Rapid Enzymatic Assay of Insoluble and Soluble Dietary Fiber

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A gravimetric, enzymatic method for the determination of both soluble and insoluble dietary fiber is presented. This method makes it possible to analyze 10–15 duplicate samples in 1 day. The procedure includes the following main steps: gelatinization by boiling 15 min in the presence of a heat-stable α-amylase, incubation with pepsin at acid pH for 1 h, and incubation with pancreatin at neutral pH for 1 h. Insoluble dietary fiber is filtered off with Celite 545 as the filter aid. Soluble dietary fiber is precipitated from the filtrate with 4 volumes of ethanol and recovered by filtration in the same way as insoluble dietary fiber. As an alternative the alcohol precipitation can be performed immediately after the enzyme incubations. Both soluble and insoluble components can then be recovered in one single filtration. All dietary fiber components seem to be determined. Practically all starch is solubilized, but some protein remains undigested. Microbial enzymes were also tested. When the materials used in the EEC/IARC collaborative study were analyzed, the two enzyme systems gave very similar results.

Detailed information concerning amount and composition of dietary fiber in foods and raw materials can be obtained by using fractionation methods with colorimetric (Southgate, 1969) or gas chromatographic assay (Theander and Åman, 1979; Englyst, 1981) of dietary fiber monomers. These methods, however, are too laborious for routine purposes, e.g., for declaration and control of dietary fiber content of foods.

The old crude fiber method has been repeatedly abandoned, since it measures only a small and variable fraction of the total dietary fiber. More recently developed gravimetric procedures employ detergent (Van Soest and Wine, 1967), detergent and amylase (Robertson and Van Soest, 1977; Schaller, 1977), or protease and amylase (Weinstock and Benham, 1951; Hellendoorn et al., 1975; Elchazly and Thomas, 1976). A disadvantage of these methods is that only insoluble dietary fiber components are determined. Soluble polysaccharides, such as pectins, gums, and some hemicelluloses, are physiologically important and constitute a considerable fraction of the total dietary fiber in mixed diets (Asp, 1978, 1980; Asp and Johansson, 1981). Obviously, therefore they should be included in assay of total dietary fiber.

In the enzymatic gravimetric methods of Furda (1977, 1981), Schweizer and Würsch (1979), and Asp and Johansson (1981) the soluble polysaccharides are precipitated with alcohol and recovered by filtration and centrifugation. Detergent methods cannot be developed to include the soluble components in this way.

The enzymatic methods for assay of both soluble and insoluble fiber employ long (20–36 h) incubation time and/or laborious centrifugation procedures to recover the dietary fiber fractions.

The purpose of the present investigation was, first, to define enzyme systems capable of removing completely digestible protein and starch from the fiber in as short time as possible and, second, to find a simple and rapid method for separation of dietary fiber from the solubilized protein and starch.

MATERIALS AND METHODS

Samples and Sample Preparation. Wheat bran, whole grain wheat flour, wheat flour, and rye flour were of ordinary Swedish quality and obtained from Kungsörnen AB, one of the main Swedish cereal suppliers. Brown beans (*Phaseolus vulgaris*) were obtained from a local store. The "mixed diet" sample was obtained by collecting meals included in an ordinary Swedish 24-h diet. It was homogenized and freeze-dried. The samples of lactovegetarian diets were obtained from a dietary survey where six lactovegetarians collected duplicates of all their meals during 4 days. The samples from each day were homogenized and freeze-dried. Fat was extracted with chloroform. These samples were obtained from the Unit for Community Care Sciences at Dalby, University of Lund, where they had been extracted with chloroform for other purposes than fiber analysis.

The final procedures were tested by analyzing eight samples distributed in the European collaborative study on analysis of dietary fiber reported by James and Theander (1981).

All samples were milled to a particle size less than 0.3 mm in a Cyclotec mill (Tecator AB, Höganäs, Sweden). Moisture content was determined by drying to constant weight at 105 °C.

When comparing different conditions in the steps of the method, triplicate samples were run. Routinely, samples are run in duplicate.

Enzymes. The following enzymes were used for digestion of protein and starch: pepsin NF (Merck, Darmstadt, West Germany), pancreatin 4 × NF (Sigma, St. Louis, MO), Alcalase 0.6 L (Novo A/S, Copenhagen, Denmark), Termamyl 60 L (Novo), and amyloglucosidase (crystal suspension, Boehringer, Mannheim, West Germany). Incubations were performed in ordinary Erlenmeyer flasks or vessels specially fitted to the filtration equipment. All incubations were performed with horizontal agitation.

Filtration Equipment. A modified Fibertec M equipment (Tecator AB), allowing individual collection of filtrates, was used. Crucibles with porosity 2 (pore size 40–90 μm) were used with 0.5 g of Celite 545 (acid-washed and incinerated, Kebo Grave, Stockholm, Sweden) as the filter aid.

Starch Assay. The fiber residue plus Celite was removed from the crucibles and ground in a mortar. The whole sample was weighed and transferred to an Erlenmeyer flask. A total of 25–50 mL of distilled water was added to give a suspension with approximately 3 mg of fiber/mL. The suspension was stirred for at least 5 min and then gelatinized in a boiling water bath for 15 min. A 1-mL suspension was withdrawn with a pipet under simultaneous stirring and transferred to a centrifugation tube.

A total of 0.5 mL of acetate buffer, pH 4.75, and 5 μL of amyloglucosidase (crystal suspension, 10 mg/mL) was

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Table I. Neutral Sugars in Filtrate after Alcohol Precipitation at Different Conditions during the Pepsin Incubation[a]

material	conditions for pepsin digestion	rhamnose	arabinose	xylose	mannose	galactose	glucose
brown beans	pH 1.0, 18 h	0	2.5	0	0.7	0	27.7
brown beans	pH 1.0, 18 h, dialyzed	0	1.9	0	0.2	0	1.0
brown beans	pH 1.5, 18 h	0	0.7	0	0.2	0	28.7
brown beans	pH 1.5, 18 h, dialyzed	0	0.8	0	0.2	0	1.0
wheat bran	pH 1.0, 18 h	0	0.5	0.2	0.1	0	11.1
wheat bran	pH 1.5, 1 h	0	0	0	0	0	9.2
rye flour	pH 1.0, 18 h	0	0.4	0	0.1	0	46.4
rye flour	pH 1.5, 1 h	0	0	0	0	0	39.7
wheat flour	pH 1.0, 18 h	0	0.7	0	0	0	44.0
apple pulp	pH 1.0, 18 h	0	0.5	0	0	0	4.5
mixed diet	pH 1.0, 18 h	0	0.2	0	0	0	17.0

[a] The results are expressed as percent of the original material (moisture-free basis) analyzed for dietary fiber and corrected for alcohol-extractable sugars present in that material.

added, and the tubes were incubated at 60 °C for 30 min and then centrifuged at 4000 rpm for 10 min. The supernatants were transferred to 5-mL volumetric flasks. The sediment was washed twice with 1.5 mL of water and centrifuged. The washing water was combined with the original supernatant which was then diluted to 5 mL with water. One milliliter of this was mixed with 2 mL of glucose oxidase peroxidase reagent [5.6 g of GLOX-Novum (Kabi-Diagnostica, Stockholm) in 100 mL of 0.5 M Tris-buffer, pH 7.0] and incubated at 37 °C for 1 h. Absorbance was measured at 450 nm. Glucose, 25 μg/mL, was used as the standard and distilled water as the blank.

Protein Assay. Nitrogen was assayed with the Kjeldahl procedure in Kjeltec System 1003 (Tecator AB) according to the manual. Protein was calculated as $N \times 6.25$.

Assay for Monosaccharides from Hydrolyzed Dietary Fiber. The filtrate was evaporated to dryness in a Rotavapor (40 °C), hydrolyzed with sulfuric acid, and derivatized to alditol acetates according to the procedure of Theander and Åman (1979). The hydrolytic conditions have been extensively elucidated by Bethge et al. (1971). Correction factors for losses during hydrolysis and derivatization were determined and used in the calculations. Allose was used as the internal standard. The derivatives were separated by gas chromatography (Varian 3700) with a glass column (0.2 × 180 cm) packed with 3% SP2340 on Supelcoport (100–120 mesh). The temperature program was 180–225 °C (3 °C/min). Argon was used as the carrier gas (30 mL/min). Free monosaccharides were determined in the same way (without hydrolysis) after extraction of the original samples with 78% ethanol at room temperature overnight.

Fat Extraction. Fat was extracted according to the procedure of Saunders and Hautala (1979). The sample was weighed and transferred to an Erlenmeyer flask. Forty milliliters of petroleum ether (bp 60–71 °C) was added per gram of sample. The suspension was agitated with a magnetic stirrer at room temperature for 15 min and was then allowed to settle. The solvent was withdrawn with a pipet and the sample was air-dried at room temperature. In the experiments where the importance of fat extraction was studied, fat was also extracted with $CHCl_3$–CH_3OH (2:1) in a Soxhlet apparatus for 5 h.

Fat Determination. Fat was determined gravimetrically by extraction in diethyl ether and petroleum ether (bp 40–60 °C; 1:1) after hydrolysis with 7.7 N HCl at 70–80 °C for 30–40 min (Association of Official Analytical Chemists, 1980).

RESULTS AND DISCUSSION

Separation of Dietary Fiber Fractions by Filtration. Precipitation of soluble dietary fiber components by adding 4 volumes of 95% ethanol to the filtrate (final ethanol concentration 78% v/v), as also used by Furda (1977) and Schweizer and Würsch (1979), appeared to be a practicable way to recover the soluble dietary fiber components. Dialysis was considered too slow a procedure. Ultrafiltration was tried but the flow rate through the commercial filters tested was very low. Furthermore, the retention of dietary fiber components was incomplete.

To speed up the separation of both insoluble and soluble dietary fiber from digested nonfiber material, it was essential to avoid the repeated centrifugations of large volumes used in earlier modifications of the Hellendoorn method (Asp and Johansson, 1981; Schweizer and Würsch, 1979).

Filtration in glass-filter crucibles for recovery of both insoluble and alcohol precipitated soluble fiber was used by Furda (1977), with compressed glass wool as the filter aid. We found that addition of Celite 545, 0.5 g, to crucibles with porosity 2, diameter 30 mm, prevented clogging when filtering both insoluble and precipitated soluble fiber.

Optimization of Digestion with Physiological Enzymes. In view of the encouraging results obtained by the two groups using modified Hellendoorn procedures in the EEC/IARC collaborative study (Asp and Johansson, 1981; Schweizer and Würsch, 1981), we decided to try in the first place to reduce incubation time with the physiological enzymes pepsin and pancreatin used in the Hellendoorn type of methods.

Loss of Dietary Fiber Constituents in Filtrate after Alcohol Precipitation. Two of the enzymes used in the method, pepsin and pancreatin, are physiological digestion enzymes from hog and are free of any polysaccharidase activity besides that of α-amylase. The heat-stable α-amylase, Termamyl, which is used in the gelatinization step, is of technical grade and may contain other enzymes than α-amylase. However, Theander and Åman (1981) have found that it does not liberate any detectable amounts of sugars from β-glucan, arabinoxylan, and cotton-linter cellulose when used under the same conditions as for our starch hydrolysis. In the original procedure of Hellendoorn et al. (1975) the pH during the pepsin incubation was 1.0 and the incubation time 18 h. It has been pointed out repeatedly (Schweizer and Würsch, 1979; James and Theander, 1981) that acid-labile dietary fiber components, especially arabinose-containing side chains, might be hydrolyzed under such conditions and thus lost. To find out whether such losses are quantitatively important, the filtrates after alcohol precipitation were analyzed for sugars with GLC. The samples were acid hydrolyzed prior to GLC analysis to estimate oligo- and polysaccharides that might not have precipitated, as well as free monosaccharides.

Table II. Efficiency of Protein Digestion with Various Conditions at the Pepsin Incubation and a Following 1-h Pancreatin Digestion (Percent of Original Material on a Moisture-Free Basis) for Brown Beans (*P. vulgaris*)

conditions for pepsin step	insoluble dietary fiber			soluble dietary fiber			total dietary fiber (corrected for blank and in vitro undigestible protein)
	dietary fiber (corrected for blank)	protein (in addition to blank)	dietary fiber (corrected for protein)	dietary fiber (corrected for blank)	protein (in addition to blank)	dietary fiber (corrected for protein)	
pepsin, 18 h, pH 1.0	12.2	2.1	10.1	7.3	1.5	5.8	15.9
pepsin, 18 h, pH 1.5	13.4	2.2	11.2	7.3	2.1	5.2	16.4
pepsin, 1 h, pH 1.5	17.9	4.5	13.4	7.1	1.8	5.3	18.7

Table III. Dietary Fiber and in Vitro Undigestible Protein in 24 1-Day Samples of Lactovegetarian Diets

	total dietary fiber, % of moisture and fat-free substance		in vitro undigestible protein, % of total dietary protein
	corrected for blank	corrected for blank and undigestible protein	
mean	12.6	10.7	13.4
SD	2.5	2.5	2.7
range	7.48-17.14	5.71-15.24	7.3-18.2

Table IV. Residue after Incubation of Various Amounts of Casein with the Proposed Method

amount of casein, mg	protein, mg, remaining in insoluble + soluble fraction (blank subtracted)
100	0.0
200	1.1
300	2.4
500	10.4
750	15.6

Table I shows that the conditions in the pepsin step used in the original Hellendoorn method (pH 1.0, 18 h) caused considerable losses of arabinose and mannose in beans and losses of arabinose in the cereal samples. Since the glucose in the filtrates comes mainly from starch, possible losses of nonstarch glucans could not be detected. Such glucans, however, are not known to be particularly acid labile. When the pH was raised to pH 1.5, as also suggested by Schweizer and Würsch (1979), the losses were smaller in beans. Most of the arabinose and mannose losses in the filtrate from beans was not dialyzable, indicating that the lost sugars occurred as dietary fiber fragments with at least about 20–30 monomeric units. With pepsin incubation at pH 1.5 for 1 h, as in the recommended procedure, no dietary fiber monomers were detectable in the second filtrate. This also shows that the precipitation with 4 volumes of ethanol can be considered complete in practice.

Efficiency of Protein Digestion. Among several different foods analyzed, soy meal and brown beans gave the highest protein residues. The in vivo digestibility of proteins from *P. vulgaris* is very low, ranging from 0.65 to 0.88 in different varieties (Tobin and Carpenter, 1978). Heating in a boiling water bath for 15 min, which should inactivate protease inhibitors, did not improve protein digestion. The effect of increased pH and shortened incubation time in the pepsin step on the protein removal was tested on brown beans as shown in Table II. Increasing the pH from 1.0 to 1.5, but keeping the 18-h incubation time, did not affect the protein residue but increased the insoluble fiber by 1 g/100 g. Soluble fiber was not affected. Decreasing the incubation time to 1 h doubled the protein residue and increased the insoluble fiber by another 2 g/100 g. Still the soluble fiber was not affected. Thus, the total dietary fiber corrected for undigestible protein was 3 g/100 g higher with 1-h pepsin incubation at pH 1.5 than with 18-h incubation at pH 1.0. This is in agreement with the loss of arabinose and mannose in the filtrate after alcohol precipitation of brown bean samples digested with the last-mentioned conditions as shown above (Table I).

Table III shows the mean content of dietary fiber in 24 1-day samples of lactovegetarian diets. The in vitro undigestible protein in the dietary fiber fractions, i.e., the protein (Kjeldahl $N \times 6.25$) associated with the dietary fiber in addition to the protein in an enzyme blank (see below), was 13.4% (range 7.3–18.2%) of the total protein—a reasonable figure for what can be expected to be undigestible also in vivo. The usefulness of this in vitro undigestible protein assay should be further evaluated by comparison with the in vivo assay.

The efficiency of the protein digestion with the short incubation time was also tested by incubation of casein in various amounts. Up to 300 mg of casein, corresponding to 30% protein in a sample for dietary fiber assay, was almost completely removed. With larger amounts, about 2% was left (Table IV). This experiment also shows that the enzyme protein left in the blank is rather constant, regardless of the protein content of the sample.

Efficiency of Starch Digestion. The original Hellendoorn procedure as well as our first modification with a short pepsin step gave starch residues in the dietary fiber fraction, measurable with the amyloglucosidase method (see Materials and Methods). For instance, when different wheat and rye flours were analyzed, the starch residue increased the fiber value by about 1 g/100 g. Addition of amyloglucosidase to the pancreatin step, or introduction of a separate 30-min incubation with amyloglucosidase at pH 4.5, did not remove this residual starch.

Addition of the extremely heat-stable α-amylase Termamyl (Novo A/S, Copenhagen, Denmark) in the gelatinization step, however, completed the starch solubilization. This enzyme was also used by Theander and Åman (1979). With this modification only traces of starch ($\leq$0.1 g/100 g) remained in the fiber fractions (Table V). It should be noted, however, that both Termamyl and the amylase in pancreatin are α-amylases which break down starch only to maltose and dextrins. If starch is to be determined by filtrate analysis for glucose, an amyloglucosidase treatment is necessary to complete the starch hydrolysis.

Autoclaving has been suggested for complete gelatinization of starch prior to starch degradation (Schweizer and Würsch, 1979). Table VI shows that autoclaving for 1 h

Table V. Effect of Addition of Termamyl to the Gelatinization Step and Amyloglucosidase either to the Pancreatin Step or in an Additional Incubation Step on Starch Residue in Dietary Fiber of a Mixed Wheat/Rye Flour (the Figures Are in Percent of the Flour)

	total dietary fiber	starch residue	protein residue (in addition to blank)
no Termamyl			
(1) no amyloglucosidase	12.6	0.9	1.3
(2) amyloglucosidase added in pancreatin step	12.6	0.8	1.3
(3) separate amyloglucosidase incubation; pH 4.5, 60 °C, 30 min	12.4	0.8	ND[a]
Termamyl in gelatinization step			
(1) no amyloglucosidase	11.4	0.1	1.4
(2) amyloglucosidase added in pancreatin step	11.6	0.1	1.3
(3) separate amyloglucosidase incubation; pH 4.5, 60 °C, 30 min	11.4	0.1	ND

[a] Not determined.

Table VI. Effect of Autoclaving (125 °C for 1 h) and/or Additional Amyloglucosidase Digestion (pH 4.5, 60 °C, 30 min) on Dietary Fiber Value Assayed with the Proposed Method (the Figures Are in Percent of the Flour)

mixed wheat/rye flour		dietary fiber (including undigestible protein)		
amyloglucosidase	autoclaving	insoluble	soluble	total
no	no	9.0	2.6	11.6
yes	no	8.7	2.5	11.2
no	yes	8.5	3.3	11.8
yes	yes	8.7	3.1	11.8

at 125 °C of a mixed wheat and rye flour did not change the total dietary fiber value but tended to render a larger fraction of the fiber soluble. The efficiency of the starch digestion also without autoclaving is further illustrated by the fact that analysis of wheat starch gave a dietary fiber value of only 0.7 g/100 g.

Fat Extraction. It was recommended at the Cambridge meeting that samples should be fat extracted prior to fiber analysis (James and Theander, 1981). Two different fat extraction procedures were tested—a 15-min petroleum ether extraction at room temperature expected to remove the bulk of the fat and a more extensive extraction with chloroform–methanol (2:1) in a Soxhlet apparatus for 5 h (see Materials and Methods). Three materials with different fat contents were used: whole grain wheat flour, wheat bran, and a mixed diet.

As shown in Table VII dietary fiber was the same without fat extraction as with any of the two fat extraction methods. This was true even for the mixed diet samples with 22% fat. Obviously, fat is efficiently removed in one or several steps of the dietary fiber method itself. The fat can be expected to be solubilized during the gelatinization and incubation steps as well as by the 78% ethanol used to precipitate soluble fiber. Any fat retained at the filtration is obviously removed by the final washing with acetone.

Thus, fat extraction does not seem necessary. A simple extraction of the bulk of the fat, however, for example, with petroleum ether, is recommended in samples containing more than 6–8% fat to ensure optimal enzyme digestion.

Blanks. Running the fiber determination without sample gave blank values as shown in Table VIII. Of the enzyme preparation used (about 250 mg) 16.9 mg of protein remained (7.5 mg of insoluble and 9.4 mg of soluble). Thus, more than 90% of the added enzymes were autodigested enough to stay in solution in 78% alcohol. In addition to the remaining enzyme protein small tare changes in the Celite also contributed somewhat to the blank value, corresponding to 0.2% insoluble and 0.1% soluble dietary fiber.

Subtraction of a blank assayed without sample would be a suitable correction to obtain dietary fiber with associated undigestible protein from the sample, provided that the enzyme protein residue is not affected by the sample protein content. The experiment shown in Table IV where increasing amounts of casein were added indicates that sample protein does not influence the blank values to any practically important extent.

Direct Precipitation. In many instances separate assay of soluble and insoluble dietary fiber components is not necessary. Alcohol precipitation immediately after the enzyme digestion was therefore tried to recover both insoluble and soluble fiber in one single filtration. As shown in Table IX, the dietary fiber values were the same with this procedure as with the separate assay of insoluble and soluble components. The coprecipitation of starch, protein, and ash was also similar.

Recommended Method for Enzymatic Dietary Fiber Assay. *Reagents.* The following reagents were used: (1) 0.1 M sodium phosphate buffer, pH 6.0; (2) 4 M HCl; (3) 4 M NaOH; (4) 95% ethanol, technical grade; (5) 78% ethanol; (6) acetone, puriss.

Enzymes used are as follows: (1) Termamyl 60 L (a higher grade, 120 L, is now available and preferred); (2) pepsin NF; (3) pancreatin 4 × NF.

Procedure. (1) Wet samples are homogenized and lyophilized. All samples are milled in a laboratory mill with 0.3-mm screen. (2) Fat extraction is carried out, with petroleum ether at room temperature for 15 min, when the fat content exceeds 6–8% or whenever needed for proper milling (see Materials and Methods). (3) Weigh 1 g of sample with 0.1-mg accuracy (W) and transfer to an Erlenmeyer flask. Add 25 mL of 0.1 M sodium phosphate buffer, pH 6.0, and suspend the sample thoroughly. (4)

Table VII. Effect of Fat Extraction with Various Methods before Dietary Fiber Analysis with the Proposed Method (the Figures Are in Percent of the Original Material)

	whole grain wheat flour			wheat bran			mixed diet		
	no fat extraction	petroleum ether[a]	Soxhlet[b]	no fat extraction	petroleum ether[a]	Soxhlet[b]	no fat extraction	petroleum ether[a]	Soxhlet[b]
remaining fat in sample, analyzed after acid hydrolysis,[c] %	3.5	1.8	0.7	6	3.2	1.3	22.2	5.9	1.4
dietary fiber (corrected for undigestible protein), %	12.5	12.4	13.1	47.4	47.4	47.0	6.4	6.5	6.4

[a] Extraction at room temperature with 40 mL of petroleum ether/g of sample for 30 min. [b] Extraction with chloroform-methanol, 2:1, in a Soxhlet apparatus for 5 h. [c] Association of Official Analytical Chemists (1980).

Table VIII. Enzyme Blanks with the Proposed Method (Mean ± SD, $n = 6$)

	insoluble	soluble	total
mg	10.4 ± 1.23	12.8 ± 0.73	23.2 ± 1.9
% of 1 g	1.0 ± 0.12	1.3 ± 0.07	2.3 ± 0.19
protein ($N \times 6.25$), mg	7.5 ± 0.59	9.4 ± 0.55	16.9 ± 0.80

Add 100 μL of Termamyl. Cover the top of the flask with Al film and incubate in a boiling water bath for 15 min (with occasional shaking). (5) Allow to cool. Add 20 mL of distilled water and adjust the pH to 1.5 with HCl. Rinse the electrode with a few milliliters of water. (6) Add 100 mg of pepsin. Cover the flasks and incubate in a 40 °C water bath with agitation for 60 min. (7) Add 20 mL of distilled water and adjust the pH to 6.8 with NaOH. Rinse the electrode with 5 mL of water. (8) Add 100 mg of pancreatin. Cover the flasks and incubate in a 40 °C water bath with agitation for 60 min. (9) Adjust the pH to 4.5 with HCl. (10) Filter through a dry and weighed crucible (porosity 2) containing 0.5 g of dry Celite (exact weight known) as the filter aid. Wash with 2 × 10 mL of distilled water.

(*A*) *Residue* (*Insoluble Fiber*). (11) Wash with 2 × 10 mL of 95% ethanol and 2 × 10 mL of acetone. (12) Dry at 105 °C to constant weight (or overnight). Weigh, after cooling in a desiccator (D_1). (13) Incinerate at 550 °C for at least 5 h. Weigh, after cooling in a desiccator (I_1).

(*B*) *Filtrate* (*Soluble Fiber*). (14) Adjust the volume of the combined filtrate and washing waters to 100 mL. (15) Add 400 mL of warm (60 °C) 95% ethanol. Allow to precipitate for 1 h (time can be shortened). (16) Filter through a dry and weighed crucible (porosity 2) containing 0.5 g of Celite. (17) Wash with 2 × 10 mL of 78% ethanol, 2 × 10 mL of 95% ethanol, and 2 × 10 mL of acetone. (18) Dry at 105 °C overnight. Weigh, after cooling in a desiccator (D_2). (19) Incinerate at 550 °C for at least 5 h. Weigh, after cooling in a desiccator (I_2).

Blank. Insoluble and soluble blank values are obtained by running the procedure without sample (B_1 and B_2). The blank values should be checked occasionally and when new batches of enzymes are used.

Calculation. W = sample weight (g). D = weight after drying (g). I = weight after incineration (g). B = weight of ashfree blank (g).

$$\% \text{ insoluble dietary fiber} = \frac{D_1 - I_1 - B_1}{W} \times 100$$

$$\% \text{ soluble dietary fiber} = \frac{D_2 - I_2 - B_2}{W} \times 100$$

Correction for in Vitro Undigestible Protein. (1) Nitrogen in the dietary fiber fractions was determined with the Kjeldahl method and transfered to protein by multiplication with 6.25. (2) The protein content of the corresponding blank was assayed in the same way. (3) The difference, i.e., the in vitro undigestible protein, is subtracted from the dietary fiber values. In practice duplicate samples are run. One is used for incineration (steps 13 and 19, respectively) and the other one for protein determination.

Total Fiber Determination. Total fiber can be precipitated directly by adding 4 volumes of 95% alcohol to the whole digest after step 9 and separated with a single filtration, performed as described above for the soluble fiber (steps 15–19).

Precision of Dietary Fiber Assay. Table X shows standard deviation of insoluble, soluble, and total dietary fiber in different samples. The samples with low to moderate fiber content were cereal and potato samples, some of which were thermally processed. The high fiber samples were industrially purified wheat bran samples. The SD's of total dietary fiber—0.32 g/100 g in samples with low to moderate dietary fiber content and 0.46 g/100 g in samples with very high dietary fiber content—correspond to coefficient of variations of 3% and 0.5%, respectively, which must be considered satisfactory. These values indicate that the errors in the method are rather constant and do not depend on the dietary fiber content of the sample.

Digestion with Alternative Enzymes. A number of other enzymes have been tested and compared with the pepsin–pancreatin system. A bacterial, rather heat-stable, protease Alcalase 0.6 L (Novo A/S, Copenhagen, Denmark) was found useful in combination with Termamyl and amyloglucosidase (Boehringer, Mannheim, West Germany).

The gelatinization–Termamyl step was performed at pH 6.0 as in the recommended procedure above, steps 1–4. pH was then adjusted to 8.5 and 100 μL of Alcalase added. The protease treatment, which was optimal at this pH, was performed at 60 °C for 60 min with horizontal agitation. Longer incubation time did not improve the protein removal.

Starch digestion was completed by incubation with 25 μL of amyloglucosidase (crystal suspension, 10 mg/mL) at pH 4.5 at 60 °C for 30 min.

Filtration, washing of residues, drying, incineration, and correction for in vitro undigestible protein are carried out according to the recommended procedure for the physiological enzymes.

The blank value (insoluble plus soluble) was considerably lower with the Alcalase–amyloglucosidase system (5.8 ± 1.3 mg, 1.0 ± 0.3 mg of protein) than with the physiological enzymes (23.2 ± 1.9 mg, 16.9 ± 0.8 mg of protein).

Application to EEC/IARC Samples. The samples extensively investigated in the EEC/IARC collaborative study (James and Theander, 1981) were analyzed with both the physiological enzymes and the Alcalase–amyloglucosidase system as shown in Table XI. The two en-

Table IX. Comparison of Two Precipitation and Filtration Procedures: (A) Filtration, Precipitation, and Filtration; (B) Direct Precipitation and One Filtration (All Figures in Percent on a Moisture-Free Basis)

materials analyzed	method	total dietary fiber (corrected for blank)	residual starch	residual protein (in addition to blank)	dietary fiber (corrected for residual starch and protein)	ash
brown beans (*P. vulgaris*)	A	22.2	0.3	5.3	16.6	2.6
	B	22.5	0.4	5.9	16.2	3.0
wheat flour	A	3.1	0.1	0.5	2.5	1.0
	B	2.7	<0.1	0.2	2.4	0.9
whole grain wheat flour	A	12.1	<0.1	1.5	10.5	1.2
	B	11.8	0.1	1.4	10.3	1.8

Table X. Precision of Dietary Fiber Analysis with the Proposed Method (Calculated from Duplicate and Triplicate Analyses of Samples with Various Dietary Fiber Content)

	n	SD	mean	range of dietary fiber content of samples
samples with low or intermediate dietary fiber content				
insoluble fiber	14	0.26	7.36	1.3-26.1
soluble fiber	14	0.23	3.96	1.1-8.3
total fiber	14	0.32	11.32	2.4-30.5
samples with high dietary fiber content				
insoluble fiber	14	0.33	86.05	83.7-88.3
soluble fiber	14	0.40	2.28	1.8-3.7
total fiber	14	0.46	88.32	85.8-91.2

Table XI. Comparison of Dietary Fiber Analysis with Physiological and Microbial Enzymes, Respectively[a]

material	physiological enzymes	microbial enzymes
wheat bran	49.2	48.9
rye flour A	17.5	19.0
rye biscuit A	16.3	16.4
rye flour B	18.1	18.5
rye biscuit B	18.3	17.7
potato powder	7.6	7.1
apple pulp	16.1	14.5
soya flour	21.6 (13.8)[b]	19.1 (12.4)

[a] The materials analyzed are those used in the EEC/IARC collaborative study (James and Theander, 1981). The figures given are corrected for enzyme blank (percent of moisture-free material). [b] Values in parentheses are corrected for in vitro undigestible protein.

zyme systems gave very similar results. Correction for undigestible protein was essential in the soy flour sample but quantitatively less important in other samples. Due to the very large range of results from different laboratories in the EEC/IARC collaborative study, it is hardly possible to judge the accuracy of the methods from analyses of these samples.

DISCUSSION

In view of the complex and variable composition of dietary fiber, gravimetric methods seem to be the only alternative in practice for simple and rapid analysis for declaration and control purpose. The proposed method offers a convenient way of analyzing both soluble and insoluble dietary fiber. All fiber components seem to be recovered with the method. The time for the manual handling when analyzing a series of 10–15 duplicate samples is 1 day and thus comparable to the crude fiber and detergent fiber methods. No sophisticated equipment is necessary nor any special experience in carbohydrate chemistry. The Tecator filtration equipment used in this study, however, speeds up the method and probably also improves the precision.

The alterations of the original method of Hellendoorn et al. (1975) that have been made concern the recovery of soluble fiber components and the conditions for hydrolysis of protein and starch, some of which need some further comments.

The change of the conditions for the pepsin incubation was done in order to minimize the losses of acid-labile fiber components. As a result, the amount of undigested protein in the residues increased. The nature of this undigestible protein is not known, for example, if it is cell wall protein and if it is undigestible in vivo as well. However, it is known that especially vegetable protein has low in vivo digestibility; the protein in beans, for example, can have as low a digestibility as 65% (Tobin and Carpenter, 1978). Whether this undigestible protein should be regarded as a part of the fiber complex or not is a matter of discussion; some authors (Saunders and Hautala, 1979) claim that it should. According to the definition of Trowell et al. (1976), dietary fiber is the parts of the vegetable cells that are not digested by the alimentary enzymes and thus includes cell wall associated substances like cutin, tannins, waxes, and cell wall proteins as well as undigestible polysaccharides and lignin in the fiber complex. However, for analytical purposes another working definition is often used: "Dietary fiber is the sum of undigestible polysaccharides plus lignin" (Southgate et al., 1978). As long as this latter definition is the one accepted among analysts, the undigested protein in the fiber residues of our method has to be corrected for. This could be done by a Kjeldahl N analysis. Although the conversion factor to protein might vary, a standard 6.25 factor would give a satisfactory protein correction. However, in subtraction methods where the bulk of the protein is not removed as in our method, variations in the true factor may cause considerable error in the dietary fiber value.

In all fiber methods, an incomplete starch hydrolysis will give too high a fiber values. Termamyl during the gelatinization seems to eliminate almost all starch in the fiber residues that is available to amyloglucosidase. An obvious advantage with gravimetric methods is the possibility of checking the efficiency of the hydrolyses by analyzing the residues. The Termamyl in combination with the α-amylase activity in the pancreatin in the present method seems to give an almost complete solubilization of starch available for α-amylase and amyloglucosidase.

Registry No. α-Amylase, 9000-90-2; pepsin, 9001-75-6; pancreatin, 8049-47-6; starch, 9005-25-8; alcalase, 9014-01-1; termamyl, 9000-85-5; amyloglucosidase, 9032-08-0.

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Synthesis and Olfactory Properties of Some Thiazoles with Bell Pepper Like Odor

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Ten new cycloalkanethiazoles have been synthesized and their olfactory properties studied in relation to 4-butyl-5-propylthiazole. Only derivatives containing eight carbon atoms attached to the thiazole ring have the characteristic bell pepper odor. The study of olfactory thresholds has shown that such odor is most pronounced with 4-isopropyl-7-methylcyclohexathiazole; clear differences, both in odor quality and in olfactory threshold, have been found between stereoisomers of this odorant.

A number of pyrazine and thiazole derivatives exhibit a characteristic bell pepper like odor, associated with extremely low olfactory thresholds. These compounds seem to stimulate a common specific receptor, recently identified in the olfactory mucosa of several mammals (Pelosi et al., 1982). Several authors (Seifert et al., 1970, 1972; Pittet and Hruza, 1974; Parliment and Epstein, 1973; Buttery et al., 1976) have investigated the relationship of bell pepper odor and low olfactory threshold to chemical structure and have recognized common parameters in the molecules of pyrazines and thiazoles with such an odor. It is evident from their results that the lipophile part of the molecule plays an important role in the odor of the compound. However, the odorants examined so far bear hydrocarbon chains of medium length and therefore can adapt to the specific receptor in a great number of different conformations. In order to define with a greater accuracy the shape that the hydrocarbon side of bell pepper odorants must have for best fitting into the olfactory receptor and therefore for eliciting the characteristic odor, we have synthesized 10 new cycloalkanethiazoles that can be regarded as conformational models for 4,5-dialkylthiazoles.

EXPERIMENTAL SECTION

Synthesis of Compounds. All the thiazoles have been prepared according to two general routes, both starting from ketones. Route a involves bromination of the ketone, according to Catch et al. (1948), and cyclization of the bromo ketone with thioformamide, following the method of Kurkjy and Brown (1952). Route b involves direct synthesis of the 2-aminothiazole by reaction of the ketone with thiourea and iodine, according to the procedure of Hurd and Wehrmlister (1949), followed by deamination of the corresponding diazonium salt in the presence of hypophosphorous acid, as described by Roussel and Metzeger (1962). L-4-Isopropyl-7-methylcyclohexathiazole was prepared from L-menthone, obtained by oxidation of natural L-menthol with sodium dichromate, following the procedure reported in Vogel (1957). The optical activity of L-menthone was $[\alpha]^{25}_{D}$ −25.6°, corresponding to an optical purity better than 90%. All the thiazoles were purified by distillation to a grade better than 99.9%, as checked by GLC, by using a 3 mm × 1.5 m column, packed with 5% OV-17 on Anakrom. Mass spectra were recorded on a Hewlett-Packard 5992 B GC–MS, equipped with a jet separator and by using an ionization voltage of 70 eV. Odor threshold were measured in aqueous Tris-HCl, 0.05 M, buffer, at pH 7.0, by using panels of 18–25 subjects, following the method described by Amoore et al. (1968, 1975). Odor quality was judged informally by the authors and few other subjects in the laboratory.

RESULTS AND DISCUSSION

Synthesis of Compounds. Figure 1 shows the structures of the compounds prepared, while the data on their synthesis, structure, and olfactory properties are reported in Table I. Two derivatives, cyclohexathiazole and cyclooctathiazole have been prepared with both methods, to compare yields and purity of the final products. Although, on the average, both routes were equally satisfactory and gave easily purifiable products, the cyclization of the bromo ketones proved difficult in the cases of strained or large rings; in fact, it failed with 2-bromocyclopentanone and 2-bromocyclododecanone and gave poor yield with 2-bromocyclooctanone.

4-Isopropyl-7-methylcyclohexathiazole obtained from commerical menthone, i.e., from a mixture of the four possible stereoisomers, appeared to be constituted by a single pair of enantiomeric compounds, showing a single peak, when gas chromatographed on several different columns (packed OV-1 and OV-17 and capillary OV-1 and FFAP). The retention time is the same as that shown by

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