

Determination of Dietary Fibre as Non-starch Polysaccharides With Gas-Liquid Chromatographic, High-performance Liquid Chromatographic or Spectrophotometric Measurement of Constituent Sugars

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Methods for the measurement of dietary fibre as non-starch polysaccharides (NSP) are described. A common enzymic removal of starch and acid hydrolysis of the NSP to their constituent sugars are followed by one of three alternative techniques, gas-liquid chromatography, high-performance liquid chromatography or spectrophotometry, for measurement of the released sugars. The results obtained by the three methods are in good agreement for a wide range of raw and processed foods. NSP compose approximately 90% of the plant cell-wall material and are therefore a good index of this material. Values for NSP therefore provide a good marker for a diet rich in fruit, vegetables and high-extraction cereal products associated with health and recommended in dietary guidelines. Values for total, soluble and insoluble NSP may be obtained with any of the end-point techniques, and the detailed information obtained from the chromatographic methods is useful in studies of the relationship between the intakes of various types of NSP and health. The causes of some potential interferences in the spectrophotometric assay, especially from processed foods, have been identified and eliminated. The rapid spectrophotometric version is suitable for food labelling purposes and for quality control, and the changes described have made it more robust.

Keywords: Non-starch polysaccharide; gas-liquid chromatography; high-performance liquid chromatography; spectrophotometry; neutral sugar

Introduction

The original dietary fibre hypothesis was simply that eating a diet rich in unrefined plant foods was protective against a range of diseases that were common in Western societies, where much of the 'dietary fibre' was removed by food refining techniques. Over the years, a large body of evidence has accumulated that tends to support the hypothesis.¹ It is now clear that the component of the diet for which the term 'dietary fibre' was coined is largely plant cell-wall material, and that the several physiological effects of eating this material depend on both its chemical identity and its physical structure. This does not lead easily to a definition that will allow direct quantification by analysis. However, recognition that the majority (approximately 90%²) of the plant cell-wall material in human foods can be defined chemically as non-starch polysaccharides offers a solution. Plant foods contain a wide variety of polysaccharides (polymers of sugars joined by glycosidic linkages), which may be separated into two groups on the basis of both structure and function. The starches are storage polysaccharides and are chemically identifiable as

polymers of glucose with α -glucosidic linkages. The remainder, which contain no α -glucosidic linkages, are mostly structural components of the plant cell walls, and may be referred to conveniently as the non-starch polysaccharides (NSP). The direct measurement of NSP in plant foods thus provides a good index of the plant cell-wall material present in the food. Dietary fibre measured as NSP provides useful values for food labelling, because NSP are markers for the type of diet rich in fruit, vegetables and high-extraction cereal products advocated in dietary guidelines, thus providing the consumer with valuable information. NSP values are obtained by chemical analysis of a defined group of carbohydrates and are therefore suitable for regulatory purposes.

The procedure is for dietary fibre measured as NSP, using enzymic-chemical methods, and has evolved from the principles laid down by Southgate³ in 1969. Starch is completely removed enzymically and NSP are measured as the sum of the constituent sugars released by acid hydrolysis. The sugars may be measured by gas-liquid chromatography (GLC)⁴ or by high-performance liquid chromatography (HPLC),⁵ giving values for individual monosaccharides, or a single value for total sugars may be obtained by spectrophotometry.⁶ Values may be obtained for total, soluble and insoluble NSP, and a small modification allows cellulose to be measured separately.

The procedure allows the measurement of NSP within a working day (8 h elapsed time) by the spectrophotometric version, or within 1.5 working days with the chromatographic assays. Preparation of the hydrolysate for analysis is virtually a single-tube procedure, which minimizes analytical losses, and no special skill or equipment is needed for the spectrophotometric version.

The methods have been used successfully for the analysis of a very wide range of foods.^{7,8} The information obtained by the chromatographic procedures (Table 1) is valuable for interpreting the results of physiological and epidemiological studies where disease may be related to the type of dietary fibre. When detailed information is not required, however, values for NSP can be obtained by spectrophotometry. This paper presents some recent improvements to the Englyst NSP method.

Experimental

Modification to the Procedure as Previously Described^{4-6,9-12}

Precipitation at pH 5.2 and 2

Samples of free galacturonic acid (GalA) (10, 20, 30, 40, 50 and 100 mg) were weighed into four sets of test-tubes. Sodium acetate buffer (10 cm³) containing calcium chloride (pH 5.2, see Reagents) was added to two sets of tubes (A and B), and acetate buffer without additional calcium chloride was added

to the other two sets (C and D). Ethanol was added to tubes A and C to a final concentration of 80%. Hydrochloric acid (5 mol dm⁻³, 0.15 cm³) was added to the remaining tubes (B and D) followed by acidified ethanol to a final concentration of ethanol of 80% v/v (as described below). The tubes were centrifuged for 10 min at 1500g and the supernatants were removed by aspiration. The residues were washed with ethanol and dried with acetone as described below and then dissolved in 30 cm³ of 2 mol dm⁻³ sulfuric acid. The amount of GalA recovered was determined by the reducing sugar method as described below.

The effect of pH on coprecipitation of GalA was investigated by adding 10, 20, 50 and 100 mg of GalA to 300 mg of sugar beet fibre. Following dispersion in acetate buffer, the beet fibre was precipitated in 80% v/v ethanol at either pH 2 or 5.2 and then washed with ethanol and dried with acetone as described below. The dried residue was subjected to treatment with sulfuric acid and the NSP content was determined using the reducing sugar technique as described below.

Hydrolysis of NSP

Fibrim (purified soy fibre), α-cellulose and Fiberform (purified wheat bran) were subjected to treatment with 12 mol dm⁻³ sulfuric acid for 5, 10, 30 and 60 min at 35 °C followed by dilution to 2 mol dm⁻³ and kept at 100 °C for 1 h.

Samples of Fibrim, pectin and sugar beet were subjected to treatment with 12 mol dm⁻³ sulfuric acid for 60 min at 35 °C followed by dilution to 2 mol dm⁻³ and kept at 100 °C for 1 h. The hydrolysates were cooled to room temperature and to 0.5 cm³ portions were added 0.5 cm³ of dimethyl glutarate

solution and 4.2 cm³ of 1 mol dm⁻³ NaOH, as described below.

To test for the optimum concentrations of pectinase, 0.1 cm³ of diluted pectinase solutions (1 cm³ of pectinase solution plus 5, 10, 15, 20, 25, 50, 100 or 200 cm³ of water) was added. The samples were kept at 50 °C for 20 min and the recovery of NSP was measured using the reducing sugar technique (see below). The time course (5, 10, 15, 20, 25, 30 and 60 min) for the maximum release of NSP by pectinase was tested at dilutions of 1 + 10, 1 + 25 and 1 + 50.

Interference in spectrophotometry

Portions (0.5 cm³) of solutions (1.5 mg cm⁻³) of the Maillard reaction products 5-hydroxymethyl-2-furaldehyde and 2-furaldehyde and of xylose, glucose and galacturonic acid were analysed by the reducing sugar method (see below), with heating times of 5, 10, 15 and 20 min.

Cornflakes, white bread and wholemeal bread were subjected to NSP analysis as described below with end-point determination by GLC, and by the reducing sugar method with heating times of 5, 10, 15 and 20 min.

Materials and Methods

High-purity reagents and distilled, de-ionized water or water of equivalent purity should be used throughout the method.

Reagents common to the GLC, HPLC and spectrophotometric procedures

Acidified ethanol, absolute and 85% v/v. Add 1 cm³ of 5 mol dm⁻³ hydrochloric acid per dm³ of ethanol.

Table 1 Values for the mean (g per 100 g of sample), the minimum difference from the mean (Diffmin), the maximum difference from the mean (Diffmax) and the standard deviation (s) for the NSP constituent sugars of cabbage, wheat bran and haricot beans from individual analytical runs of portion A only (total NSP)

Sample	Parameter	Neutral sugars*							Total sugars by GLC	Uronic acids	Total NSP
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc			
Cabbage		0.4	0.1	2.8	1.2	0.7	2.0	8.4	15.5	7.9	23.4
		0.3	0.1	2.6	1.2	0.7	1.7	7.8	14.2	7.9	22.1
		0.3	0.0	2.7	1.3	0.7	1.7	8.4	15.1	8.1	23.2
		0.3	0.1	2.4	1.1	0.7	1.9	7.4	13.9	6.6	20.6
	Mean	0.3	0.1	2.6	1.2	0.7	1.8	8.0	14.7	7.6	22.3
	Diffmin	0.0	0.1	0.2	0.1	0.0	0.1	0.6	0.8	1.0	1.8
	Diffmax	0.0	0.0	-0.1	-0.1	0.0	-0.1	-0.4	-0.4	-0.5	-0.9
	s	0.05	0.05	0.19	0.06	0.01	0.14	0.47	0.73	0.68	1.31
Bran		0.1	0.0	8.4	15.5	0.3	1.2	11.3	36.7	2.0	38.7
		0.0	0.0	8.8	16.0	0.3	1.1	10.7	37.0	2.0	38.9
		0.0	0.0	8.6	16.2	0.3	0.9	10.9	36.8	1.3	38.1
		0.0	0.0	8.5	15.7	0.3	1.0	10.9	36.4	1.5	37.9
		0.0	0.0	8.7	16.9	0.3	1.0	11.1	38.1	1.6	39.7
	Mean	0.0	0.0	8.6	16.0	0.3	1.1	11.0	37.0	1.7	38.7
	Diffmin	0.0	0.0	0.2	0.6	0.0	0.1	0.2	0.6	0.3	0.8
	Diffmax	0.0	0.0	-0.2	-0.9	0.0	-0.1	-0.1	-1.1	-0.3	-1.0
Beans		0.03	0.02	0.17	0.57	0.02	0.11	0.22	0.64	0.28	0.72
		0.2	0.4	6.0	1.8	0.4	1.4	4.6	14.8	4.4	19.2
		0.2	0.4	6.3	2.0	0.5	1.4	4.6	15.2	4.3	19.5
		0.2	0.4	6.0	2.0	0.5	1.2	4.4	14.6	3.6	18.2
		0.2	0.3	6.2	2.2	0.6	1.1	4.3	14.8	3.6	18.4
		0.2	0.4	6.5	2.2	0.5	1.1	4.6	15.6	3.9	19.5
		0.1	0.3	5.9	1.9	0.3	1.1	4.7	14.3	4.0	18.3
		0.0	0.3	6.2	1.9	0.4	1.2	4.7	14.6	3.4	18.1
		0.1	0.4	5.8	1.9	0.5	1.2	4.4	14.2	3.8	18.0
	Mean	0.1	0.4	6.1	2.0	0.5	1.2	4.5	14.8	3.9	18.6
	Diffmin	0.1	0.0	0.3	0.1	0.1	0.1	0.2	0.6	0.5	0.6
	Diffmax	-0.1	0.0	-0.4	-0.3	-0.1	-0.2	-0.2	-0.8	-0.4	-0.9
	s	0.07	0.02	0.23	0.15	0.07	0.12	0.15	0.46	0.34	0.65

* Rha = Rhamnose; Fuc = fucose; Ara = arabinose; Xyl = xylose; Man = mannose; Gal = galactose; and Glc = glucose.

Dimethylphenol solution. Dissolve 0.1 g of 3,5-dimethylphenol $[(\text{CH}_3)_2\text{C}_6\text{H}_3\text{OH}]$ in 100 cm^3 of glacial acetic acid.

Enzyme solution I. Take 2.5 cm^3 of Termamyl (Novo Nordisk Bioindustries, Farnham, Surrey, UK), dilute to 200 cm^3 with pre-equilibrated sodium acetate buffer, mix and keep the solution in a 50 °C water-bath. Prepare the solution immediately before use.

Enzyme solution II. Place 1.2 g of pancreatin (Pancrex V powder from Paynes and Byrne, Greenford, Middlesex, UK) in a 50 cm^3 tube, add 12 cm^3 of water, vortex mix initially and then mix for 10 min with a magnetic stirrer. Vortex mix again, then centrifuge at 1500g for 10 min. Take 10 cm^3 of the (cloudy) supernatant, add 2.5 cm^3 of pullulanase (Promozyme, Novo Nordisk Bioindustries) and vortex mix. Prepare the solution immediately before use and keep it at room temperature.

Glass balls, 2.5–3.5 mm diameter. BDH, Poole, Dorset, UK.

Sand, acid-washed 50–100 mesh. BDH.

Sodium acetate buffer, 0.1 mol dm^{-3} , pH 5.2. Dissolve 13.6 g of sodium acetate trihydrate ($\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$) in and dilute to 1 dm^3 with water. Adjust to pH 5.2 with 0.1 mol dm^{-3} acetic acid. To stabilize and activate enzymes, add 4 cm^3 of 1 mol dm^{-3} calcium chloride to 1 dm^3 of buffer.

Sodium chloride solution–boric acid solution. Dissolve 2 g of NaCl and 3 g of boric acid in 100 cm^3 of water.

Sodium phosphate buffer, 0.2 mol dm^{-3} , pH 7. Adjust 0.2 mol dm^{-3} Na_2HPO_4 to pH 7 with 0.2 mol dm^{-3} NaH_2PO_4 .

Sulfuric acid, concentrated.

Sulfuric acid, 12 mol dm^{-3} . *N.B.* Caution should be taken in making this reagent. Measure 280 cm^3 of water into a 2 dm^3 thermally resistant beaker. Place the beaker into a bowl of ice–water in a fume cupboard and slowly add 390 cm^3 of concentrated sulfuric acid, with stirring.

Sulfuric acid, 2 mol dm^{-3} . Add 5 cm^3 of 12 mol dm^{-3} sulfuric acid to 25 cm^3 of water. Allow to cool to room temperature before use.

Sulfuric acid, 2.4 mol dm^{-3} . Add 5 cm^3 of 12 mol dm^{-3} sulfuric acid to 20 cm^3 of water and mix. Allow to cool to room temperature before use.

Reagents used only in the GLC procedure

Ammonia solution–sodium tetrahydroborate solution. Prepare 6 mol dm^{-3} ammonia solution containing 200 mg cm^{-3} of sodium tetrahydroborate (NaBH_4). Prepare immediately before use.

Bromophenol Blue solution, 0.4 g dm^{-3} . BDH.

GLC internal standard solution, 1 mg cm^{-3} . Weigh 500 mg of allose (dried to constant mass under reduced pressure with phosphorus pentoxide) to the nearest 1 mg. Dilute to 500 cm^3 with 50% saturated benzoic acid to give a 1 mg cm^{-3} solution. The solution is stable at room temperature for several months.

GLC stock sugar mixture. Weigh (all sugars dried to constant mass under reduced pressure with phosphorus pentoxide), to the nearest 1 mg, 0.52 g of rhamnose, 0.48 g of fucose, 4.75 g of arabinose, 4.45 g of xylose, 2.3 g of mannose, 2.82 g of galactose, 9.4 g of glucose and 2.79 g of galacturonic acid. Place them in a 1 dm^3 calibrated flask and dilute to volume with 50% saturated benzoic acid. The solution is stable at room temperature for several months.

Reagents used only in the HPLC procedure

Internal standard solution. Deoxygalactose, 10 mg dm^{-3} in water.

HPLC stock sugar mixture. Weigh (all sugars dried to constant mass under reduced pressure with phosphorus pentoxide), to the nearest 1 mg, 0.52 g of rhamnose, 0.48 g of

fucose, 4.75 g of arabinose, 4.45 g of xylose, 2.3 g of mannose, 2.82 g of galactose and 9.4 g of glucose. Place them in a 1 dm^3 calibrated flask, dissolve them in water, add 10 mg of thiomersal as a preservative and dilute to volume with water. Store at 4 °C.

Sodium hydroxide solution, 50% m/v. Low in carbonate (BDH).

Solution I. Prepare a 1.6 $\text{cm}^3 \text{ dm}^{-3}$ solution of the 50% m/v NaOH solution in high-purity water. Sparge the water with helium for 5 min before and during addition of the NaOH solution.

Reagents used only in the spectrophotometric procedure

Chromogenic reagent (DNS). Dissolve 10 g of 3,5-dinitrosalicylic acid and 300 g of potassium sodium tartrate in approximately 300 cm^3 of high-purity water plus 400 cm^3 of 1 mol dm^{-3} NaOH. Dissolve by stirring (overnight) and dilute to a final volume of 1 dm^3 with high-purity water. Sparge for 10 min with helium or nitrogen, or de-gas using an ultrasonic bath. Store in well capped opaque bottles and keep for 2 d before use. The reagent is stable at room temperature for at least 6 months.

Dimethylglutaric acid solution, 0.5 mol dm^{-3} . Add 98.5 g of 5 mol dm^{-3} NaOH to 8.0 g (weighed to the nearest 1 mg) of dimethylglutaric acid and dilute to 100 cm^3 with water at room temperature. Add 1 volume of the solution to 1 volume of 2 mol dm^{-3} sulfuric acid; the pH should be between 3.6 and 3.8 at room temperature.

Stock sugar mixture. Make the stock sugar solution by weighing (all sugars dried to constant mass under reduced pressure with phosphorus pentoxide), to the nearest 1 mg, 10.185 g of arabinose, 5.145 g of glucose and 2.16 g of galacturonic acid. Place them in a 1 dm^3 calibrated flask and dilute to volume with 50% saturated benzoic acid. The solution is stable at room temperature for several months.

Pectinase solution. EC 3.2.1.15 (Novo Nordisk Bioindustries). Add 9 volumes of water to 1 volume of pectinase solution.

Method

The procedure as described here provides the following options (Fig. 1).

(1) GLC procedure: measures NSP as the sum of neutral sugars obtained by GLC and uronic acids measured separately.

(2) HPLC procedure: measures NSP as the sum of neutral sugars and uronic acids.

(3) Spectrophotometric procedure: measures NSP as reducing sugars.

(4) Spectrophotometric procedure with separate measurement of uronic acids.

Treatments Common to the GLC, HPLC and Spectrophotometric Procedures

Sample preparation

All samples should be finely divided (to pass a 0.5 mm mesh) so that representative sub-samples may be taken. Foods with a low water content (<10 g of sample) may be milled, and foods with a higher water content may be homogenized wet or milled after freeze-drying. Analysis of three sub-samples, A, B and C, allows separate values to be obtained directly for total NSP, insoluble NSP and cellulose, respectively. Soluble NSP is determined as the difference between total and insoluble NSP. Portions A and B are treated identically throughout the procedure, except in steps 3 and 4. The third portion, C, is needed only if a separate value for cellulose is

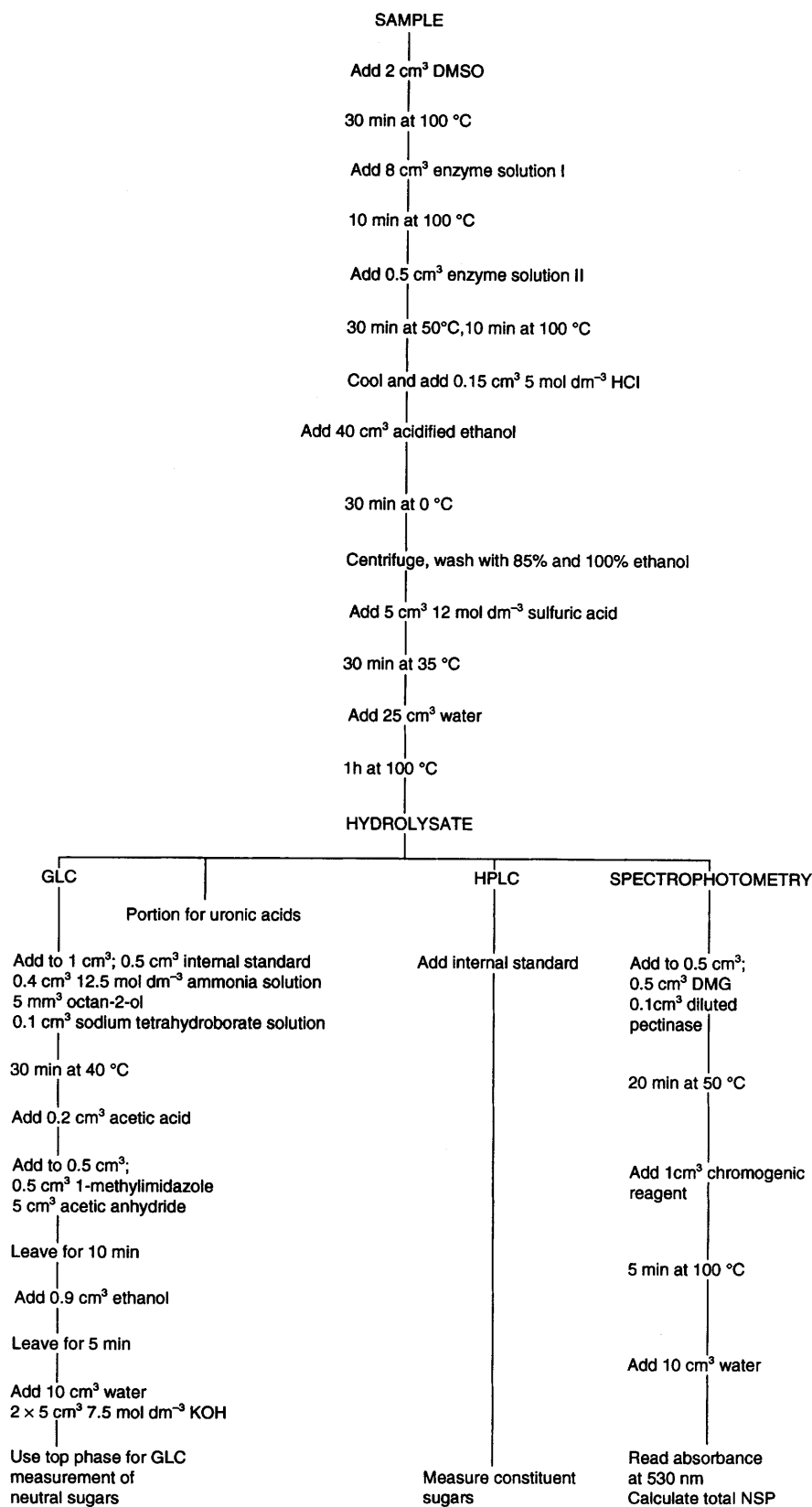


Fig. 1 Flow chart illustrating the common hydrolysis steps and the optional end-points of the analytical scheme. DMG, dimethylglutaric acid solution. Soluble dietary fibre (DF) is calculated as total DF – insoluble DF. For the measurement of insoluble dietary fibre, replace the 40 cm³ of ethanol with 40 cm³ of the 0.2 mol dm⁻³ sodium phosphate buffer (pH 7) and extract for 30 min at 100 °C.

required; it is carried through steps 1, 2 and 3 of the procedure, then one proceeds to step 5.2.

Step 1.1. Weigh, to the nearest 1 mg, between 50 and 1000 mg depending on the water and NSP content of the sample (to give not more than 300 mg of dry matter; *e.g.*, 300 mg are adequate for most dried foods but smaller amounts should be used for bran and purified fibre preparations) into 50–60 cm³ screw-topped glass tubes. Add 300 (± 20) mg of acid-washed sand and approximately 15 glass balls to each. If the sample is dry (85–100 g of dry matter per 100 g of sample) and contains less than 10 g of fat per 100 g of sample, proceed to step 2; otherwise, go to step 1.2. (It is recommended that all analyses be carried out in duplicate.)

Step 1.2. Add 40 cm³ of acetone, cap the tubes and mix several times over a 30 min period. Centrifuge at 1000g for 10 min to obtain a clear supernatant and remove by aspiration as much of the supernatant liquid as possible without disturbing the residue. Vortex mix vigorously to ensure that the residue is dispersed thinly around the bottom 5 cm of the tube. Place the rack of tubes in a pan of water at 75 °C in a fume-cupboard. Remove the tubes singly and vortex mix vigorously at frequent intervals until the tubes and residues are dry.

Dispersion and enzymic hydrolysis

Pre-equilibrate sufficient acetate buffer at 50 °C (8 cm³ required per sample).

Step 2.1. Add 2 cm³ of dimethyl sulfoxide (DMSO) to the dry sample, cap the tube, and *immediately* mix the contents using a vortex mixer, treating each tube in turn. It is essential that all the sample is wetted and no material is encapsulated or adhering to the tube wall before proceeding. When DMSO has been added to all the tubes, vortex mix three or four times during a 5 min period. Vortex mix and immediately place two tubes in a boiling water-bath. Remove after 20 s, vortex mix and immediately replace the tubes in the bath. Repeat this for subsequent pairs of tubes until all the tubes are in the bath and leave them for 30 min from that time. During this period, prepare enzyme solutions I and II (the volumes given are suitable for 24 samples).

Step 2.2. Remove *one tube at a time*, vortex mix, uncap and *immediately* add, by positive displacement 8 cm³ of enzyme solution I (kept at 50 °C), cap the tube, vortex mix thoroughly, ensuring that no material adheres to the tube wall, and replace it in the boiling water-bath. Leave the tubes there for 10 min, timed from the last addition of enzyme. Transfer the rack of tubes to the 50 °C water-bath. After 3 min, add 0.5 cm³ of enzyme solution II to each tube and mix the contents thoroughly to aid distribution of the enzyme throughout the sample. Replace the tubes in the 50 °C water-bath and leave them there for 30 min. Mix the contents of each tube continuously or after 10, 20 and 30 min. Transfer the rack of tubes to the boiling water-bath and leave them there for 10 min.

Precipitation and washing of the residue for measurement of total NSP

Only sample portion A is given this treatment.

Step 3.1. Cool the samples by placing in ice–water. Add 0.15 cm³ of 5 mol dm^{−3} hydrochloric acid and vortex mix thoroughly two or three times during a 5 min period with samples being replaced in the ice–water. Add 40 cm³ of acidified absolute ethanol and mix well by repeated inversion, then leave in ice–water for 30 min. Centrifuge at 1500g for 10 min to obtain a clear supernatant liquid. Remove by decanting or by aspiration as much of the supernatant liquid as possible, without disturbing the residue, and discard it.

Step 3.2. Add approximately 10 cm³ of acidified 85% v/v ethanol to the residue and vortex mix. Dilute to 50 cm³ with

acidified 85% v/v ethanol and mix thoroughly by repeated inversion. Centrifuge and remove the supernatant liquid as above. Repeat this stage using 50 cm³ of absolute ethanol.

Step 3.3. Add 30 cm³ of acetone to the residue and vortex mix thoroughly to form a suspension. Centrifuge and remove the supernatant liquid as in step 3.1.

Step 3.4. Vortex mix vigorously to ensure that the residue is dispersed thinly around the bottom 5 cm of the tubes. Place the rack of tubes in a pan of water at 75 °C in a fume-cupboard. Remove the tubes singly and vortex mix vigorously at frequent intervals, to ensure that the residue in each tube is finely divided, until the tube and residue are dry. Place the rack of tubes in a fan oven at 80 °C for 10 min to remove any trace of acetone. It is essential that the residues and tubes are completely free of acetone.

Extraction and washing of the residue for measurement of insoluble NSP

Only sample portion B is given this treatment.

Step 4.1. After the treatment with enzymes in step 2, add 40 cm³ of sodium phosphate buffer. Place the capped tubes in a boiling water-bath for 30 min. Mix continuously or a minimum of three times during this period. Remove the tubes and equilibrate to room temperature in water. Centrifuge and remove the supernatant liquid as described in step 3.1.

Step 4.2. Add approximately 10 cm³ of water and vortex mix. Dilute to approximately 50 cm³ with water and mix well by repeated inversion. Centrifuge and remove the supernatant liquid as above. Repeat this stage using 50 cm³ of absolute ethanol. Proceed as described for steps 3.3 and 3.4.

Acid hydrolysis of the residue from enzymic digestion

Step 5.1. Add 5 cm³ of 12 mol dm^{−3} sulfuric acid to one tube and immediately vortex mix vigorously; ensure that all the material is wetted. Repeat this for each tube in turn. Once the acid has been added to all the tubes, vortex mix again and place all the tubes into a water-bath at 35 °C. Leave the tubes at 35 °C for 30 min with vigorous vortex mixing after 5, 10 and 20 min to disperse the cellulose. Add 25 cm³ of water rapidly and vortex mix. Place into a boiling water-bath and leave for 1 h, timed from when boiling recommences; mix after 10 min. Cool the tubes in tap water.

Step 5.2. This modification allows the separate measurement of cellulose and non-cellulosic polysaccharides (NCP). To portion C treated in steps 1–3, add 30 cm³ of 2 mol dm^{−3} sulfuric acid and mix. Place in a boiling water-bath and leave for 1 h, timed from when boiling recommences, stirring continuously or after 10 min. A value for cellulose may be obtained as the difference between glucose (measured by GLC or by glucose oxidase) for sample portions A and C. NCP is calculated as the difference between total NSP and cellulose.

Breaks in the procedure

The procedure may be halted at either of the following stages: (1) after precipitation, washing and drying the starch-free residue in steps 3 and 4; the residue may be stored for long periods; or (2) after the hydrolysis with 2 mol dm^{−3} sulfuric acid in step 5; the hydrolysate may be kept at 4 °C for 48 h.

GLC Procedure

This assay includes the measurement of neutral sugars by GLC and the separate measurement of uronic acids by spectrophotometry.

Measurement of neutral NSP constituents by GLC

To prepare the standard sugar mixture, mix 1.0 cm³ of the GLC stock sugar solution and 5 cm³ of 2.4 mol dm⁻³ sulfuric acid. Treat 2 × 1.0 cm³ of this standard sugar mixture for calibration of GLC in parallel with the hydrolysates from step 5 of the procedure.

Prepare the alditol acetate derivatives for chromatography as follows. Add 0.50 cm³ of internal standard (1 mg cm⁻³ allose) to 1.0 cm³ of the cooled hydrolysates from step 5 and to 2 × 1 cm³ of the standard sugar mixture; vortex mix. Place the tubes in ice-water, add 0.4 cm³ of 12 mol dm⁻³ ammonia solution and vortex mix. Test that the solution is alkaline (add a little more ammonia solution if necessary but replace the ammonia solution if more than 0.1 cm³ extra is required), then add approximately 5 mm³ of the antifoam agent octan-2-ol and 0.1 cm³ of the ammonia solution-sodium tetrahydroborate solution and vortex mix. Leave the tubes in a heating block or in a water-bath at 40 °C for 30 min, then remove, add 0.2 cm³ of glacial acetic acid, and mix again. Transfer 0.5 cm³ to 30 cm³ glass tubes, add 0.5 cm³ of 1-methylimidazole to each. Add 5 cm³ of acetic anhydride and vortex mix immediately. Leave the tubes for 10 min for the reaction to proceed (the reaction is exothermic and the tubes will become hot). Add 0.9 cm³ of absolute ethanol, vortex mix and leave for 5 min. Add 10 cm³ of water, vortex mix and leave for 5 min. Add 0.5 cm³ of Bromophenol Blue solution. Place the tubes in ice-water and add 5 cm³ of 7.5 mol dm⁻³ potassium hydroxide; a few minutes later add a further 5 cm³ of 7.5 mol dm⁻³ potassium hydroxide, cap the tubes and mix by inversion. Leave until the separation into two phases is complete (10–15 min) or centrifuge for a few minutes. Draw part of the upper phase into the tip of an automatic pipette; if any of the blue phase is included, allow it to separate, then run it out of the tip before transferring a portion of the upper phase alone into a small (autoinjector) vial.

Carry out conventional GLC measurement of the neutral sugars. At the beginning of each batch of analyses, equilibrate with the isothermal elution conditions for at least 1 h. Carry out several calibration runs to check that the response factors are reproducible. Inject 0.5–1 mm³ of the alditol acetate derivatives.

GLC conditions

The following conditions are used: injector temperature, 275 °C; column temperature, 220 °C; detector temperature, 275 °C; carrier gas, helium; and flow rate, 8 cm³ min⁻¹. Under these conditions, a chromatograph fitted with a flame-ionization detector and, preferably, autoinjector and computing integrator, using a Supelco SP-2330 wide-bore capillary column (30 m × 0.75 mm i.d.) or a Supelco SP-2380 wide-bore capillary column (30 m × 0.53 mm i.d.) at 210 °C will allow accurate determination of the individual sugars in the standard sugar mixture within 8 min. For calibration, use the following ratios for the combination of the standard sugar mixture and internal standard (allose):

Sugar	Sugar mixture actual/ mg dm ⁻³	NSP recovery (%)	Sugar mixture apparent/ mg dm ⁻³	Calibration ratio
Rhamnose	520	52	1000	1
Fucose	480	96	500	0.5
Arabinose	4750	95	5000	5
Xylose	4450	89	5000	5
Mannose	2300	92	2500	2.5
Galactose	2820	94	3000	3
Glucose	9400	94	10000	10
Allose (int. std.)				3

The 'actual' column shows the amount of each sugar in the mixture and the 'apparent' column shows the values to be used for calibration taking into account the recovery of NSP constituents. The calibration ratio column gives the ratio of sugars to the internal standard after the addition of allose to the standard sugar mixture as described in the text (the experimental evidence for the recovery values has been published⁴).

Calculation of neutral sugars

The amount of individual sugars (expressed as grams of polysaccharide per 100 g of sample) is calculated as

$$\text{Amount} = \frac{A_T M_I R_F \times 100}{A_I M_T} \times 0.89$$

where A_T and A_I are the peak areas of the sample and the internal standard, respectively, M_I is the mass (in mg; here 15 mg; total hydrolysate 30 cm³ × 0.5 mg of allose) of the internal standard, M_T is the mass (in mg) of the sample, R_F is the response factor for individual sugars obtained from the calibration run with the sugar mixture and internal standard (allose) treated in parallel with the samples and 0.89 is the factor for converting experimentally determined values for monosaccharides to polysaccharides. All the calculations may be performed with a computing integrator.

Measurement of uronic acids by spectrophotometry

Make the standard solutions as follows. The standard sugar mixture in 2 mol dm⁻³ sulfuric acid, prepared as described for the GLC assay, contains, for the purpose of calibration, 500 µg cm⁻³ of galacturonic acid. To prepare the uronic acid standard solutions, place 0.5, 2.0 and 3.0 cm³ of this sugar mixture into separate tubes and dilute to 10 cm³ with 2 mol dm⁻³ sulfuric acid to give standards of 25, 100 and 150 µg cm⁻³ of galacturonic acid. Only the 100 µg cm⁻³ standard is required for routine analysis, and it may be kept at 5 °C for several weeks.

Place into separate tubes (40–50 cm³ capacity) 0.3 cm³ of blank solution (2 mol dm⁻³ sulfuric acid), 0.3 cm³ of each of the standard solutions and 0.3 cm³ of the sample hydrolysates, diluted if necessary (with 2 mol dm⁻³ sulfuric acid) to contain no more than 150 µg cm⁻³ of uronic acids (*e.g.*, no dilution for flour, 1:2 for bran and 1:5 for most fruits and vegetables). Add 0.3 cm³ of sodium chloride solution-boric acid solution and mix. Add 5 cm³ of concentrated sulfuric acid and vortex mix immediately. Place the tubes in a heating block at 70 °C and leave for 40 min. Remove the tubes and cool to room temperature in water (the tubes may be kept in the water for up to 1 h). Add 0.2 cm³ of dimethylphenol solution and vortex mix immediately. After 15 min measure the absorbance at 400 and 450 nm in the spectrophotometer against the blank solution. The timing for measurement of the absorbance of standards and samples should be identical. In practice, this is achieved by adding the chromogenic reagent at 1 min intervals. Subtract the reading at 400 nm from that at 450 nm, to correct for interference from hexoses. A straight line should be obtained when the differences in absorbance for the standards are plotted against concentration.

Calculation of uronic acids

The amount of uronic acids (expressed as grams of polysaccharide per 100 g of sample) is calculated as

$$\text{Amount} = \frac{A_T V_T DC \times 100}{A_S M_T} \times 0.91$$

where A_T is the difference in absorbance of the sample solution, V_T is the total volume of sample solution (in cm^3 ; here 30 cm^3), D is the dilution of the sample solution, C is the concentration of the standard (here 0.1 mg cm^{-3}), A_S is the difference in absorbance of the $100 \mu\text{g cm}^{-3}$ standard, M_T is the mass (in mg) of the sample and 0.91 is the factor for converting experimentally determined values for monosaccharides to polysaccharides.

Calculation of NSP

The amounts of total, soluble and insoluble NSP (in grams per 100 g of sample) are calculated as

Total NSP = neutral sugars calculated for portion A + uronic acids calculated for portion A

Insoluble NSP = neutral sugars calculated for portion B + uronic acids calculated for portion B

Soluble NSP = total NSP – insoluble NSP

It is recommended that results are expressed as grams of polysaccharides per 100 g of dry matter.

Breaks in the GLC procedure

The procedure may be halted at either of the following stages: (1) after acidification of the reduced samples; the samples may be stored at room temperature for 2–3 d; or (2) the acid hydrolysate in step 5 may be kept at 5°C for several weeks before the measurement of uronic acids.

Trouble-shooting for the GLC procedure

(1) Extra peaks on the chromatogram may be due to incomplete reduction of monosaccharides. Ensure an alkaline pH before adding NaBH_4 . Replace old NaBH_4 ; do not compensate for loss of activity by adding more NaBH_4 .

(2) Variations between replicate analyses may be caused by non-reproducible pipetting of the internal standard or hydrolysates. Test or calibrate dispensers by weighing 1 cm^3 replicates of water.

(3) If the response factors are not reproducible, this may be due to inaccurate pipetting of the sugar mixture and/or internal standard. Test or calibrate dispensers by weighing replicates of water.

(4) If values for glucose for samples of known composition are too high and are variable for replicates, this may be due to incomplete wetting of samples with DMSO in step 2.1. Vortex mix vigorously after adding DMSO and follow the protocol exactly.

(5) If values for glucose and uronic acids for samples of known composition are too low and are variable for replicates, this may be due to incomplete wetting of samples with 12 mol dm^{-3} sulfuric acid in step 5. Vortex mix vigorously before and after addition of sulfuric acid and at intervals during the incubation.

Measurement of NSP Constituents by HPLC

The NSP constituents in the hydrolysates may be separated and measured by HPLC with pulsed amperometric detection (PAD).⁵ If hexosamines are not present, a Dionex Carbpac PA-1 ($10 \mu\text{m}$) column ($250 \times 4 \text{ mm i.d.}$) is the column of choice for measuring neutral sugars; however, for mushrooms, mycoproteins and ileostomy effluents, where hexosamines are present, a Dionex Carbpac PA-100 ($10 \mu\text{m}$) column ($250 \times 4 \text{ mm i.d.}$) is the column of choice.⁵ In either instance, column switching should be used to avoid contamination of the analytical column with sulfate ions.⁵

Uronic acids may also be measured by HPLC, and this procedure is described in detail in the accompanying paper.¹³

Neutral sugars

To prepare the standard sugar mixture, mix 1.0 cm^3 of the HPLC stock sugar mixture and 5 cm^3 of 2.4 mol dm^{-3} sulfuric acid. Treat $2 \times 0.15 \text{ cm}^3$ of this standard sugar mixture for calibration of HPLC in parallel with the hydrolysates from step 5 of the procedure.

To 0.15 cm^3 of hydrolysate or the HPLC standard sugar mixture add 5 cm^3 of internal standard solution and mix well. Inject 25 mm^3 for analysis. Carry out several calibration runs to check that the response factors are reproducible.

Removal of sulfate ions

Under the conditions described here, sulfate ions are retained for 80 s on a Dionex AG5 guard column. Column switching after 60 s prevents sulfate ions from reaching the analytical column. Sulfate ions are purged from the guard column within 19 min, well within the total run time.

Detection

A Dionex Model PAD 2 detector was used for HPLC of neutral sugars and amino sugars. Monosaccharide detection was carried out with the following pulse potentials and durations: $E_1 = 0.05 \text{ V}$ ($t_1 = 300 \text{ ms}$); $E_2 = 0.60 \text{ V}$ ($t_2 = 120 \text{ ms}$); and $E_3 = -0.60 \text{ V}$ ($t_3 = 60 \text{ ms}$). The response time was 1 s and the detector output was set at 1000 nA .

Separation of neutral sugars (PA-1 column)

Elute with 23% v/v solution 1 from 0 to 3.5 min, a gradient from 23 to 1% v/v solution 1 from 3.5 to 4.5 min and with 1% v/v solution 1 from 4.5 to 30 min at a flow rate of $1 \text{ cm}^3 \text{ min}^{-1}$. Re-equilibrate with the starting conditions for at least 6 min between runs. Add (Dionex DQP-1 single-piston pump) 300 mmol dm^{-3} NaOH at a flow rate of $0.5 \text{ cm}^3 \text{ min}^{-1}$ to the column effluent before the PAD cell to minimize baseline drift and increase the analytical signal. Saturate the eluent with helium (Dionex eluent de-gas module) to minimize CO_2 absorption.

Regeneration of analytical and guard columns

Wash with 100 mmol dm^{-3} NaOH– 600 mmol dm^{-3} sodium acetate for 1 h at a flow rate of $1 \text{ cm}^3 \text{ min}^{-1}$. Then wash with 1 mol dm^{-3} NaOH ($1 \text{ cm}^3 \text{ min}^{-1}$) for 1 h but, to avoid contamination of the internal reference solution, do not allow this solution to pass through the detector.

Calculations

For calibration, use the following ratios for the combination of the standard sugar mixture and deoxygalactose, the internal standard (see step 6.2):

Sugar	Sugar mixture actual/ mg dm^{-3}	NSP recovery (%)	Sugar mixture apparent/ mg dm^{-3}	Calibration ratio
Rhamnose	520	52	1000	1
Fucose	480	96	500	0.5
Arabinose	4750	95	5000	5
Xylose	4450	89	5000	5
Mannose	2300	92	2500	2.5
Galactose	2820	94	3000	3
Glucose	9400	94	10000	10
Deoxygalactose (int. std.)				2

The ‘actual’ column shows the amount of each sugar in the mixture and the ‘apparent’ column shows the values to be used for calibration taking into account the recovery of NSP constituents.

The amounts of individual sugars (in grams per 100 g of sample) are calculated as

$$\text{Amount} = \frac{A_T M_I R_F \times 100}{A_I M_T} \times 0.89$$

where A_T and A_I are the peak areas of the sample and the internal standard, respectively, M_I is the mass of the internal standard if added to the whole sample (in mg; here 25 mg), M_T is the mass of the sample (in mg), R_F is the response factor for individual sugars obtained from a calibration run with the sugar mixture treated in parallel with the samples and 0.89 is the factor for converting experimentally determined values for monosaccharides to polysaccharides. All the calculations may be performed conveniently with a computing integrator. It is recommended that results are expressed as grams of polysaccharides per 100 g of dry matter.

Measurement of NSP Constituents by Spectrophotometry

To prepare the standard sugar mixture, place 0.50 cm³ of the spectrophotometric stock sugar mixture in a glass tube, add

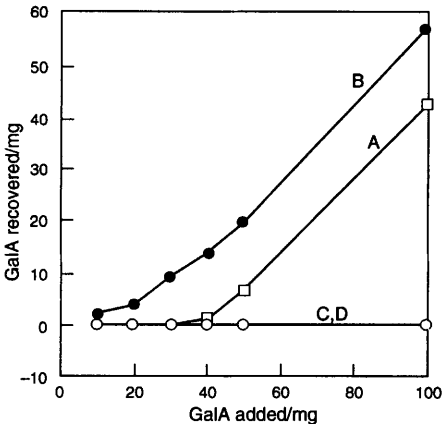


Fig. 2 Effect of precipitation of free GalA in 80% ethanol: at pH 5.2 (A) and at pH 5.2 with added calcium (B); at pH 2 and at pH 2 with added calcium (C and D).

2.50 cm³ of 2.4 mol dm⁻³ sulfuric acid and mix to give 3 cm³ of 3 mg cm⁻³ sugars standard solution in 2 mol dm⁻³ sulfuric acid. The chromogenic reaction is linear up to 3 mg cm⁻³ sugar. The absorbance of the test samples should not exceed that of the standard.

For measurement of total reducing sugars, place in separate glass tubes 0.5 cm³ of the standard sugar solution, 0.5 cm³ of the hydrolysate from step 5 and, in each of two tubes (blanks 1 and 2), 0.5 cm³ of 2 mol dm⁻³ sulfuric acid. Add 0.5 cm³ of dimethyl glutarate solution and vortex mix. Check the pH of one drop of blank 1; it should be between 3.5 and 4. If it is different from this, check the preparation of the 2 and the 12 mol dm⁻³ sulfuric acid and the dimethyl glutarate solution. If the pH is correct, add 0.1 cm³ of diluted pectinase solution, vortex mix and place all the tubes into a water-bath at 50 °C for 20 min. Cool the tubes to room temperature, add 0.1 cm³ of 3 mol dm⁻³ sodium hydroxide, vortex mix and leave for 5 min. Check that the pH of blank 1 is between 7 and 8. Add 1 cm³ of the dinitrosalicylate reagent to each tube and vortex mix. Place the tubes all at the same time into a briskly boiling water-bath, leave for 5 min. Remove the rack of tubes and cool to room temperature in water. Add 10 cm³ of water (at room temperature) and mix well by inversion (do not use a vortex mixer at this stage). Measure the absorbance in a spectrophotometer at 530 nm against blank 2.

[Sample blanks may be prepared by diluting the hydrolysates as described above, replacing the chromogenic reagent with water and reading the absorbance against water.

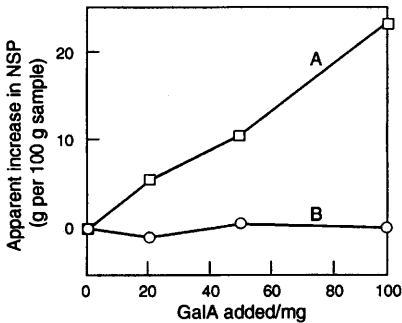


Fig. 3 Effect of coprecipitation of free GalA on the apparent recovery of NSP in sugar beet after precipitation with 80% ethanol at pH 5.2 (A) and pH 2 (B).

Table 2 Hydrolysis of NSP with 2 mol dm⁻³ sulfuric acid for 1 h after pre-treatment with 12 mol dm⁻³ sulfuric acid for the times indicated

Sample	Pre-treatment time/min	NSP/g per 100 g of sample*								
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UAc	Total
Fibrim	5	1.3	1.7	11.1	4.1	1.2	24.1	12.1	11.6	67.1
	10	1.3	1.7	11.5	4.3	1.2	25.2	12.7	11.8	69.7
	20	1.2	1.7	11.3	4.2	1.2	25.2	12.7	12.3	69.8
	30	1.2	1.8	11.2	4.1	1.2	24.8	12.5	11.6	68.3
	60	1.1	1.7	11.3	4.0	1.2	25.0	12.6	12.1	69.0
α -Cellulose	5	—	—	0.2	2.3	1.3	—	58.5	—	62.3
	10	—	—	0.2	2.2	1.4	—	92.8	—	96.6
	20	—	—	0.2	2.2	1.3	—	90.7	—	94.5
	30	—	—	0.2	2.5	1.4	—	92.5	—	96.6
	60	—	—	0.2	2.5	1.4	—	91.2	—	94.9
Fiberform	5	—	—	13.5	27.1	0.4	1.1	17.5	NM†	59.6
	10	—	—	13.5	27.1	0.4	1.1	17.9	NM	59.9
	20	—	—	13.5	27.2	0.4	1.1	17.8	NM	59.9
	30	—	—	13.7	26.8	0.4	1.1	18.0	NM	60.0
	60	—	—	13.2	25.4	0.4	1.1	17.6	NM	57.7

* Rha = Rhamnose; Fuc = fucose; Ara = arabinose; Xyl = xylose; Man = mannose; Gal = galactose; Glc = glucose; and UAc = uronic acids.
† NM, not measured.

Alternatively, and more conveniently, the absorbance of the undiluted hydrolysate can be measured against water and the value divided by 24.4 (the dilution of the hydrolysate after

Table 3 Time course of the release of uronic acids from polymers by treatment with pectinase. Time zero values are those after the treatments with sulfuric acid. The four test samples were a fibre extract from soya beans (Fibrim), sugar beet and two samples of citrus pectin from commercial sources. Each result is the mean of three samples (standard deviation in parentheses); all values are g per 100 g of dry matter

Time/ min	Fibrim	Sugar beet	Pectin 1	Pectin 2
0	70.6 (0.06)	70.6 (0.48)	69.9 (0.15)	73.6 (0.74)
5	71.1 (0.14)	71.4 (0.15)	70.7 (2.07)	75.3 (1.47)
10	71.3 (0.34)	73.3 (0.38)	75.4 (0.66)	80.6 (0.51)
15	72.0 (0.23)	72.8 (0.18)	76.2 (0.25)	80.5 (1.28)
20	72.0 (0.16)	72.7 (0.27)	75.9 (0.62)	80.9 (0.86)
30	71.8 (1.04)	73.0 (0.21)	75.9 (0.60)	80.4 (0.47)
60	72.1 (0.34)	73.0 (0.41)	76.1 (0.39)	80.4 (0.39)

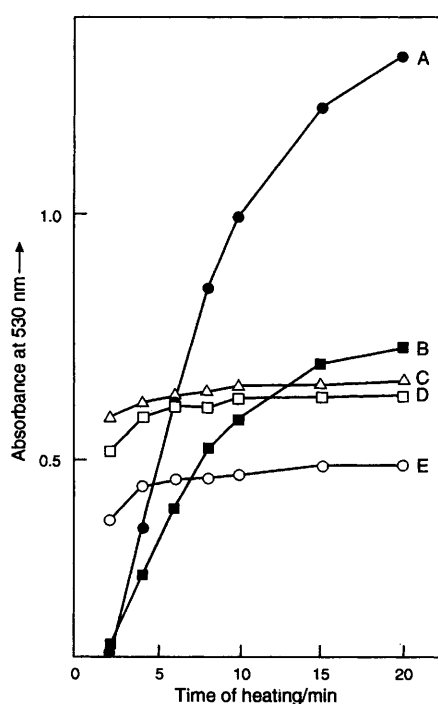


Fig. 4 Effect of heating time with DNS chromogenic reagent on solutions of: A, 5-hydroxymethyl-2-furaldehyde; B, 2-furaldehyde; C, xylose; D, glucose; and E, galacturonic acid.

Table 4 Effect of heating time with the chromogenic reagent (DNS) on measurements of total NSP for duplicate analyses of corn flakes, wholemeal bread and white bread. The values obtained by the GLC procedure are shown for comparison. All values are g per 100 g of dry matter

Sample	Time of heating with DNS/min				GLC
	5	10	15	20	
Corn flakes	0.9	1.2	1.3	1.4	0.9
	0.9	1.2	1.3	1.4	0.9
Wholemeal bread	10.1	10.3	10.2	10.4	9.8
	10.2	10.5	10.6	10.8	9.7
White bread	3.5	3.4	3.3	4.6	3.3
	3.5	3.8	3.9	4.2	3.4

addition of the chromogenic reagent and water). The absorbance of the test sample is then calculated by subtracting this value. When the hydrolysate is colourless and the NSP content is more than 5%, the sample blank is not required.]

Calculation of NSP

The amount of total NSP (portion A) and of insoluble NSP (portion B), in grams of polysaccharide per 100 g of sample, is calculated as

$$\text{Amount} = \frac{A_T V_T D F C \times 100}{A_S M_T} \times 0.89$$

where A_T is the absorbance of the sample solution (minus the absorbance of the hydrolysate blank if measured), V_T is the total volume of the sample solution (in cm^3 ; here 30 cm^3), D is the dilution of the sample solution ($D = 1$ if no dilution), F is the factor correcting the difference between the composition of monosaccharides in the standard sugar mixture and that in NSP of various types of plant foods [for the calculation of NSP in cereals (except oats), $F = 0.95$, in fruit and non-starchy vegetables, $F = 1.05$, and in starchy vegetables, oat products and unknown samples, $F = 1$; using the standard sugar mixture as specified and these factors make corrections for the 2–4% hydrolytic losses], C is the concentration (in mg cm^{-3} sugars) of the standard, A_S is the absorbance of the standard, M_T is the mass (in mg) of sample taken for analysis and 0.89 is the factor for converting experimentally determined monosaccharides to polysaccharides. The amount of soluble NSP is calculated as the difference between total NSP and insoluble NSP. It is recommended that results are expressed as grams of polysaccharides per 100 g of dry matter.

Correction Based on Separate Measurement of Uronic Acids

When a sample has a high content of uronic acids, e.g., pectin, a more accurate value for NSP may be obtained if uronic acids are measured separately. The standard sugar mixture for spectrophotometry as described here contains 12.5% of uronic acids, and it has been determined (data not shown) that this leads to a 17% underestimation of NSP when the NSP contains only uronic acids (using $F = 1$). Correction for the underestimation is straightforward if a separate value for uronic acids is obtained. If the NSP contains 12.5% of uronic acids, the standard sugar mixture is entirely appropriate and no correction is required; otherwise, the correction required is an increment or decrement to the NSP value obtained by spectrophotometry equivalent to 17% of the uronic acid content that is in excess of or less than 12.5% of the NSP, respectively.

The percentage of the NSP value to be corrected for is the difference (Δ) between the value for uronic acids (Z ; expressed as a percentage of the NSP value, X) and 12.5, and is calculated as

$$\Delta = [(Z/X) \times 100] - 12.5$$

and the correction factor Y is calculated as

$$Y = 0.17 [(X/100) \times \Delta]$$

which may be reduced to $0.17Z - 0.021X$, and then the value for total NSP after correction is $X + Y$.

Breaks in the spectrophotometric procedure

The procedure may be halted at either of the following stages: (1) after precipitation, washing and drying the starch-free residue; the residue may be stored for long periods; or (2) after the hydrolysis with 2 mol dm^{-3} sulfuric acid; the hydrolysate may be kept at 4°C for 48 h.

Trouble-shooting for the spectrophotometric procedure

(1) Variations between replicate analyses may be due to inaccurate pipetting (test or calibrate dispensers by weighing replicates of water) or to incomplete removal of acetone in step 3.4.

(2) If values are too high for samples of known composition and variable for replicates, this may be due to incomplete wetting of sample with DMSO in step 2.1. Mix vigorously immediately after addition of DMSO.

(3) If values are too low for samples of known composition and variable for replicates, this may be due to incomplete wetting of sample with 12 mol dm⁻³ sulfuric acid in step 5. Vortex mix vigorously both before and immediately after the addition of sulfuric acid, and at intervals during the incubation.

(4) If no colour is produced for standards and/or samples, this may be due to an error in the preparation of the sulfuric acid or sodium hydroxide solutions. Make new reagents. Test that the pH of the solution is between 7 and 8 before adding the chromogenic reagent solution.

Results and Discussion

Precipitation of NSP at pH 5.2 and 2

Mono- or oligosaccharides may be linked by non-covalent bonds, such as calcium bridges,¹⁴ to polysaccharides, forming complexes that are insoluble in 80% ethanol at pH 5.2, and therefore falsely included in values for NSP. These bonds are not present, however, in 80% ethanol at pH 2. Lowering the pH of the 80% ethanol has a further advantage, because some free galacturonic acid (GalA) is insoluble in 80% ethanol in the presence of sodium acetate buffer (0.1 mol dm⁻³, pH 5.2) but the GalA is resolubilized on addition of HCl to a final pH of 2 (Fig. 2). More GalA was precipitated when calcium ions were added to the sodium acetate buffer but again all the GalA was soluble when the buffer was adjusted to pH 2 (Fig. 2). Similarly, when pH 5.2 was used for the precipitation of sugar beet NSP in the presence of added free GalA, coprecipitation led to a falsely elevated value for NSP. However, NSP values were unaffected by addition of free GalA when the polysaccharides were precipitated at pH 2 (Fig. 3). When tested in this study, values for the total NSP

content of a polysaccharide mixture (duplicate analyses on four different occasions) were 91.2 ± 2.8 and 91.9 ± 2.1% m/m when the precipitation in ethanol was carried out at pH 5.2 and 2, respectively. These results show that the precipitation of NSP is as complete at pH 2 as it is at pH 5.2.

Hydrolysis of NSP

We have shown⁴ that cellulose and non-cellulosic NSP are completely dispersed and hydrolysed by treatment with 12 mol dm⁻³ sulfuric acid for 1 h at 35 °C, followed by treatment with 2 mol dm⁻³ sulfuric acid for 1 h at 100 °C. The treatment with 12 mol dm⁻³ sulfuric acid for 1 h was previously used to ensure the complete hydrolysis of samples that had been subjected to the destarching and washing steps, as it was found that some samples aggregate during the final drying with acetone, hindering the access of 12 mol dm⁻³ sulfuric acid, which therefore required prolonged treatment to ensure complete dispersion and hydrolysis. Independently, Hoebler *et al.*¹⁵ showed that a wide range of samples, including microcrystalline cellulose and sunflower husks, could be dispersed completely by treatment with 12 mol dm⁻³ sulfuric acid for 30 min at 25 °C. Table 2 gives the results for a range of samples treated with 12 mol dm⁻³ sulfuric acid for various times at 35 °C, followed by treatment with 2 mol dm⁻³ sulfuric acid for 1 h at 100 °C.

Fibrim [purified soy fibre that was found to contain uronic acid polymers that were difficult to hydrolyse enzymically (see later)], Fiberform (highly lignified, purified wheat bran) and α-cellulose were chosen for analysis. Complete hydrolysis of these purified polysaccharide preparations was obtained after only 10 min of treatment with 12 mol dm⁻³ sulfuric acid. In order to decrease the time of treatment with 12 mol dm⁻³ sulfuric acid, for samples that have been subjected to the washing and drying procedures it is essential that the sample is finely divided before addition of the acid. The grinding action of the sand and the glass balls prevents aggregation and ensures that samples are finely divided after the drying procedure. Identical NSP values were obtained for a wide range of samples taken through the washing and drying procedures when either 30 min or 1 h of treatment with 12 mol dm⁻³ sulfuric acid was used (data not shown). Treatment with 12 mol dm⁻³ sulfuric acid for 30 min at 35 °C has

Table 5 Values for NSP (g per 100 g of sample) predicted to be obtained by the spectrophotometric procedure, based on measurements of NSP constituent sugars by GLC. These values were calculated in four ways: Pred(1) on the basis of the colour yields of individual sugars, using the mixture of NSP constituent sugars determined for each food by the GLC procedure; Pred(2) using the standard sugar mixture as described here for the spectrophotometric procedure, with no correction ($F = 1.0$); Pred(3) with correction of values based on the mean ratio [the ratio of the mean values of Pred(1) and Pred(2)], $F = 1.05$ for vegetables and fruits, $F = 0.95$ for cereal products other than oats, $F = 1.0$ for starchy legumes and oat products; and Pred(4) using the standard sugar mixture with no correction factor ($F = 1.0$) but incorporating separate values for uronic acids. The predicted values calculated as Pred(2) to Pred(4) above for individual foods are compared, as percentage differences (%Δ), with those given as Pred(1)

	Pred(1)	Pred(2)		Pred(3)		Pred(4)	
		Abs.	%Δ	Abs.	%Δ	Abs.	%Δ
Vegetables—							
Mean	22.6	21.5	-6.4	22.6	0.3	22.5	-0.3
Range			-9.5 to 0.9		-3.0 to 7.9		-1.6 to 2.6
Fruits—							
Mean	11.5	10.9	-6.7	11.5	0	11.4	-0.1
Range			-10.1 to -1.1		-3.6 to 5.8		-2.1 to 1.1
Cereal products (other than oats)—							
Mean	8.3	8.8	2.8	8.3	-0.4	8.4	0.6
Range			0 to 7.2		-3.0 to 3.9		-0.9 to 2.5
Starchy legumes and oat products—							
Mean	12.2	12.2	-1.6	12.3	0.4	12.3	0.2
Range			-4.1 to 0		-2.1 to 2.0		-2.8 to 3.4

therefore been incorporated into the procedure as this ensures complete dispersion of NSP and allows a shorter hydrolysis reaction time.

As a test for completeness of depolymerization, HPLC-PAD was used to detect any oligosaccharides remaining in solution after the acid hydrolysis steps. No residual oligomers were detected for cellulose or other non-cellulosic neutral sugar-containing NSP, indicating complete hydrolysis to monosaccharides. Oligosaccharides were found, however, for uronic acid-containing polymers, such as pectin, indicating incomplete depolymerization. This incomplete hydrolysis is not important when the uronic acid content is determined by the modified Scott procedure as used in the GLC analysis of NSP, which measures both free uronic acids and uronic acid polymers in solution. Special treatment of the hydrolysate is required, however, when using the spectrophotometric or HPLC procedures. Prolonged treatment (3 h) with 2 mol dm⁻³ sulfuric acid is required to obtain maximum values using HPLC but such a treatment is time consuming and results in further undesirable losses. The use of pectinase, after the treatments with sulfuric acid, to complete the hydrolysis of uronic acid-containing polymers is described in

detail in the accompanying paper for the determination of uronic acids by HPLC.¹³ Similarly, the addition of pectinase after adjustment of the pH of the hydrolysate to between 3.5 and 4 can be used to ensure complete recovery of uronic acids when using the spectrophotometric procedure. Results for two citrus pectins with various degrees of methoxylation, sugar beet and a Fibrim sample (Table 3) show that complete hydrolysis is readily achieved within the 20 min of treatment with the pectinase now used before addition of the chromogenic reagent. In addition, the enzyme preparation has sufficient reducing groups that, when used at the concentration described here (0.1 cm³ of pectinase diluted 1:10 v/v), the glucose solution previously used to compensate for dissolved oxygen is not required.

Interference in the Reducing Sugar Assay

Mineral ions have been reported to increase the intensity of the colour formed on heating dinitrosalicylic acid with reducing groups.^{16,17} The extent of the interference is variable and dependent on the concentrations of both mineral ions and reducing sugars. In the determination of NSP, the presence of mineral ions may explain the small differences obtained by GLC and spectrophotometry for fortified products such as corn flakes and wholemeal bread. To investigate the effects of mineral ions on NSP analysis, the commercial mineral mixture Aminogran (Allen and Hanbury, London, UK) was chosen. It contains various salts of potassium, calcium, phosphorus, sodium, iron, zinc, copper and manganese and trace amounts of cobalt, aluminium and molybdenum. A small proportion of the minerals was insoluble in 80% v/v ethanol at pH 5 and may increase the colour yield. However, all the minerals were soluble in acidified 80% v/v ethanol (pH 2.0), and the use of acidified ethanol as described here completely removed this type of interference. In addition, the presence of mineral ions during the drying procedure results in residues that are more prone to aggregation, which hinders subsequent hydrolysis with sulfuric acid. Removal of the mineral ions as described here obviates this problem.

Advanced Maillard reaction products such as 5-hydroxymethyl-2-furaldehyde and 2-furaldehyde have reducing aldehyde groups and their effect on spectrophotometry is shown in Fig. 4. The results show a time-dependent increase in absorbance for 2-furaldehyde and 5-hydroxymethyl-2-furaldehyde, whereas virtually maximum values are obtained for glucose (and the other NSP monosaccharides; data not shown) after 5 min of reaction. The absorbance values for 5-hydroxymethyl-2-furaldehyde after 5 min are approximately 30% of the values obtained after 15 min and similar to those obtained for sugars when present in the same concentration. The concentrations of 5-hydroxymethyl-2-furaldehyde and of 2-furaldehyde used in this experiment were chosen to be the same as those of the sugars simply to illustrate the effects of the interference; the amounts of these compounds in foods are normally very small. The results in Table 4 for three different cooked foods show the effect of increasing the time of reaction with the chromogenic reagent. As the colour yields for monosaccharides reach a plateau after 5 min of reaction (Fig. 4), the small increase in values obtained between 10 and 20 min must be due to interfering substances. Shortening the time of reaction with the chromogenic reagent from 15 to 5 min therefore minimizes the interference from the products formed during food processing.

Colour Yields

Previous studies have shown that different colour yields are obtained for hexoses, pentoses and uronic acids with the chromogenic reagent used in the reducing sugar method.^{6,18,19}

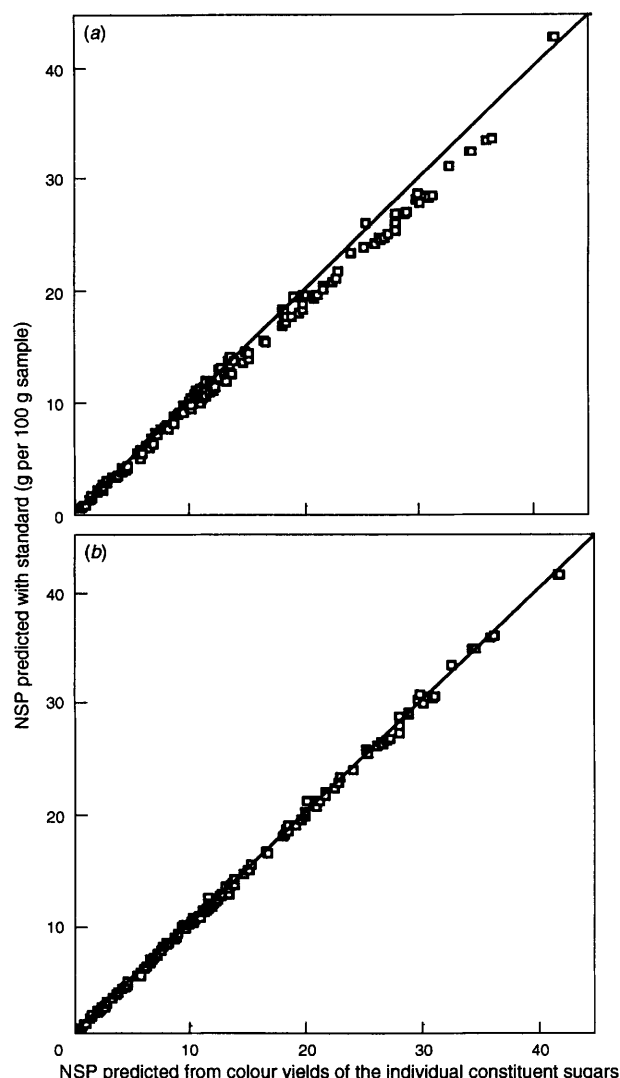


Fig. 5 Values predicted to be obtained by the spectrophotometric procedure on the basis of analysis by the Englyst GLC procedure for a range of foods: (a) without using correction factors, and (b) using the correction factors described in the text for groups of foods. The line of unity is shown.

In practice, a single standard may be used for spectrophotometry, especially if correction is made for the colour yield from different types of NSP, *e.g.*, cereals contain a high proportion of pentoses, whereas fruits and vegetables are high in cellulosic and uronic acid-containing polymers.

Table 5 shows the NSP values that are predicted to be obtained by the spectrophotometric procedure for 43 vegetables, 40 fruits, 52 cereal products (other than oats) and 17 starchy legume and oat product samples, calculated on the basis of the colour yields of individual sugars. These values were calculated in four ways: (1) on the basis of using a standard sugar mixture of composition identical with the values for NSP constituent sugars determined for each food by the GLC procedure; (2) using the standard sugar mixture as described here, with no correction ($F = 1.0$); (3) with correction of values based on the mean ratio [the ratio of the mean values predicted from the 'correct' sugar mixture (1) and those predicted using the standard sugar mixture (2) without correction]; and (4) using the standard sugar mixture with no correction factor ($F = 1.0$) but incorporating separate values for uronic acids. The predicted values calculated as described for (2)–(4) above are compared as percentage differences (% Δ) with those predicted as described for (1) above.

Vegetables

The range of % Δ values using $F = 1.0$ is asymmetric around the target value of zero. Application of the mean ratio as the correction factor, $F = 1.05$, yields a more symmetrical distribution of the % Δ values around zero, largely removing the negative bias of the results seen with $F = 1.0$. When calculations are performed incorporating separate values for uronic acids and using $F = 1.0$, the same improvement in symmetry around zero is achieved but the range of values is markedly reduced, to -1.6 (for yam) to $+2.6$ (for maize). Of the 43 different foods, the % Δ values using the uronic acid data lie between -0.5 and $+0.5$ for 20 samples and

numerically between 0.5 and 1.0 for a further 11 foods. Only the extreme values (for yam and for maize) lie outside % $\Delta = \pm 1.5$, a range of absolute differences of only -0.1 g NSP per 100 g of dry matter (yam) to $+0.3$ per 100 g of dry matter (for maize).

Fruits

As for the vegetables, application of the mean ratio makes the distribution of % Δ values more nearly symmetrical around zero. Incorporation of separate uronic acid data has the same effect and reduces the range to -2.1 (for red currants) to $+1.1$ (for olives and pineapple). Of the 40 individual % Δ values, 23 are zero, 6 are numerically between 0.5 and 1.0 and only 2, red currants (-2.6) and pawpaw (-1.7), exceed 1.5.

Cereals other than oats

Again, application of the mean ratio as a correction factor ($F = 0.95$) improves the symmetry of % Δ values around zero. Incorporation of separate uronic acid values reduces the range of values but the asymmetry around % $\Delta = 0$ is not improved. For the 52 products, 47 of the % Δ values with uronic acids lie between the single negative value (-0.9 for pearled barley) and $+1.5$, with 24 values of zero. Only four products have % Δ values larger than 1.5: brown bread, 1.6; rye flakes and Original Ryvita, 1.8; and wheat cracottes, 2.5.

Starchy legumes and oat products

This small group of foods shows the same improvement in symmetry around zero for % Δ values calculated on the basis of the mean ratio. However, there is no evidence that the use of separate values for uronic acids represents any advantage over this.

In summary, the use of correction factors based on the mean ratio (calculated as described above) has the advantage of making the likely error in the values obtained by the

Table 6 Values used in the analysis of variance to examine the effects of precipitation at pH 2 and 5.2, and method type on values for total NSP obtained by the GLC procedure and by the spectrophotometric procedure with 5 min (with pectinase) and 15 min (without pectinase) of heating with the DNS chromogenic reagent

Spectrophotometry					Spectrophotometry					Spectrophotometry				
Sample	pH	GLC	5 min	15 min	Sample	pH	GLC	5 min	15 min	Sample	pH	GLC	5 min	15 min
Haricot beans	5.2	18.4	19.0	18.6	Biscuit	5.2	14.4	13.7	14.0	White bread	5.2	2.9	2.9	3.2
		19.5	19.8	19.0			14.6	14.1	13.0			2.8	2.8	2.8
		2	18.9	18.7	17.8		2	15.2	14.3	13.5		2	3.0	2.9
Brussels sprout	5.2	19.0	19.2	18.1	Cornflakes	5.2	15.0	14.3	14.1	Rye flour	5.2	2.9	2.8	3.1
		27.3	28.3	27.0			1.2	0.9	1.1			14.8	14.8	15.4
		2	27.6	28.6	27.8		2	1.1	1.0	1.2		2	14.8	14.9
Cabbage	5.2	26.6	26.7	25.6	Wheat bran	5.2	1.1	0.9	1.2	Rice	5.2	15.2	14.9	15.4
		26.6	26.4	24.4			1.0	0.9	0.9			15.1	14.9	15.3
		2	26.8	27.0	24.9		2	40.5	39.9	38.4		2	0.9	0.9
Parsnip	5.2	25.8	26.6	25.6	Porridge oats	5.2	40.9	39.6	38.4	Linseed	5.2	0.9	1.0	1.0
		2	26.0	25.2	24.2		2	41.0	39.5	38.0		2	0.8	0.8
		2	25.1	25.0	24.7		2	41.9	40.0	39.0		2	0.8	0.8
Wholemeal bread	5.2	21.4	20.4	20.0	Peas	5.2	7.8	8.2	8.3	Carrots	5.2	20.0	20.1	21.2
		2	20.7	20.4	19.9		2	7.9	8.3	8.4		2	20.7	20.4
		2	20.5	19.9	18.5		2	7.7	7.9	7.7		2	20.3	20.2
Polysaccharide mixture	5.2	20.3	19.8	18.2	Rhubarb	5.2	7.7	7.8	7.6	Potato	5.2	20.4	20.3	20.9
		9.2	9.8	10.4			15.0	15.4	15.4			19.8	18.4	18.1
		2	9.5	9.9	10.5		2	14.6	15.3	15.9		2	19.5	18.6
	5.2	9.9	9.7	10.4		5.2	14.2	14.5	15.3		5.2	18.8	18.1	17.6
		9.4	9.6	10.0			13.6	14.1	14.8			18.8	18.4	17.6
		2	95.0	89.4	88.2		2	30.8	28.9	29.2		2	5.8	6.3
	5.2	94.1	91.0	89.8		5.2	31.1	28.9	27.7		5.2	6.0	6.2	6.4
		2	95.2	90.8	87.8		2	31.0	28.5	29.0		2	5.7	5.7
		2	93.4	90.3	88.0		2	30.7	28.3	29.0		2	5.4	5.8

spectrophotometric procedure smaller and more nearly symmetrical around zero for all the foods examined here. The incorporation of separate values for uronic acids generally has the same effect, and has the advantage of reducing the likely error for vegetables, fruit and cereal products. However, the difference in the results obtained by these two approaches is not large for most foods. There is a clear need for the separate assay of uronic acids only for the analysis of purified materials such as pectins. Fig. 5 shows a comparison of the values predicted for all foods using a standard sugar mixture identical with the values obtained by GLC [calculation (1), above] with those predicted using the sugar mixture as described here and a correction factor of $F = 1$ [calculation (2), above], and with the values predicted using the appropriate F values for each group of foods but without correction for uronic acids [calculation (3), above].

Sources of potential interferences in the measurement of NSP have been identified and eliminated by the modifications described here. These changes have made the NSP procedure more accurate and robust. Virtually identical values for NSP are obtained by GLC and by the more rapid spectrophotometric assay of constituent sugars following the isolation and hydrolysis procedures as described.

Statistical Comparison of the Results Obtained by the Three Assays

Data for 18 samples with a wide range of NSP content were obtained in duplicate by GLC and by spectrophotometric procedures. All the procedures were carried out with an 80% ethanol (pH 2) and with an 80% ethanol (pH 5.2) precipitation step. Two versions of the spectrophotometric procedure were used: heating for 5 min with pectinase (as recommended) and 15 min without pectinase (as used previously) with the chromogenic reagent.

The analytical data for total NSP (see Table 6) were submitted to analysis of variance, conducted on the assumption that any differences were proportional. With 18 samples, in duplicate, at two pH values, analysed by three methods, there were 215 degrees of freedom for the analysis. The samples were deliberately chosen to represent a wide range of NSP contents, so the majority (on average, 99.8%) of the variance in the results was accounted for by differences between foods. There was no evidence for a difference ($P = 0.085$) between the results obtained over all samples by the

GLC and the spectrophotometric procedures, and 76% of the difference over all methods was accounted for by the difference ($P = 0.003$) between the 5 and 15 min spectrophotometric procedures. There was a significant effect ($P < 0.001$) of pH over all methods but this was not associated ($P = 0.273$) with the interaction of pH and any one method.

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