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Colorimetric Method for Routine Measurement of Dietary Fibre as Non-Starch Polysaccharides. A Comparison with Gas–Liquid Chromatography

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ABSTRACT

A method is described for the measurement of dietary fibre as the non-starch polysaccharides in plant material. The basic fractionation procedure from a previously validated gas–liquid chromatographic method has been combined with colorimetric measurement of constituent sugars. The method is shown to produce accurate quantitative data by comparison with the previous gas–liquid chromatographic procedure. Although detailed qualitative information is not obtained, the method described offers the opportunity to measure soluble and insoluble dietary fibre, without the need for expensive gas–liquid chromatography equipment.

INTRODUCTION

A method has been reported for the determination of dietary fibre as non-starch polysaccharides (NSP) using gas–liquid chromatography (GLC) for the measurement of constituent sugars (Englyst *et al.*, 1982; Englyst & Cummings, 1984; Englyst, 1985). This technique permits the determination of total NSP and the measurement and characterization of various

components of NSP from analysis of their constituent sugars. This detailed information is essential for the investigation of the physiological significance of various types of NSP, and for establishing food Tables with values for the components of NSP.

For routine analysis, where details of composition are not required, soluble and insoluble NSP can be measured colorimetrically. The technique is simple and rapid, and obviates the need for expensive GLC equipment.

Using an established colorimetric method (Dahlqvist, 1962) we have developed a modification of the GLC procedure (Englyst *et al.*, 1982) that allows the constituents of NSP to be measured as reducing sugars. The results obtained with the colorimetric procedure for a range of foodstuffs are compared with those obtained by the previously validated GLC procedure. Finally, we demonstrate that the colorimetry is suitable for automation.

MATERIALS AND METHODS

Samples

Cereal products were purchased locally and milled, when necessary, to provide homogeneous samples to be taken for analysis. Fruits, nuts and vegetables were purchased locally and were freeze-dried before milling.

Reagents

All reagents used were of AnalaR quality.

Pullulanase (EC 3.2.1.41) was purchased from Boehringer-Mannheim. Pullulanase solution: dilute the enzyme suspension 1:100 with water immediately before use.

Alpha-amylase (EC 3.2.1.1) was purchased as Pancrex V capsules (approximately 9000 BP units per capsule) from Paines & Byrne Ltd. (Any source of pancreatin of equivalent activity may be used.) Alpha-amylase solution: empty two capsules (approximately 350 mg of powder per capsule) into a centrifuge tube, add a stirrer and 9 ml of water, and vortex mix. Then leave stirring on a magnetic stirrer for 10 min. Centrifuge for 10 min at 1500 *g*. Use the supernatant solution. Prepare immediately before use.

Procedure

Figure 1 is a summary of the complete analytical procedure, which is described in detail below.

Weigh to the nearest 0·1 mg not more than 200 mg of dry sample (< 10% water) containing not more than 50 mg of NSP; i.e. 200 mg for most

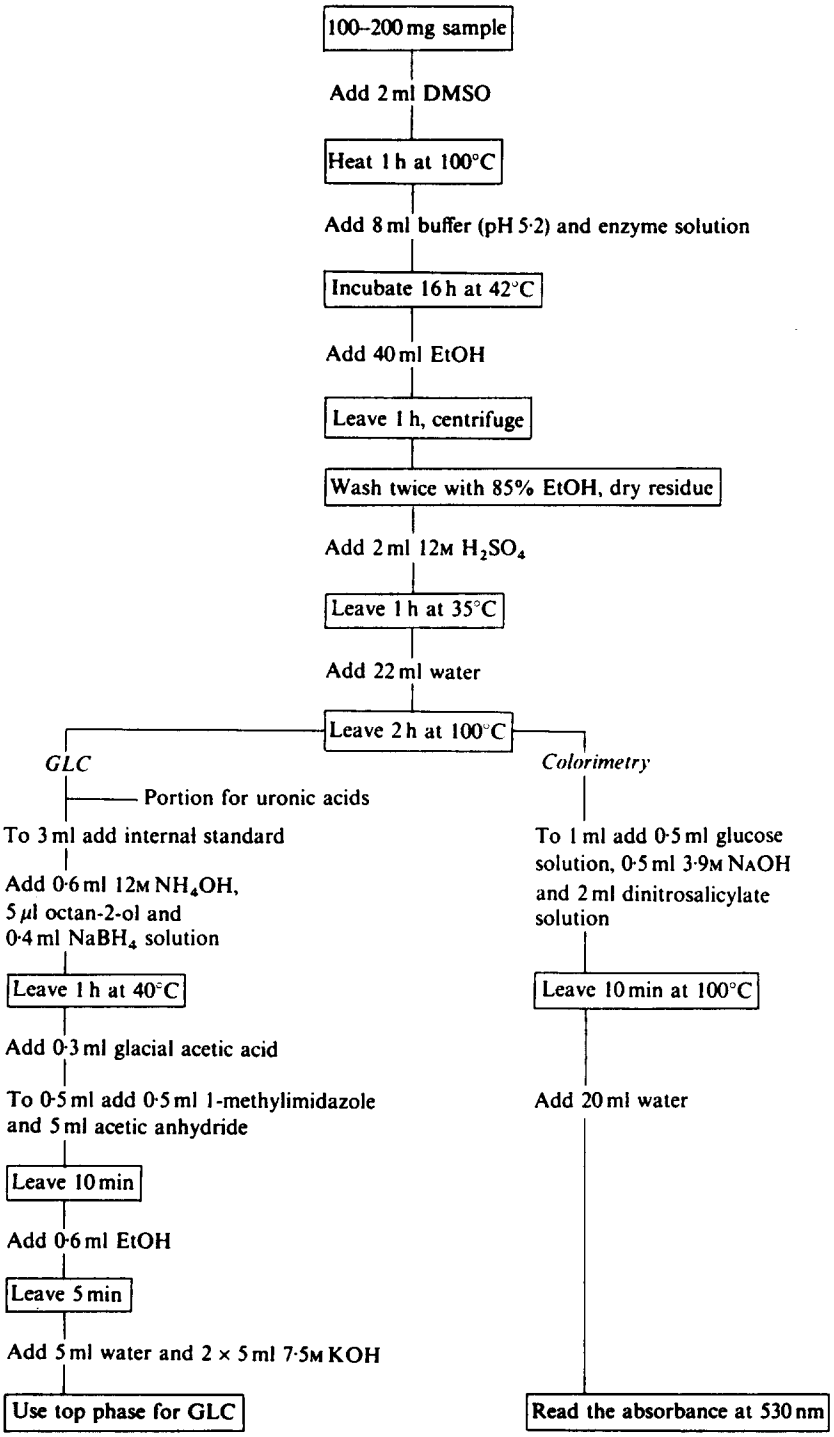

Fig. 1. A flow-chart of the complete analytical procedures described in this paper. The sample preparation is common, followed by GLC or colorimetric measurement of component sugars. For full details, see the text.

samples, 100 mg for bran. For samples containing more than 10% water and/or more than 5% fat, add 40 ml of acetone, mix for 30 min using a magnetic stirrer, centrifuge and remove by aspiration as much of the supernatant as possible without disturbing the residue. Place the tube in a beaker of water at 65°C on a stirrer/hotplate and mix the residue for a few minutes until it appears dry. The beaker may be covered and the acetone vapour removed with a water pump.

Dispersion and hydrolysis

Add 2 ml of dimethyl sulphoxide, cap the tube and mix for about 2 min at room temperature using the magnetic stirrer. Place the tube in a beaker of boiling water on a stirrer/hotplate with the stirrer switched on. (Note that gel formation may later occur so that the stirrer is prevented from moving, but this does not affect the procedure.) After 1 h remove the tube and immediately, without cooling, add 8 ml of 0·1M sodium acetate buffer (pH 5·2) pre-equilibrated at 50°C and vortex mix. Place in a waterbath at 40°C; remove the tube after 2 to 3 min and immediately add 0·5 ml of alpha-amylase solution followed by 0·1 ml of pullulanase solution and vortex mix (do not mix the enzyme solutions beforehand). Incubate the capped sample at 42°C, mix after the first hour, and leave for about 16 h.

Add 40 ml of ethanol, mix well by inversion and leave for 1 h at room temperature. Centrifuge at 1500 *g* for 10 min, then remove as much of the supernatant as possible without disturbing the residue. Wash the residue twice, using 50 ml of 85% ethanol each time. Mix by inversion and then use a magnetic stirrer to form a suspension of the residue, centrifuge and remove the supernatant as before.

Add 40 ml of acetone to the washed residue, stir for 5 min, then centrifuge at 1500 *g* for 10 min. Remove the supernatant by aspiration and discard it. Place the tube in a beaker of water at 65°C on a stirrer/hotplate and mix the residue for a few minutes until it appears dry. The beaker may be covered and the acetone vapour removed with a water pump.

Add 2 ml of 12M sulphuric acid to the dried residue and immediately disperse it by vortex mixing or with a magnetic stirrer. Leave at 35°C for 1 h, with mixing (occasional or continuous), to disperse cellulose. Rapidly add 22 ml of water, cap the tube and mix. Place in boiling water for 2 h from reboiling, stirring continuously. Cool to room temperature, and take a portion for the measurement of uronic acids (see below).

GLC determination of neutral sugars

From the cooled hydrolysate (final volume 24 ml) take 3·0 ml and accurately add about 0·5 ml of internal standard (a concentration of 1 mg

allose per millilitre in 50% saturated benzoic acid is suitable for the majority of analyses). When using a computer integrator, the internal standard may be added as a constant percentage of the sample weight, allowing all calculations to be done by the computer. Add 0·6 ml of 12M ammonium hydroxide, mix and test that the solution is alkaline, add a little more ammonium hydroxide if necessary. Then add 0·4 ml of a freshly prepared solution of 3M ammonium hydroxide containing 50 mg of sodium borohydride per millilitre and about 0·005 ml of octan-2-ol as a surfactant. Mix, leave for 1 h at 40°C, add 0·3 ml of glacial acetic acid and mix. To 0·5 ml of the acidified solution, add 0·5 ml of 1-methylimidazole and 5 ml of acetic anhydride, mix. After 10 min add 0·6 ml of ethanol, mix and leave for 5 min. Then add 5 ml of water, mix and leave for a further 5 min. Place in cold water to aid the dispersal of heat and add 5 ml of 7·5M potassium hydroxide; a few minutes later, add a further 5 ml. Cap the tube and mix by inversion. Leave until the separation into two phases is complete. Draw some of the upper phase into a pipette. Some of the lower phase may be drawn up at the same time; run some of the liquid out of the pipette to ensure that none of the lower phase remains. Transfer the upper phase to a vial for storage at 5°C (stable for at least 3 weeks).

Carry out conventional GLC determination of the alditol acetate derivatives, using 0·001 or 0·002 ml for injection onto the chromatography column (Englyst & Cummings, 1984; Englyst, 1985; Englyst *et al.*, 1987).

Determination of uronic acids

Mix 0·3 ml of the hydrolysate (see above), diluted if necessary to contain no more than 0·10 mg of uronic acids per millilitre (e.g. no dilution for flour; 1:5 for bran), with 0·3 ml of a solution containing 2 g of sodium chloride and 3 g of boric acid/100 ml in a 50-ml tube. Add 5 ml of concentrated sulphuric acid and vortex mix. Place in a heating block at 70°C, leave for 40 min, then cool to room temperature in water.

When cool, add 0·2 ml of dimethylphenol solution (0·1 g in 100 ml of glacial acetic acid) and vortex mix immediately. Between 10 and 15 min later, read the absorbance at 400 nm and 450 nm against a water reference. Subtract the reading at 400 nm from that at 450 nm. Plot the difference in absorbance obtained for glucuronic acid standards over the range 0·025 to 0·125 mg/ml. Calculate sample concentrations or read them from the graph.

Colorimetric measurement of total reducing sugars

Standard solutions

Prepare solutions of 1·0, 2·0, 3·0 and 4·0 mg of total sugar per millilitre in 50% saturated benzoic acid. Immediately before use, dilute 1:1 with 2M

sulphuric acid to provide standards of 0·5, 1·0, 1·5 and 2·0 mg of sugar per millilitre in 1 M sulphuric acid. A mixture of arabinose/xylose/glucose in the proportions 3:4:3 (by weight) was used for cereals. A mixture of arabinose/glucose/galacturonic acid in the proportions 1:2:1 (by weight) was used for fruit and vegetables (only small differences are seen if glucose is used as sole standard, see below).

Dinitrosalicylate solution

Dissolve 10 g of 3,5-dinitrosalicylic acid, 16 g of sodium hydroxide and 300 g of sodium/potassium tartrate in deionized water to a final volume of 1 litre. Store in a well-capped dark bottle and keep for 2 days before use (stable at room temperature for at least 6 months).

Manual. Place into separate test-tubes, 1 ml of blank solution (1:1 (v/v) 50% saturated benzoic acid, 2M sulphuric acid), 1 ml of each of the standard sugar solutions and 1 ml samples of the hydrolysates to be tested. Add 0·5 ml of 0·5 mg glucose per millilitre and 0·5 ml of 3·9M sodium hydroxide to each tube and vortex mix. Add 2 ml of the dinitrosalicylate solution to each tube and vortex mix. Place all the tubes, at the same time, into a briskly boiling waterbath and leave for 10 min. Cool in water to room temperature. Add 20 ml of deionized water and mix thoroughly by inversion. Measure the absorbance at 530 nm and calculate the concentration of total sugars by reference to the standards.

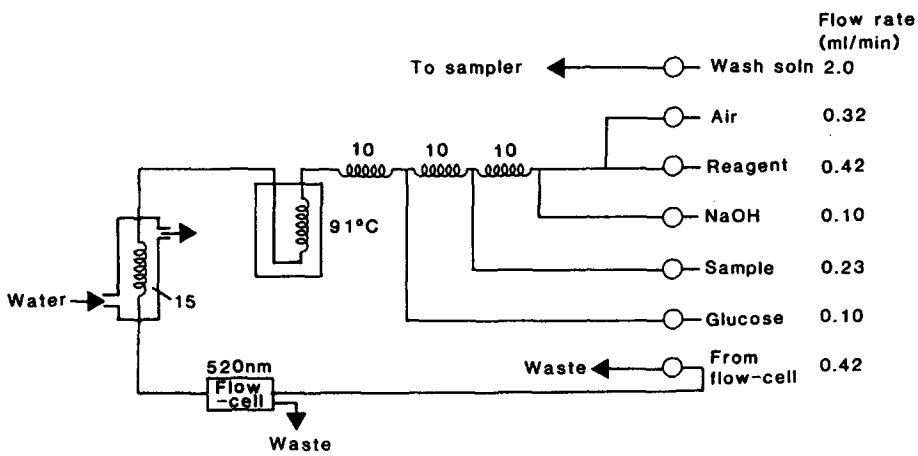

Fig. 2. A diagram of the configuration used with Technicon continuous-flow apparatus. The number of turns in each mixing coil, the temperature of the heating block and the wavelength of the interference filter are indicated. Wash soln; water with 0·5 ml of 30% (w/v) Brij 35 (Technicon) per litre as wetting agent. Reagent; 1% (w/v) 3,5-dinitrosalicylic acid, 1·6% (w/v) NaOH, 30% (w/v) potassium/sodium tartrate (see 'Materials and Methods'). NaOH; 3·9M NaOH. Glucose; 0·5 mg glucose per millilitre in 50% saturated benzoic acid. Sampling rate, 60/h. Wash time, 8 s.

Automated. The dinitrosalicylate reaction is suitable for automation and Fig. 2 shows the configuration used with Technicon continuous-flow apparatus. Although wetting agent is included only in the wash solution, a satisfactory stability of the trace was achieved. The heating coil has a capacity of approximately 3·5 ml, which results in a delay time of about 4 min with the configuration shown.

Further applications

Measurement of soluble and insoluble NSP

The procedure for the measurement of insoluble NSP is identical with that described above for total NSP up to and including the overnight incubation. Then, omitting the precipitation with ethanol, add 40 ml of 0·2M phosphate buffer (pH 7), place the tubes in a beaker of boiling water on a stirrer/hotplate and stir for 1 h. Cool, centrifuge, remove the supernatant and discard it. Wash the residue once with 50 ml of the phosphate buffer, and once with 85% (v/v) ethanol. Dry the residue and treat it as described for total NSP.

Soluble NSP is calculated simply as total NSP minus insoluble NSP.

Measurement of NSP plus resistant starch

Starch made resistant to digestion by pancreatic amylase by food processing, previously described (Englyst *et al.*, 1982, 1983) as resistant starch (RS), is dispersed by dimethyl sulphoxide (DMSO) and, correctly, is not included in the measurement of NSP. If a value for NSP plus RS is required, this may be obtained by omitting the treatment with DMSO and the subsequent dilution with 8 ml of buffer described for total NSP. Instead, add 10 ml of 0·1M sodium acetate buffer (pH 5·2) and place in boiling water for 1 h.

Measurement of resistant starch

Resistant starch can be measured as the difference in values obtained when using DMSO and when using aqueous buffer for the dispersion of starch.

A more accurate measurement is obtained if the determination of RS is based on the difference between glucose in total NSP plus RS and that in total NSP. After neutralization of the hydrolysate with sodium hydroxide, glucose can be measured colorimetrically using glucose oxidase.

RS can be measured as glucose after dispersion with 2M potassium hydroxide, as described previously (Englyst *et al.*, 1982).

Separation of total NSP into cellulose and NCP

If dispersion of cellulose with 12M sulphuric acid is omitted from the procedure for total NSP, and is replaced with direct hydrolysis with 1M

sulphuric acid, only non-cellulosic polysaccharides (NCP) will be hydrolyzed. Using glucose oxidase, a value for cellulose may be obtained as the difference between the glucose content of total NSP and that of NCP.

RESULTS AND DISCUSSION

In the method described, the fractionation technique from a GLC procedure (Englyst *et al.*, 1982, 1986; Englyst & Cummings, 1984) has been combined with colorimetric measurement of reducing sugars (Dahlqvist, 1962). This technique permits the determination of soluble and insoluble NSP but does not characterize these fractions by measurement of individual sugars, in contrast with the GLC procedure. However, such detailed information is not always required for routine analyses. The colorimetric procedure is simple and rapid, and obviates the need for expensive GLC equipment. The reducing sugar method is the well-established technique developed by Sumner & co-workers (Sumner & Graham, 1921; Sumner & Noback, 1924) and later investigated and modified by von Hostettler *et al.* (1951), Miller *et al.* (1959) and Dahlqvist (1962). The dinitrosalicylic acid reagent used in the procedure is inexpensive and easily prepared. The working procedure used here is essentially that described by Dahlqvist (1962) but with two small modifications. It was found necessary to neutralize the hydrolysate before applying the reducing sugar method. To avoid destruction of sugars by alkali, sodium hydroxide was added only to the point where the hydrolysate was still slightly acid. The other modification found necessary was the result of the observation that some losses of glucose occurred in the presence of sodium/potassium tartrate. The effect of such losses on the measurements may be eliminated, as described by Miller (1959), by adding a known, small amount of sugar to samples and standards.

Slight differences in colour yield were obtained for various sugars upon their reaction with dinitrosalicylic acid, in agreement with von Hostettler *et al.* (1951), who reported the following yields of chromatogen: pentoses, 0·60; hexoses, 0·56; and galacturonic acid, 0·47 (as milligrams per milligram of sugar). To compensate for these different levels of response, mixtures of sugars in proportions approximating those found in the samples (see 'Materials and Methods') were used in the present study. In practice, it is possible to use only glucose as the standard and to apply a correction factor to the results. For example, normalizing the colour yields of individual sugars to glucose leads to calculation of the following factors from the detailed composition obtained by GLC for a range of food types. Butter bean, 0·99; French bean, 1·03; cabbage, 1·01; carrot, 1·03; leek, 1·02; pea,

0·99; potato, 1·02; apple, 1·03; whole-wheat flour, 0·96; and All-Bran, 0·96. These factors are the value predicted for total sugars by colorimetry divided into that obtained from the GLC procedure, and the use of glucose as sole standard without correction would not result in errors in excess of 4% for these types of samples. However, use of a mixed sugar standard results in errors of no more than 2%.

When using the colorimetric procedure for the analysis of purified polysaccharides, the appropriate sugar should be used as sole standard; e.g. for hexans, pentosans or pectins, use glucose, xylose or galacturonic acid, respectively.

Uronic acids may be measured specifically by the technique described for the GLC procedure (see 'Materials and Methods').

A large range of food types has been analyzed by the colorimetric procedure described here, using mixed sugar standards, and by the GLC procedure. Detailed results for a representative range of foods are presented in Table 1. The results obtained for total NSP by GLC and by colorimetry are very similar. The main differences in composition of the products are: (1) a higher proportion of galactose and uronic acids in fruit and vegetables than in cereals; (2) arabinose is the main pentose in fruit and vegetables, whereas xylose predominates in cereals. The correlation between the results from the two procedures for all 232 foods analyzed (78 fruits and nuts, 64 vegetables, 90 cereals and cereal products) is illustrated by Fig. 3. The values obtained by the two methods for each sample are plotted against each other, and the line of unity is shown.

Although analysis of the data by the method of Oldham (1962) reveals that the two methods do not give numerically identical values for vegetables or for cereal products, the absolute differences are small. The average (+/−SEM) differences of the values for g total NSP/100 g of dry matter from the colorimetric procedure compared with those from the GLC procedure are: −0·46 (+/−0·21) for vegetables; +0·49 (+/−0·11) for fruits and nuts; +0·13 (+/−0·06) for cereals and cereal products and +0·09 (+/−0·08) over all foods. Expressed as a percentage of the mean, these values are equivalent to −2% for vegetables, +4·9% for fruits and nuts, +1·63% for cereals and cereal products, and only +0·69% over all foods. Paired *t*-tests confirm that these differences are not statistically significant (e.g. for cereals and cereal products, $t = 2{\cdot}06$; $n = 90$).

Dietary fibre was defined (Trowell, 1972) in terms of plant cell walls as 'the skeletal remains that are resistant to digestion by the enzymes of man'.

Different interpretations of this definition have led to the development of a number of methods, all of which claim to determine dietary fibre, but by measuring different fractions of plant material.

The lack of an agreed chemical definition has caused confusion and

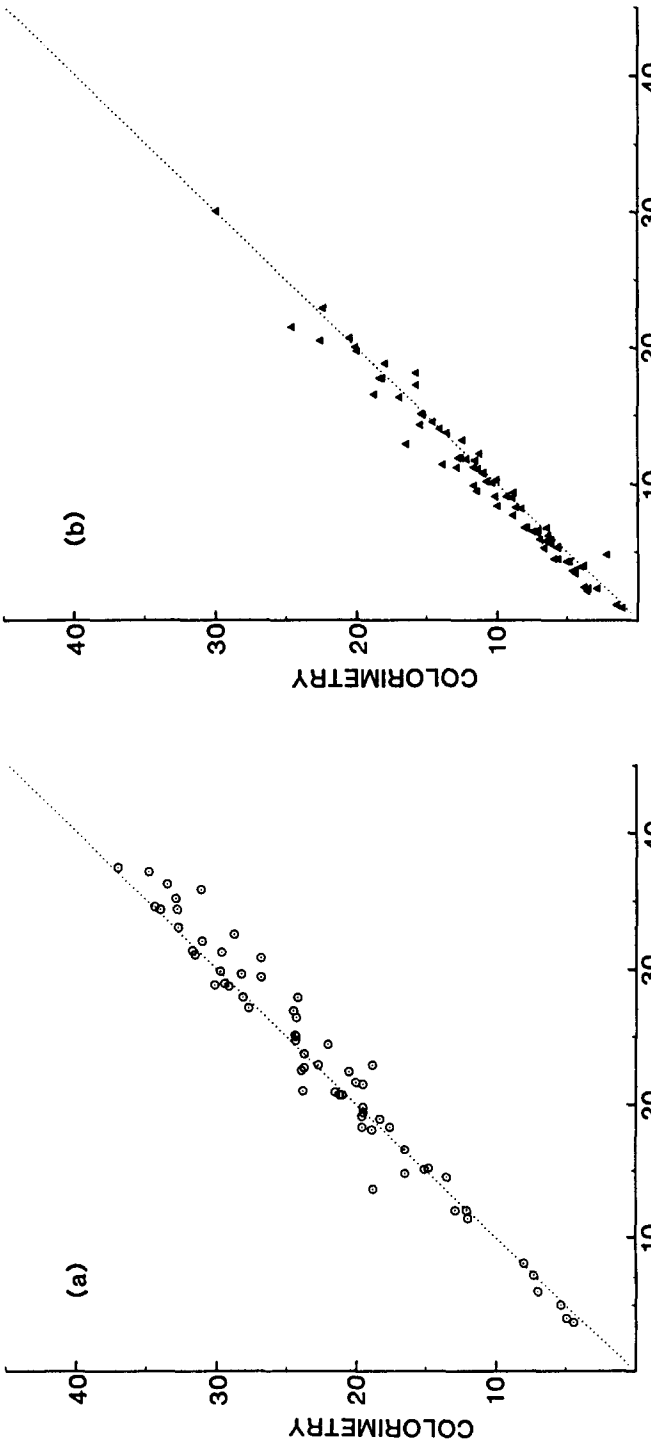

Fig. 3. The values for total NSP (g/100 g of dry matter) obtained by the GLC procedure and by the colorimetric procedure described here are compared. The comparison is shown for (a) 64 vegetables, (b) 78 fruits and nuts, (c) 90 cereals and cereal products and (d) for all 232 foods together. In each case, the line of unity is shown on the plot. For statistical analysis, see the text.

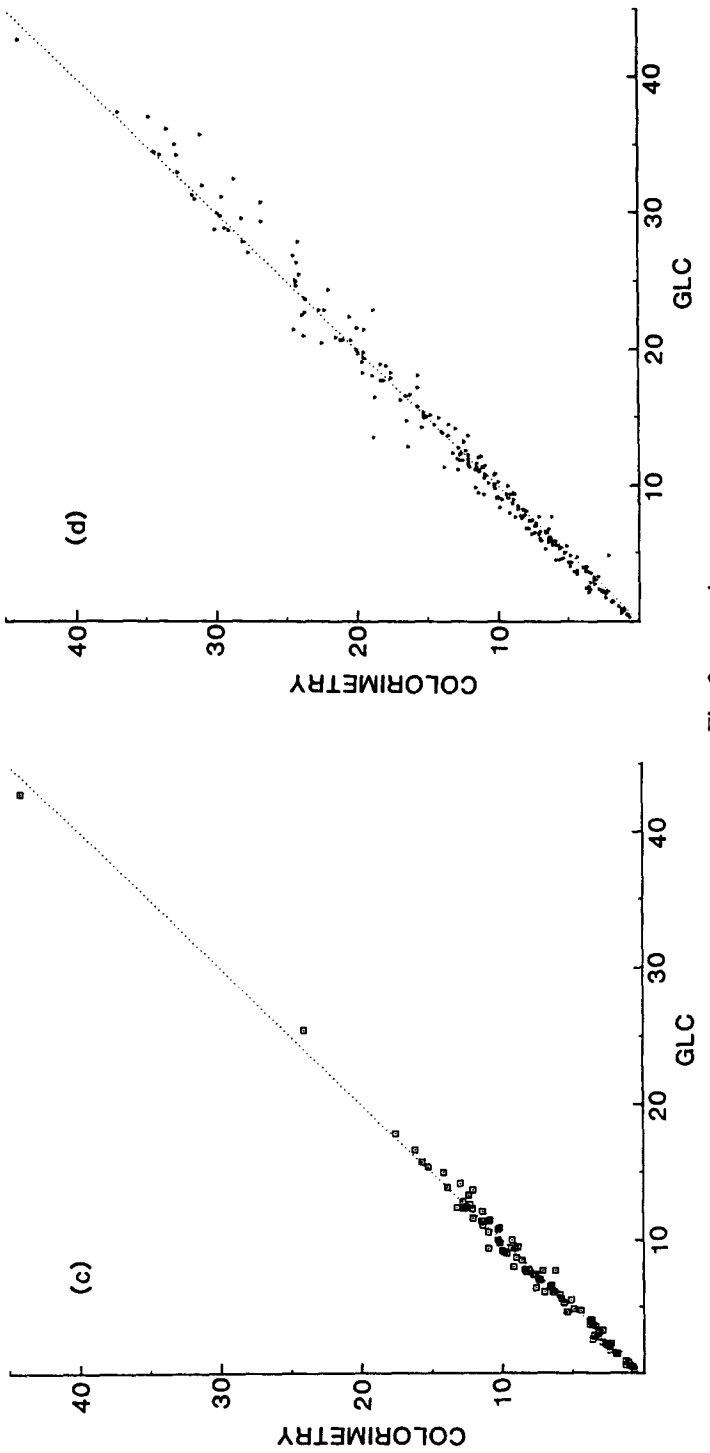

Fig. 3.—*contd.*

TABLE 1
A Comparison of Results Obtained from the GLC and Colorimetric Procedures for A Range of Foods

	Total NSP colorim.[a]	*Total NSP GLC*	*Composition of NSP*							
			Rha	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glu*	*U.Ac.*
Runner beans	29·4	28·9	0·2	t	2·3	1·8	1·0	4·3	10·8	8·5
Dried peas	15·1	15·1	0·2	t	4·1	1·4	t	0·8	6·4	2·2
Cabbage	37·0	37·4	0·7	0·1	5·7	2·0	1·1	3·6	14·9	9·3
Sprouts	29·7	29·8	1·2	0·1	7·1	1·3	0·7	3·8	9·1	6·5
Carrot	24·4	23·8	0·5	t	2·1	0·4	0·5	5·0	9·9	5·4
Lettuce	22·7	22·9	0·8	t	1·1	1·1	0·7	1·6	8·7	8·9
Onion	19·5	19·4	0·5	t	0·4	0·6	0·2	4·5	6·6	6·6
Potato	6·3	6·4	0·1	t	0·4	0·1	t	2·2	2·6	1·0
Apples, Cox	11·0	10·8	0·1	0·1	1·7	0·7	0·2	0·9	4·3	2·8
Oranges	15·2	15·0	0·3	t	2·2	0·6	0·4	1·8	3·5	6·2
Grapes, white	3·8	4·0	t	t	0·3	0·2	0·1	0·3	1·6	1·5
Strawberries	12·5	11·9	0·2	t	0·8	1·4	0·2	0·5	4·5	4·3
White flour	3·0	2·8	t	t	0·9	1·1	0·1	0·2	0·5	t
Wheat bran	44·1	42·8	0·1	t	10·5	17·1	0·3	0·8	12·8	1·2
Oats, porridge	8·4	8·0	t	t	1·1	1·3	0·1	0·2	5·1	0·2
Rye flour	14·0	13·3	t	t	3·6	5·4	0·3	0·3	3·5	0·2
Barley	12·1	11·8	t	t	2·1	3·3	0·3	0·1	5·8	0·2
Soya bran	54·0	55·7	0·8	0·3	4·6	6·4	4·0	2·7	28·2	8·7

[a] The values for total NSP obtained by the two procedures described here are given in the Table as g/100 g of dry matter.
t, trace.

delayed the development of exact methods for the measurement of dietary fibre.

No precise chemical definition of dietary fibre is yet accepted world-wide. However, Trowell (1985) concluded, 'At the present time there is considerable international agreement concerning the principal constituents of dietary fibre. They are all polysaccharides, mainly cellulose, hemicellulose and pectic substances, conveniently designated non-starch polysaccharides (NSP)'. This gives the analyst a clear task; to identify and measure the chemically well-defined non-starch polysaccharides.

The colorimetric procedure described here can accurately measure soluble and insoluble non-starch polysaccharides (NSP) as the sum of their constituent sugars. Being based on the measurement of reducing sugars released from NSP, the colorimetric procedure is more specific than gravimetric methods (e.g. Asp *et al.*, 1983).

The method is suitable for routine analysis of virtually all food products, and is unaffected by food processing techniques, storage conditions or sample pretreatment. The entire fractionation procedure for a test sample is carried out in a single tube and, unlike the gravimetric procedures, it does not include time-consuming filtration stages. It is a relatively quick procedure; 36 samples can be analyzed by one person in one day, following the overnight incubation to remove starch, and the colorimetry is suitable for automation. Finally, it has been compared with the previously validated GLC procedure for a wide range of food products, and it has been tested successfully in a collaborative trial involving 19 laboratories (Englyst *et al.*, 1987).

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