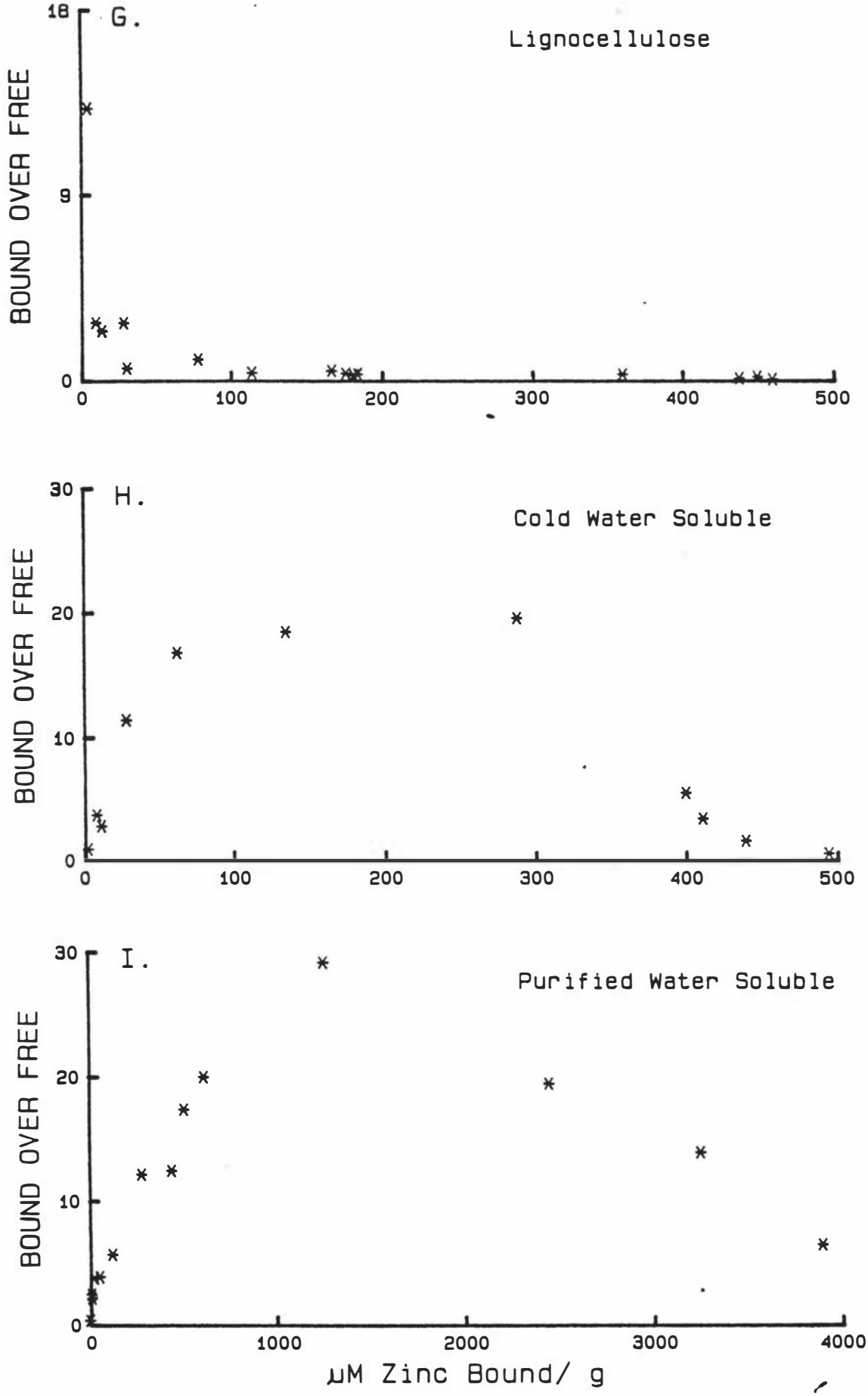
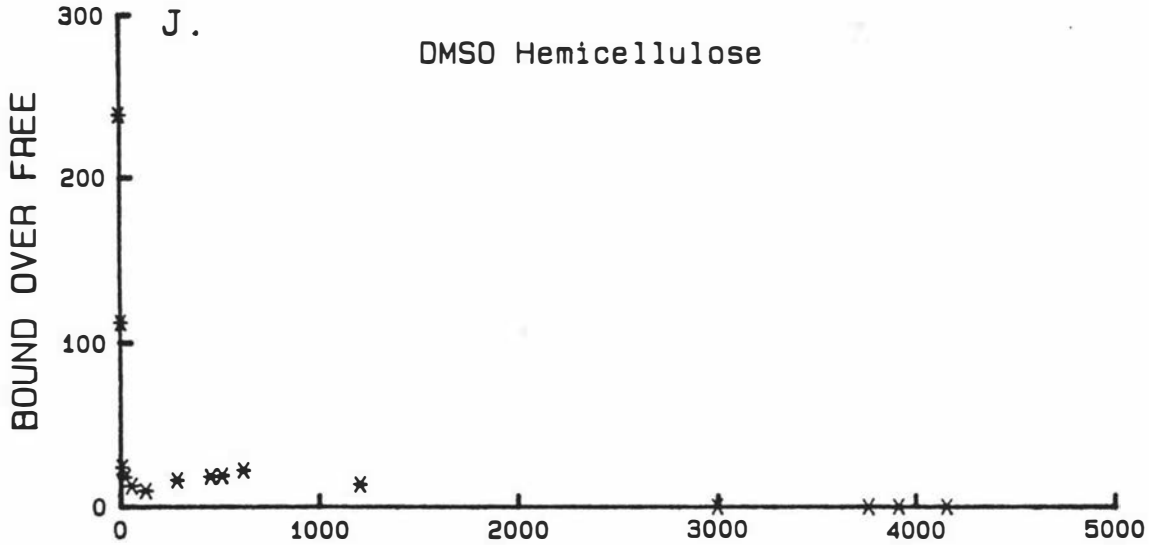
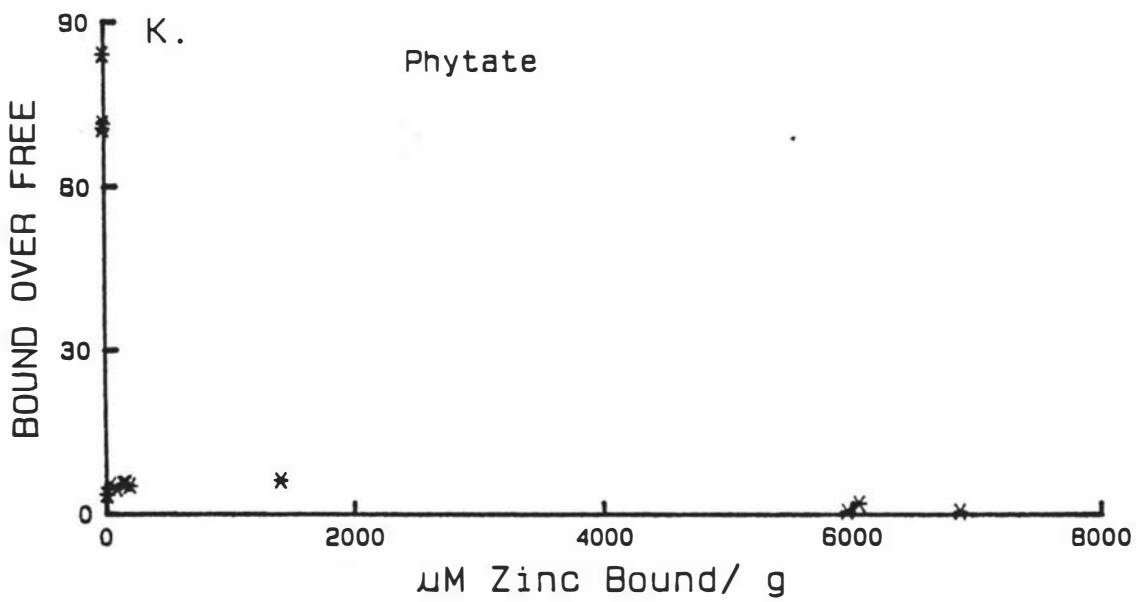


Figure 18: J to K: Scatchard Plots of Zinc Adsorption to Wheat Bran Components *





* Foot Note (as on page 117)

Table XXII

Zinc binding capacities, in μM/g, ligand 50% binding and hill coefficients of wheat bran, its various fibre components and of phytate

Name	Binding Capacity	Ligand 50%	Hill Coefficient
Bran	167.7 ± 12.7	43.1 ± 7.3	1.4 ± 0.3
Bran ex ethanol	142.3 ± 4.4	11.4 ± 0.8	3.0 ± 0.6
Bran ex oxalate	148.3 ± 50.0	264.3 ± 211	0.92 ± 0.2
Cellulose	57.4 ± 5.3	110.2 ± 32.4	0.99 ± 0.2
Cell walls	1012.6 ± 193	765.3 ± 327	1.02 ± 0.1
Hemicellulose	227.9 ± 61.4	304.4 ± 154	0.7 ± 0.1
Lignocellulose	510 ± 41.9	726.9 ± 140	1.2 ± 0.2
Cold water soluble fibre	440.0 ± 15.3	170.8 ± 151	2.14 ± 0.4
Purified water soluble fibre	4038 ± 216	94.6 ± 1.1	1.65 ± 0.2
DMSO hemicellulose	5089 ± 921	3584.3 ± 4200	0.5 ± 0.8
Phytate	6582 ± 192	582.4 ± 101	1.26 ± 0.2

Discussion

A comparison of the composition of the two water soluble extracts from wheat bran, given in Table XIX, shows that the ammonium sulphate procedure of Neukom and Markwalder (1975) gave an extract higher in; rhamnose, mannose, galactose and glucose. Both methods of extraction, cold water and ammonium sulphate, probably extract a mixture of polysaccharides. The highest yields being obtained for the cold water extract (Table XX).

Water soluble arabinoxylans have been isolated from wheat flour (Perlin, 1951) and it has been shown (Ewald and Perlin, 1959) that the arabinose side chains are a positive factor in solubility. Low ferulic acid content of arabinoxylans has also been associated with water solubility (Markwalder and Neukom, 1976). A water soluble arabinogalactan-peptide from wheat endosperm (Fincher et al. 1974) and an arabinogalactan (Neukom and Markwalder, 1975) from wheat flour have been isolated.

The polysaccharides obtained by the cold water and the ammonium sulphate procedure (hereafter referred to as purified water soluble) were found to contain low levels of protein, 3.3% and 2.9% respectively. This content is similar to the 5.0%, that Neukom and Markwalder (1975) obtained from wheat flour using the ammonium sulphate procedure. However, the mannose and xylose contents of the purified water soluble extract was considerably higher at 16.2% and 18.6% respectively than the 1.[illegible] d 0.7% that Neukom and Markwalder (1975) obtained from flour. Pos[illegible]ly a mannose containing polysaccharide is associated pr[illegible]ntly with the bran fraction

rather than with flour, as has been found in rice bran by Mod *et al.* (1979).

The results of an evaluation of the procedure used to prepare the lignin enriched fraction, by analysing the products, using the 72% sulphuric acid method, is shown in Table XXI. Wheat bran was found to contain 3.6% lignin, this is in agreement with previously published values (Holloway *et al.* 1977; Southgate *et al.* 1976).

As shown in Table XXI the lignin content of the lignocellulose fraction was 30.4%, the alkali soluble hemicellulose extracted before delignification, 1.8%, and the alkali soluble hemicellulose, extracted after delignification, was found to be devoid of lignin.

The enzymatic procedure used in this study appears to have enabled a lignin enriched fraction to be prepared. However, possible chemical and physical changes within the lignin structure cannot be excluded. Methods of analysis for this complex and variable material appear to have not reached a sufficient stage of development to enable detailed assessment of the validity of the commonly used 72% sulphuric acid procedure. It was therefore concluded that within the limitations of the analysis procedure, a lignin enriched fibre fraction was successfully prepared.

The assessment of the analytical errors involved in the measurement of binding of zinc to fibres showed that at the low range of zinc concentrations used in the binding studies, a coefficient of variation of 4.0% of the free zinc level was obtained. An assessment of the variability of zinc binding at high concentrations and the

establishment that equilibrium was reached with the other fibres, would have enabled the determination of experimental errors to be obtained.

The zinc binding data obtained in these series of experiments were analysed in terms of Scatchard Plots, shown in figures 18A to K. Unusual binding curves were obtained for the binding of zinc to dietary fibre components of wheat bran. The meaning of which is unclear, as impure polymers were used in the experimental system. Hence, the observations made are the summation of metal binding characteristics such as ion-exchange, changes in configuration, specific and non-specific binding sites of multicomponent systems. Initial interpretation was made using idealised systems for Scatchard Plot analysis. However this was done with caution and with due recognition of the considerable complexities of metal binding properties of plant cell wall components.

All the binding data of zinc to bran, its fibre fractions and to phytate, expressed in Scatchard plots, were markedly non-linear as seen in figure 18A to K. The curvature of the Scatchard Plots implies either that there is more than one group of binding sites, with each group characterised by a different intrinsic association constant, or that the binding of each successive molecule alters the association constant for the next binding molecule (Scatchard et al 1950; Scheinberg, 1982).

The Scatchard Plots of bran and bran ex ethanol, as seen in figures 18A and B are complex, possibly indicating that zinc binding occurred to more than one component. After treatment with water (see extraction sequence, Figure 2) the shape of the Scatchard plots changed. Complex plots showing positive and negative cooperativity have been described (Klotz, 1982). However negative cooperativity is the most commonly observed (Dahlquist, 1978). The notable exception is that of the interaction of oxygen with haemoglobin (Dahlquist, 1978).

The shape of the Scatchard plots of the two water soluble extracts suggest positive cooperativity as the binding mechanism (Dahlquist, 1978). The cooperativity being more pronounced in the purified water soluble polysaccharide (Neukom and Markwalder, 1975, extraction procedure) as indicated by a shift in the maximum binding along the abscissa at high degrees of saturation (Dahlquist, 1978) as shown in Figures 18H and I. These curves suggest that in order to bind the first ligand, the energetically unfavourable step of conversion of the polymer from the low-affinity to the high-affinity form is required. Subsequent zinc molecules bind without the necessity of performing this conversion and therefore have a higher affinity.

The Scatchard Plots of all the bran fractions, after removal of the water soluble fibre, all had concave and markedly nonlinear Plots as seen in Figures 18C, D, E, F, G, J and K. Phenomenologically this implies that the first zinc molecules bind with a higher affinity than subsequent molecules. This could result from induced changes in the

other binding sites resulting from the first bound ligand. An alternative explanation is that the architecture of the polymer has at least two distinct environments of binding sites (Bernhard, 1971). Site heterogeneity can not be easily distinguished from negative cooperativity. However, a qualitative indicator of the phenomenon of cooperativity can be obtained from Hill Plots (Dahlquist, 1978). Further experimentation would be required to establish that equilibrium had always been reached and that the fibre components were not slowly dissolving or coagulating thereby altering the macromolecular system.

The Hill coefficients of zinc binding to wheat bran, its fibre fractions and of phytate were calculated using Hardman's (1983) adaption of the computer program of Daniel and Wood (1980) (See Appendix E). The Hill Coefficients, shown in Table XXII, of wheat bran and its fractionated components showed considerable variability. The large experimental error associated with the determination of the Hill coefficients and Scatchard plots, which are but the summation of the various mechanisms of binding to the complexes of cell components, it is not possible to assign definitive binding mechanisms. As is, indicated by the similarity of the Hill Coefficients obtained for bran (1.4 ± 0.3) and for phytate (1.3 ± 0.2). However, the strikingly different shapes of the Scatchard plots for zinc binding to the various wheat bran fractions is suggestive of different binding mechanisms being operative. While it is conceded that there is considerable experimental error involved in this data, the observable trends of the curves of the Scatchard plots as shown in Figures 18A to

k is suggestive of different binding mechanisms occurring. It is therefore hypothesised that the occurrence of different shapes of the Scatchard plots of zinc binding to wheat bran fractions, is indicative of different binding mechanisms. This hypothesis will require further experimentation to establish its validity.

The use of Scatchard Plots to summarise and analyse binding data has been criticised (Klotz, 1982), as the level of maximum binding was often not reached. He suggested that a semilog plot of the amount bound versus the log concentration of the free ligand has a number of useful features; an inflection point at half-maximum binding, the S-shaped curve is symmetric about the inflection point and a plateau is reached asymptotically as the concentration of the free ligand approaches very large (infinite) values, and the slope decreases to the plateau value as the maximum binding is approached.

Attempts were also made to use Hill and Klotz plots. However they did not reveal any insight into the mechanisms of zinc binding to wheat bran and its fractions, and were not reported within this thesis.

In response to Klotz (1982) criticism of the use of Scatchard Plots and his advocate of semilog plots. Munson and Rodbard (1983) stated that while transforming the data does not change the information content, the altered shape of the curve results in a corresponding change in the shape of the envelope of uncertainty around the curve. Large scatter occurs in both Scatchard and Klotz Plots, as one approaches the axis for the concentration of bound ligand, especially in the presence of significant nonspecific binding.

In Munson and Rodbard's (1983) opinion "In dealing with whole tissues and therefore numerous impurities, at large free ligand concentrations, nonspecific binding will inevitably become larger than specific saturable binding". Near the upper plateau region of the binding isotherm, specific binding is often measured as a small difference between two large numbers. Hence, attempts to measure the maximum ligand bound by simply using large free-ligand concentrations may be unreliable and that the presence of nonspecific binding magnifies the problem.

Munson and Rodbard (1983) did however, agree with Klotz (1982), that extrapolation of Scatchard plots is fraught with difficulties, and that severe problems can arise in extrapolation of nonlinear plots. They suggested that graphical methods serve well to provide subjective, preliminary understanding of the data and that nonlinear least-squares curve fitting procedures should be used for quantitation (Munson and Rodbard, 1983; Thakur et al. 1980). Weisiger et al. (1982) also only used Scatchard plots for the display of the binding data, the actual determination of the binding parameters being done by computer analysis.

Accordingly data obtained from the binding of zinc to bran and its components were subjected to the non-linear least-squares procedure of Daniel and Wood (1980).

The mathematical model initially used was that of one interacting site. As seen in Figures 17; A, B, D, H, I and K, where this model was used, the calculated values, shown as open circles (o), were

observed to be in reasonable agreement with the experimental values. For the zinc binding isotherms of bran ex oxalate, cell walls, lignocellulose alkali soluble hemicellulose and DMSO soluble hemicellulose, seen in Figures 17; C, E, F, G and J, a two site interacting model was found to give reasonably close agreement with the experimental data.

The fact that reasonably good fits between the experimental and calculated data are obtained, as seen in figures 17A to K, using these rather simple mathematical models does not necessarily mean that these simple models are correct. It does indicate, however, that these saturation curves *per se* do not demand at this stage of study more complex models. However, improvements in removing the inherent experimental errors in the system used and the collection of more data would enhance the probability of establishing the number of binding sites. If more data on the saturation curves were obtained, processing it, using the more powerful computer program 'Massact', developed by McIntosh and McIntosh (1980), would enable the testing of models of up to two specific binding sites and one non-specific or low affinity, unsaturable site, to be determined.

The investigations of zinc binding to wheat bran, its fibre fractions and to phytate provide the answer to the question; Is it fibre or phytate that is the main metal binder? This information can be obtained from the results of the calculations, shown in Table XXII, of the maximum amount of zinc binding to wheat bran, its various fibre fractions and to phytate.

Wheat bran, bran ex ethanol and bran ex oxalate were found not to

bind large amounts of zinc. Their zinc binding capacities were 167 ± 12.7, 142.3 ± 4.4 and 148.3 ± 50.0 µM/g respectively. The two samples, bran and bran ex ethanol, in which the water soluble component had not been removed, appear to bind zinc more strongly, as indicated by the low ligand 50% values of 43.1 ± 7.3 and 11.4 ± 0.8 µM/g respectively. In contrast, the bran ex oxalate sample, ligand 50% value of 264 ± 211 µM/g, suggest that zinc was less strongly associated than by the samples in which the water soluble components had been retained. While the zinc binding capacities of these three samples are similar, weaker metal binding is associated with the absence of the water soluble fraction.

Cellulose has been suggested to be a factor in reducing metal bioavailability (Ranhotra et al. 1979). The results from this study indicate that cellulose bound the least zinc at 57.4 ± 5.3 µM/g. The high ligand 50% value of 110.2 ± 32.4 µM/g relative to the binding capacity suggest that the binding was weak, possibly only surface adsorption occurring.

The cell wall preparation had a high zinc binding capacity of 1012.6 ± 193 µM/g. The high ligand 50% value of 765.3 ± 327 µM/g and the possibility of multicomplex composition, suggest that weakly bound, nonsaturable sites are a significant feature. Considering the cell wall surface appearance and what appear to be large surface area, as observed in Figures 12C and D, and the macromolecular structure of the walls, composed of linked polymers of cellulose, hemicellulose and lignin (Fengel, 1976), it would not be unreasonable to expect multiple binding sites and significant nonspecific binding to occur.

The wheat bran arabinoxylans; alkali soluble and DMSO soluble, and the lignocellulose fraction, had different zinc binding capacities. The alkali soluble arabinoxylan had the lowest zinc binding capacity at 227.9 ± 61.4 µM/g, lignin at 510 ± 41.9 µM/g and DMSO soluble arabinoxylan at 5089 ± 921 µM/g. The low metal binding capacity of the alkali soluble arabinoxylan is consistent with the findings of Rendleman and Grobe (1982) and Molloy and Richards (1971). The greater zinc binding capacity of the DMSO soluble polymer and the lignocellulose may be the result of a higher phenolic content. However, the greater binding capacity was not observed in the lignocellulose. This should not be taken to imply that all lignins are relatively poor metal binders. The method used to prepare the fraction may have caused degradation or induced chemical changes to the phenolics. The zinc binding by all three of the fractions, were weak, as indicated by the high ligand 50% values. The binding of zinc to these substances may be a reflection of the retained macromolecular structure from within the plant cell wall, the linking of the arabinoxylans by phenolics, such as ferulic acid, as proposed by Markwalder and Neukom (1976), forming a complex three dimensional structure with a large internal and external surface area, within which there may be multiple sites for metal entrapment. At this juncture, the relevance these polymers may have on metal bioavailability can only be speculative. However, it is suggested that these polymers appear not to hold metals sufficiently strongly to significantly impede metal bioavailability.

The water soluble components from wheat bran appear to be the major metal binding substances. The findings of Frølich and Asp

(1980) and of Rendleman and Grobe (1982) suggest that either the water soluble fibre or phytate could be the predominant metal binding substance. The results from this study have shown that both substances have a high zinc binding capacity. The 'purified' water soluble polysaccharide bound 4038 ± 216 µM of zinc/g with a low ligand 50% value of 94.6 ± 1.1 relative to the binding capacity. Phytate bound 6582.4 ± 192 µM of zinc/g with a ligand 50% value of 582.4 ± 101. Therefore, it is concluded that both substances are significant binders of zinc, phytate binding more than the fibre, but zinc being more strongly bound to the water soluble fibre.

For interpretation of the zinc binding experiments an idealised system has been employed. However due to the considerable complexity, a more detailed interpretation of the mechanisms of metal binding to components of dietary fibre should await further research.

General Discussion

From epidemiological studies, it has been suggested that the aeteology of a variety of diseases such as carcinoma of the colon could be related to the quantity of non-absorbed plant cell wall carbohydrate in the diet (Burkitt, 1973). The indigestible dietary components, which as a group are referred to as 'dietary fibre', have an extremely complex chemistry (ARC/MRC, 1974). This complexity has resulted in fibre being chemically ill-defined, thus impeding the verification or repudiation of the fibre hypothesis.

Polysaccharides of various composition, have been marketed as 'fibre' and represented as suitable for use in food manufacturing or as dietary supplements. However, it has been suggested that all types of fibre may not be equivalent (Trowell, 1973). This suggestion is supported by the findings in Part I of this thesis. It was found that of all the species of plants examined, only from wheat bran was there a high proportion of arabinose and xylose containing polysaccharides extracted with water and oxalate, whereas the polysaccharides obtained from fruits and vegetables were generally high in pectic polymers.

Previous studies have indicated that pectic polymers are digested within the human digestive tract before they reach the start of the colon (Holloway *et al.* 1978). Arabinoxylans, however, were found to be relatively resistent to digestion (Holloway *et al.* 1980), thus, supporting the hypothesis that the physiological influence of dietary fibre from wheat bran may be different than that from fruits and vegetables.

This does not imply that the physical and chemical properties of pectic polymers do not contribute to the total properties of 'dietary fibre', such as slowing or reducing intestinal absorption of other nutrients.

Cognisance must be taken of the fact that relatively crude isolation procedures have been used to obtain plant cell wall polysaccharides. This has possibly resulted in the presence of more than one population of polysaccharides being present in a particular sample (e.g. the presence of amylase resistant starch, that may have given negative KI-I_2 spot tests, particularly in the hot water and oxalate extracts). The separation of polydisperse material isolated from cell walls can be difficult, particularly if there are few distinguishing features on which to base a fractionating procedure, and the avoidance of physical or chemical modification of the polysaccharides.

A pilot investigation of hemicellulose fractionation was undertaken using a similar method as Maekawa (1975) for the fractionation of xylans on a DEAE-cellulose column, with borate and alkali as elutants. The result of this separation is shown for the lignified wheat bran hemicellulose in appendix A and the delignified hemicellulose in appendix B. It was observed that two overlapping polysaccharide peaks and three phenolic/protein peaks were obtained from the nondelignified hemicellulose, whereas multiple polysaccharide peaks and one phenolic/protein peak was obtained after delignification, confirming the polysaccharide degradation of delignification. Carbohydrate analysis by GLC of the individual

fractions showed only that they were arabinoxylans; no other distinguishing features were detected.

This method of fractionation however, was found to be unsatisfactory to obtain homogenous polysaccharide fractions from cell walls, to enable further studies to be undertaken, due to the low yields and slow flow times (> 52 hours). Please see Part I Discussion and Appendix A and B.

The fibre extracts obtained from all the foods bound copper, iron and zinc, particularly the water soluble polysaccharides. This finding supports the observations made in human and animal nutritional studies, that high fibre diets can lead to deficiencies of iron, zinc (Reinhold et al. 1973) and copper (Hartmans and Bosman, 1970). Calcium deficiencies have also been described (Ismail-Beigi et al. 1977). The mechanism for calcium's reduced bioavailability is not readily apparent from these experiments, as calcium was displaced from all the fibres by copper, iron and zinc. Possibly the mechanisms operative within the digestive tract are more complex than can be observed using rather simple in vitro experimental techniques.

The greater metal binding capacity of the water soluble polysaccharides, which contain a high uronic acid content, could be ascribed to the 'egg-box model' mechanism of Rees and his colleagues (Morris et al. 1982; Thom et al. 1982, Powell et al. 1982 and Furda, 1979). Calcium ions fitting between two or more chains of unesterified polygalacturonosyl residues in such fashion that the calcium ions chelate to the oxygen atoms of four galacturonosyl residues distributed between two galacturonan chains.

The possibility of polysaccharide conformational metal binding sites that do not involve uronic acid residues, is supported by the observation of positive cooperativity of zinc binding to the wheat bran water soluble polysaccharide. The low uronic acid content of this polysaccharide (less than 1.0%) precludes specific ionic binding to uronic acid carboxyl groups or interchain chelation in the manner proposed for calcium binding to poly-L-guluronate (Kohn, 1975) or poly-L-galacturonate (Smidsrød and Haug, 1972) as the metal binding mechanism.

Other mechanisms of metal binding to neutral polysaccharides within plant cell walls may be quantitatively more important than to pectins. The metal binding to hemicellulose fractions (i.e. alkali soluble polysaccharides) used in this study, were free from calcium pectate. As pectins were removed in the extraction sequence using the chelating agent ammonium oxalate, as shown in Figure 2 and as evidenced by the low uronic acid content of the alkali soluble fractions (Table II). It would have been advantageous to have investigated the effects of other chelating agents such as EDTA on pectin removal and to have used the chelate solubilised polysaccharides for metal binding studies. The water soluble polysaccharides were used for the metal binding study, based on the observation of their high uronic acid content and that this fraction had the least possible degradative steps applied to it. However against this, it may well be justifiably argued that the slightly higher methoxyl content in about half the foods of this fraction, would mitigate against this choice. It would be of some interest to

compare the metal binding characteristics of the water soluble and oxalate soluble extracts.

The metal binding characteristics of neutral sugars have been investigated (Angyal, 1980). It has been shown that reasonably strong calcium complexes to neutral sugars can occur. The strongest binding occurring with sugars in which the hydroxyl groups are in such an arrangement that they can form bonds of equal length with the cation, such as an axial, equatorial and axial hydroxyl groups on a six-membered ring (Angyal and Mills, 1979). Methylation of the axial or equatorial hydroxyl groups reduces complex formation; however when a methyl group is carried by an anomeric oxygen atom, as in methyl glycosides, the effect on complex formation is less severe. Presumably because the anomeric oxygen atom can draw electrons from the ring oxygen atom (Angyal and Mills, 1979). Binding of other metals and sugars was investigated by Moulik and Khan (1975). They found that reducing sugars bind more strongly than nonreducing ones and that the free-energy change is of the order of hydrogen bonding. Moulick and Khan (1975) considered the binding to be electrostatic in nature where the carbohydrate acts as the centre of positive charges. Bourne *et al.* (1971) found that copper (II) complexes were formed predominantly to glycoside aldoses and under the conditions they used, reducing sugars (except D-ribose, D-xylose and D-gulose) did not. Angyal *et al.* (1974) also investigated complex formation of alditols with metal ions in aqueous solutions by electrophoresis and then by NMR. They found that complex formation of alditols with metal ions occurs at three consecutive oxygen atoms which are in a gauche-gauche

arrangement. The energy required to form this arrangement, by rotation around carbon-carbon bonds, determining the extent of complex formation. However for substantial complex formation to occur, concentrations of sugars and cations are higher than usually found in living organisms are required. Moreover the sugars which readily form complexes rarely occur in Nature, those which are common do not complex well. In Angyal's (1980) view, most of the work on complex formation with cations has so far been carried out with monosaccharides, the behaviour of polysaccharides has not yet been systematically studied. Other possible mechanisms which may account for the high calcium content observed in the hemicellulose fractions (IF). Such as physical entrapment in the complex network of cell walls and/or by surface adhesion (Canning and Madix, 1984) may occur. Further experimentation would be required to confirm and establish the dominant binding mechanism for calcium to these water insoluble fractions.

The zinc binding capacity of the wheat bran water soluble extract was higher in the polysaccharide fraction that had the greater proportions of rhamnose, mannose and galactose. The mechanism of zinc binding to this extract may involve conformational changes as has been observed in the aggregation of soluble arabinoxylans (Dea et al. 1973; Blake and Richards, 1970) or aggregation of heteroglycans (Dea et al. 1977), possibly enhanced by the presence of divalent metal ions.

A preliminary investigation of the aggregation of the wheat bran water soluble polysaccharide, in the presence of sodium and zinc, was undertaken (Appendix C). This confirmed that aggregation occurred only in the presence of the divalent metal ions.

Further investigations of the mechanisms of divalent metal mediated aggregation of low uronic acid polysaccharides could be undertaken using a variety of methods such as; circular dichromism (Morris *et al.* 1973; Morris *et al.* 1980), determination of critical chain length (Kohn, 1975), selective removal of various sugar residues such as rhamnose, influence of ferulic acid (Markwalder and Neukom, 1976), effect of length and distribution of arabinose side chains.

Investigations of the metal binding properties of various fibre extracts from foods, reported in this thesis, has tended to emphasise the negative aspects of high fibre diets. However, studies of the fibre extracts indicate that alkali extracted hemicellulose from wheat bran may be a physiologically 'active' substance that can reduce intestinal transit times (Holloway and Greig, 1984).

At this juncture it is not readily apparent what contribution these *in vitro* metal binding studies, using isolated fibre components, will have towards the development of 'new fibre' food products in which the impact of reduced bioavailability of copper, iron and zinc are minimised. For further discussion please see appendix D.

Conclusions

1. Water soluble fibres extracted from bean, cabbage, kumera, lettuce, onion, peach, pear, pumpkin, tomato, wheat bran, white clover, lucerne and ryegrass contained significantly more galactose, uronic acid and methoxyl groups than alkali soluble fibres, which contained significantly more xylose.
2. Appreciable yields of alkali soluble fibres can be obtained from foods and grasses without prior delignification.
3. Chlorite delignification and solubilisation in N-methyl-morpholine-N-oxide results in extensive degradation of arabinoxylans.
4. Low yields of wheat bran hemicellulose are obtained on extraction with dimethylsulphoxide.
5. During extraction of plant tissue a complex transition of morphological changes, up to the addition of alkali, left cell walls intact. Contact of plant tissue with alkali resulted in complete disintegration of cell walls.
6. Water soluble fibres from all the foods bound more zinc than the water insoluble fibres. Copper binding also predominantly occurred on the water soluble fibres except in the case of lettuce, tomato and wheat bran. The binding occurred with a displacement of calcium, magnesium and manganese.
7. Phytate, a water soluble polysaccharide and a DMSO soluble arabinoxylan are the major zinc binding components of wheat bran.
8. Zinc is bound more strongly, by a mechanism of positive cooperativity, to the water soluble polysaccharide than to the DMSO soluble arabinoxylan or to phytate.

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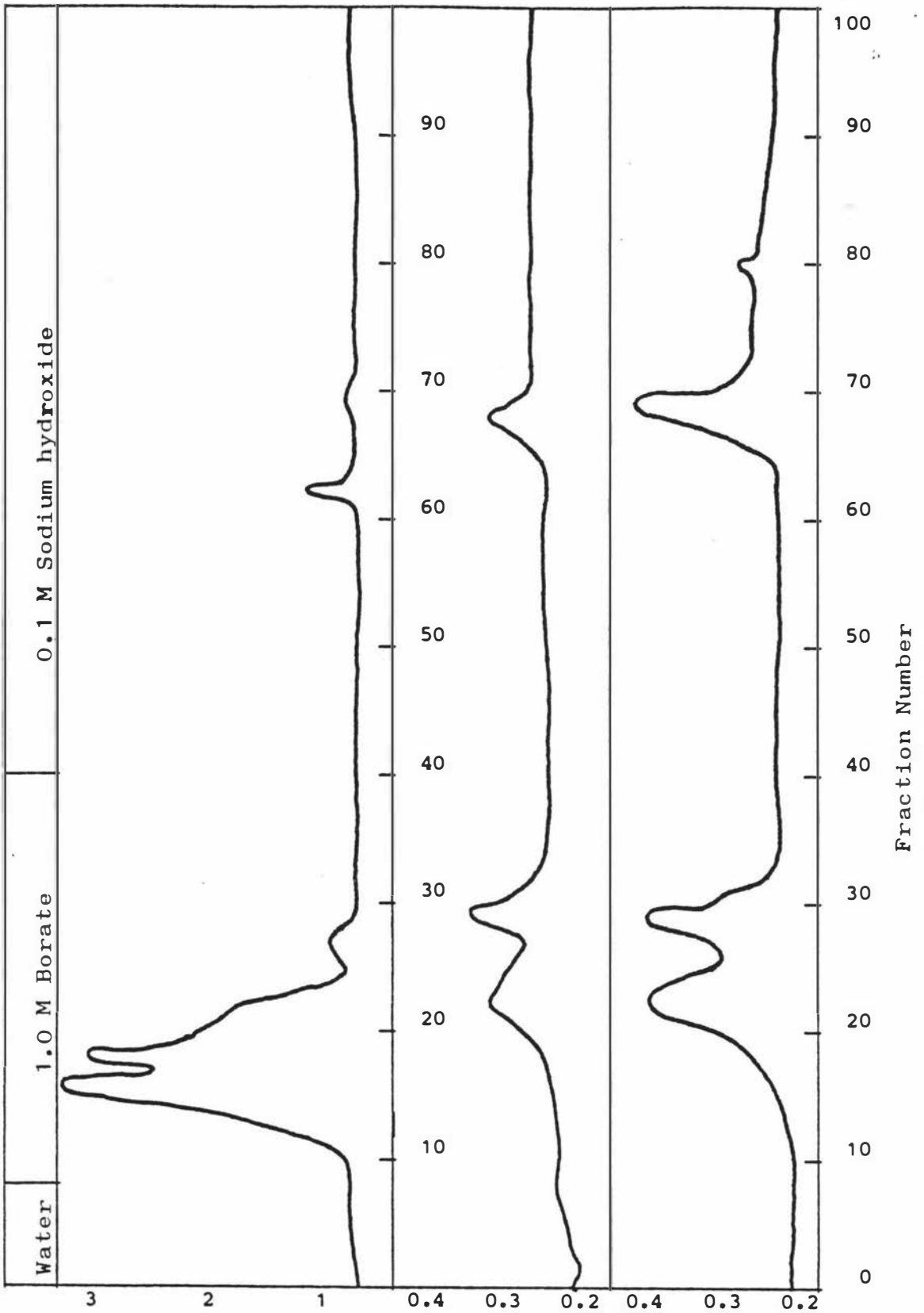
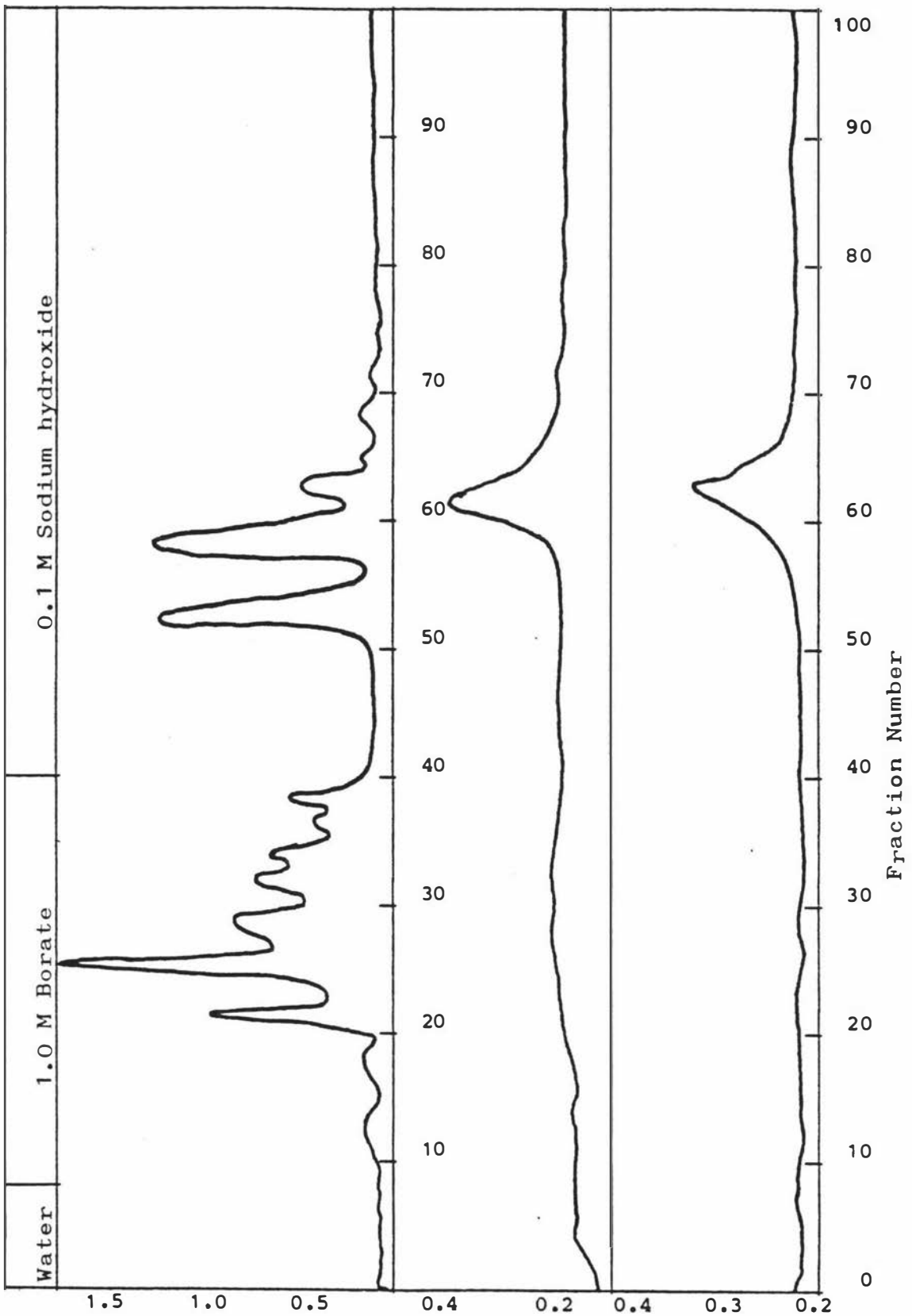


APPENDIX A

FRACTION COLLECTION OF WHEAT BRAN HEMICELLULOSE (NOT DELIGNIFIED) AFTER PASSING THROUGH DEAE-CELLULOSE COLUMN



APPENDIX B

FRACTION COLLECTION OF WHEAT BRAN HEMICELLULOSE (AFTER DELIGNIFICATION) PASSED THROUGH DEAE-CELLULOSE COLUMN

Appendix C

Investigation of the influence of sodium and zinc ions on the aggregation of the water soluble polysaccharide from wheat bran.

The possibility of metal induced precipitation of the water soluble wheat bran polysaccharide was investigated using a 5% solution of the ammonium sulphate prepared polysaccharide as described in Part V. One sample of this solution (2.5 ml) was added to a solution of pH6.2 phosphate buffer (as used in Part III) made from 0.012 ionic strength sodium chloride (0.702g/100 ml). A similar sample was also added to a pH6.2 phosphate buffer solution made from 0.012 ionic strength zinc chloride solution (0.555g/100 ml).

The solutions were mixed for 10 minutes, then allowed to stand for one hour. The pH was determined and found to have remained constant. A white fluffy precipitate was observed in the zinc solution. No precipitate was observed in the sodium chloride solution.

This brief supplementary study would appear to support the suggestion proposed in Part V, that divalent metal mediation of polysaccharide aggregation is a possible mechanism by which zinc and the wheat bran water soluble polysaccharide associate.

The possibility of other divalent metal species binding by a similar mechanism is only speculative at this juncture. It is probable however, that magnesium, calcium, manganese, iron and copper may also bind to this polysaccharide. The consequence of reduced bioavailability has, as yet, to be investigated.

APPENDIX D

Current and Future Trends in the Field of Dietary Fibre Research.

1. (a) As indicated in Part I and IV of this thesis, the uncontentious theory and experimental evidence known in 1982 can be classified into the following:

   (i) Recognition of the possible advantages of high fibre diets.

   (ii) Recognition of the complex nature of dietary fibre.

   (iii) Recognition of the difficulties in existing methods of chemical analysis of dietary fibre.

   (iv) Recognition of the possible contra-indications of high fibre diets.

   (b) Matters which were regarded as problematic or yet to be solved at that time were:

   (i) Identification of physiologically active components of dietary fibre.

   (ii) Resolution of the conflict of the whole cell wall food concept verses the polymeric components of cell walls as dietary fibre.

   (iii) Development of methods of analysis that accurately reflects the dietary fibre content of foods.

   (iv) Determination of the significance of metal binding to dietary fibre and its comparison with other food components such as phytate, in reducing metal bioavailability.

2. Development in the field of dietary fibre research from 1982 to 1985 relating to plant cell wall composition and fibre analysis.

   (i) The isolation of plant cell wall components reported in Part I of this thesis can be compared with that reported by Stevens and Selvendran (1984a and b). These authors used similar methods of extraction (hot water, ammonium oxalate and alkali) to obtain cell wall polymers from carrot and cabbage and also used similar methods of analysis as reported in this thesis. Both studies (Stevens and Selvendran 1984a and results shown in table II, p.22)

show that cabbage pectin is composed predominantly of arabinose and uronic acid. Stevens and Selvendran (1984a) however developed their study further by doing a partial acid hydrolysis and methylation to show that the major pectic polysaccharides had rhamnogalacturonan backbone to which a highly branched arabinan was linked. Simlarly the extraction and composition of wheat bran 'fibres' reported in this theses are supported by the results of the study on cereals obtained from different extraction rates (Nyman et al, 1984).

While these studies and others (reviewed by Selvendran 1984 and McNeil et al, 1984) indicate compositional differences and similarities of extractable cell wall polymers and have been used to postulate cell wall models (Keegstra et al, 1973; Monro et al, 1976). Their value in defining dietary fibre in intact cell walls remains problematical. The approach that is required to be used is to investigate cell wall components 'insitu' thus avoiding the possibility of degradation. To enable such investigations to be undertaken, new techniques are required. One strong possibility is for the development and application of solid state NMR to 'whole' plant cell walls. Such an approach would enable intact cell walls to be compared with their 'isolated' components.

(ii) The development of methods of analysis has been one of the most active areas of research on dietary fibre and is possibly the only area of significant advance in recent times. There has been a general adoption of sequential enzymatic extraction of fibre (Asp et al, 1983), which has been standardised in an International interlaboratory study (Prosky et al, 1984). This procedure also removes starch enzymaticly, however a heat stable amylase (Termamyl) in combination with the α - amylase activity of pancreatin appears to remove almost all of the starch in the fibre residues (Asp et al, 1983). The application of these enzymes in the experimental procedures described in Part I would appear to have been advantageous, particularly for the high starch containing foods such as sweet potato. The

possibility of more efficient removal of the lipid content in high lipid containing foods, with a solvent such as petroleum ether (Asp et al, 1983), instead of refluxing in ethanol, may have avoided a possibility of amylase resistant lipid-starch complexes being formed (Hanna and Lelievre, 1975).

Dietary fibre components extracted by the procedures described in Part I and by the use of pepsin and pancreatin (Asp et al, 1984) were found to contain a residual protein content. Thus supporting the suggestion that some protein could be considered as a component of dietary fibre (Dintzis, 1982).

3. In my assessment the significant publications on metal binding to dietary fibre, that have become available since this thesis was written at the end of 1984, are as follows. O'Dell's 1984 review on the bioavailability of trace elements, considered that the complexities of the interactions of trace minerals bioavailability makes this topic a 'research frontier for the indefinite future'. Among the areas of concern that he discussed was the effects of phytate, fibre and of oxalic acid on zinc bioavailability. But he left open the question of the relative importance of these substances on zinc absorption.

The significance of wheat bran on zinc absorption in the human diet was investigated by Farah et al (1984) using zinc tolerance tests and whole body monitoring to measure 7-day percentage retention of zinc in humans. They concluded that wheat bran leads to a significant reduction in zinc absorption which could eventually induce a state of zinc deficiency. What they did not determine however was the identity of the component of wheat bran that reduced zinc retention.

It has been suggested that reducing the phytate content of bran by leavening of bread, increases zinc absorption from such breads (Nävert et al, 1985). However it was not shown that all or most of the zinc in bread becomes available after the phytic acid content is reduced. For as was shown by the results described in Part V of this thesis, both phytate and the watersoluble fibre from bran are significant binders of zinc. Nävert et al, (1985) did not determine

the watersoluble fibre content of the breads, thus they would not have been able to assess the significance of the fibre content on zinc bioavailability.

The importance of brans in reducing mineral absorption is also confirmed by the study of Dintzis et al (1985). They investigated the influence of brans on the excretion of copper, iron, zinc and calcium. This was done by determining the mineral contents of corn bran, wheat bran and soyabean hulls before and after passage through the human gastrointestinal tract. They found that copper, iron and zinc increased from starting levels by factors from two to four and calcium levels increased ten fold. They however did not determine changes in magnesium, manganese, sodium or potassium levels. The increase in calcium excretion was not assessed in terms of a possible increased release of calcium by the digestive tract secretions.

The binding of minerals to fibre is supported by the findings of Whitehead et al (1985). This group investigated the distribution of ten nutrient elements in herbage. The cell wall fraction was prepared by mechanical disintegration of undried material and the mineral contents of cell walls were determined. They found that calcium comprised from 11.3 to 51.8% of the total mineral content, magnesium 6.4 to 27.6%, potassium 0.1 to 1.7% and 'substantial' though more variable proportions of the trace elements iron, manganese, zinc and copper were also present in the cell wall fractions. The extent of water and 80% ethanol solubility on the mineral content of the cell walls was determined. Their results showed that not all of the calcium and to a lesser extent magnesium present in cell walls were readily extractable in water or ethanol. This would support the findings described in Part IV that calcium is also bound to insoluble polymers such as the hemicelluloses. The location of the insoluble minerals was however not determined by Whitehead et al (1985). Also they considered that water-solubility equates with bioavailability. However the possibility of non-absorbable chelating substances such as oxalate, phytate and 'nondigestable' watersoluble polysaccharide were not considered.

The binding of minerals was however considered by Lee and Garcia-Lopez (1985). They found that iron, copper and zinc but not magnesium were bound by neutral (NDF) and acid (ADF) detergent fibre obtained from Pinto beans. They reported that iron binding increased with increasing ph, this probably reflects the formation of the insoluble ferric hydroxide which would decrease the apparent 'free' iron content. With that reservation their results would support my findings of copper, iron and zinc being the minerals predominately bound by fibre.

4. In conclusion, one of the main directions of recent research in the field of dietary fibre has been towards looking in greater detail at the composition of plant cell walls. However, as yet it has not been shown that any new or potentially unusual chemical constituent of cell walls or its components correlate with the physiological properties of dietary fibre. While it may be argued that more detailed studies of cell walls eventually lead to a greater understanding of dietary fibre, this approach is limited because the effects of 'dietary' fibre are physiological. The experimental determination of the physiological/ pharmacological aspects of specific components of plant foods that are resistant to digestion within the human small bowel is an aspect of fibre research that has been essentially ignored. It is this aspect that future research will have to be orientated towards to enable the experimental substantiation of the dietary fibre hypothesis.

The wider interdisciplinary approach that I have taken, and as documented within this thesis, has been an endeavour to cross the impediment of traditional academic boundaries and to isolate specific 'defined' components of plants and by undertaking 'invitro' experiments to determine some of their properties such as metal binding, that will form a basis for future 'invivo' studies. For at the present time we do not know which specific components are responsible for the desirable physiological actions such as faecal bulking, retention of water in the stool, binding of bile salts and alteration of intestinal flora which are attributed to dietary fibre. Until these components are identified, the dietary fibre hypothesis can not be verified.

APPENDIX E

The nonlinear curve fitting computer program used to calculate binding capacities (μM/g), ligand 50% values (point at which half maximum binding occurs in μM/g and the Hill coefficients for the binding curves were carried out using the same computer program as developed by Hardman (1983). This procedure is a development of the Daniel and Wood's (1980) method that was based on the Marquardt maximum neighbourhood method of nonlinear curve fitting program (Marquardt, 1963).

To access the general purposes program, observations were entered from the keyboard of a Cromemco (64K RAM) to set up data files. Zinc binding data, in the form of total zinc (bound zinc plus free zinc expressed in μM Zn/g of fibre) and free zinc (total zinc minus bound zinc in μM/g of fibre) were entered. The model to be used for the 'fit' had to be supplied by the user and linked with the main program and other subroutines to produce a command file. The simplest model of one interacting site was always used initially and then the more complex two interacting site model was investigated. In the case of zinc binding to bran ex oxalate, cellwalls, lignocellulose and DMSO hemicellulose, the two site interacting model gave lower residual values. Thus indicating the two site model gave a 'closer' fit to the experimental data. The user had to specify for each run the number of coefficients in the model and the number of independent variables. Starting guesses were required since this is an iterative fitting method. The initial estimates did not have to be accurate as the Marquardt procedure is sufficiently robust to obtain the 'correct' value. However process time was saved if close estimates of the maximum binding were entered. The main program handled the input control parameters, initial estimates and data. A subroutine (GAUSHS) controlled the curve fitting procedure by calling other subroutines to calculate eigenvalues, carry out matrix inversion and then to output the results of the 'fit'. The Hill coefficients and binding capacities were then checked by manually plotting and calculating using the procedures described in Dahlquist (1978).

Copies of the computer program on disk, are available from Hardman (1983) and of Wood's version from several computer libraries as listed in Daniel and Wood (1980).

A copy of the output of zinc binding to wheat bran purified watersoluble fibre is shown below.

```
PURIFIED WATER SOL
NO OF INDEPENDENT VARIABLES (X) = 1

TO CHANGE INPUT FORMAT FROM (6G,6X,6F10.2)                    ENTER 1,
OTHERWISE RETURN:
 1     .9400      -.8932      1.0000
                NON-LINEAR LEAST-SQUARES CURVE-FITTING PROGRAM


PURIFIED WATER SOL    DEP.VAR. MIN.Y=  4.300E+00  MAX.Y=  3.490E+03
RANGE Y= 3.486E+03

                                                    95%CONFIDENCE LIMITS
IND.VAR(I)  NAME        COEF.B(I)     S.E.COEF.    T-VALUE   LOWER          UPPER
     1      MWPOUND   3.55836E+03     3.59E+02       8.9     2.67E+03     4.45E+03
     2      HILL N    1.89770E+00     3.23E-01       5.9     1.18E+00     2.62E+00
     3      LIG 50%   7.60684E+01     1.58E+01       4.9     4.14E+01     1.11E+02
NO. OF OBSERVATIONS                    13
NO. OF COEFFICIENTS                     3
RESIDUAL DEGREES OF FREEDOM            10
RESIDUAL ROOT MEAN SQUARE        158.26734924
RESIDUAL MEAN SQUARE           25048.55078125
RESIDUAL SUM OF SQUARES        250485.50000000
-----ORDERED BY COMPUTER INPUT-----   ----------ORDERED BY RESIDUALS----------
OBS. NO.  OBS. Y  FITTED Y  RESIDUAL   OBS. NO. OBS. Y  FITTED Y    RESID.   SEQ.
 0001      4.300    11.280    -6.980    0011  1299.900   946.445   353.455     1
 0002     10.400    11.280     -.880    0013  3490.300  3409.596    80.704     2
 0003     15.600    24.340    -8.740    0010   645.200   576.098    69.102     3
 0004     30.600    38.554    -7.954    0009   523.900   523.767    10.133     4
 0005     66.100   142.413   -76.313    0002    10.400    11.280     -.880     5
 0006    141.200   315.555  -173.373    0001     4.300    11.280    -6.980     6
 0007    303.600   356.224   -52.624    0004    30.600    38.554    -7.954     7
 0008    473.300   704.560  -231.260    0003    15.600    24.340    -8.740     8
 0009    523.900   523.767    10.133    0007   303.600   356.224   -52.624     9
 0010    645.200   576.098    69.102    0005    66.100   142.413   -76.313    10
 0011   1299.900   946.445   353.455    0012  2576.500  2736.229  -147.829    11
 0012   2576.500  2724.329  -147.829    0006   141.200   315.555  -173.373    12
 0013   3490.300  3409.596    80.704    0008   473.300   704.560  -231.260    13

END OF PROBLEM
PURIFIED WATER SOL
WOULD YOU LIKE A PLOT (ENTER 1), TERMINAL OUTPUT RETURN
OR CRT PLOT, ENTER (ENTER 2)   1
?
```