

# Megazyme

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## TOTAL DIETARY FIBER

### ASSAY PROCEDURE

K-TDFR-100A/K-TDFR-200A 04/17

(100/200 Assays per Kit)

For use with:

**AOAC Method 985.29**

**AOAC Method 991.42**

**AOAC Method 991.43**

**AOAC Method 993.19**

**AACC Method 32-05.01**

**AACC Method 32-06.01**

**AACC Method 32-07.01**

**AACC Method 32-21.01**



## INTRODUCTION:

Dietary fiber is a mixture of complex organic substances, including hydrophilic compounds, such as soluble and insoluble polysaccharides and non-digestible oligosaccharides, as well as a range of non-swellable, more or less hydrophobic, compounds such as cutins, suberins and lignins. The procedures for the determination of total dietary fiber as outlined in this booklet are based on the methods of Lee *et al.*<sup>1</sup> and Prosky *et al.*<sup>2,3</sup> (AOAC 991.43, AOAC 985.29, AACC 32-07.01 and AACC 32-05.01). However, the enzymes in the Megazyme Total Dietary Fiber Kit can also be used in other dietary fiber analytical methods such as AACC Method 32-21.01 and AACC method 32-06.01.

## PRINCIPLE (TOTAL DIETARY FIBER):

Total dietary fiber (TDF) is determined on duplicate samples of dried and defatted (if fat content is > 10%) material. Samples are cooked at ~ 100°C with heat stable  $\alpha$ -amylase to give gelatinisation, hydrolysis and depolymerisation of starch; incubated at 60°C with protease (to solubilise and depolymerise proteins) and amyloglucosidase (to hydrolyse starch fragments to glucose); and treated with four volumes of ethanol to precipitate soluble fiber and remove depolymerised protein and glucose (from starch). The residue is filtered; washed with 78% ethanol, 95% ethanol, and acetone; dried; and weighed. One duplicate is analysed for protein and the other is incubated at 525°C to determine ash. The TDF is the weight of the filtered and dried residue less the weight of the protein and ash.

The major advantage of the Megazyme TDF Test Kit is that it contains high purity enzymes devoid of interfering activities and the activities of the enzymes are standardised. The importance of standardised  $\alpha$ -amylase activity in the measurement of resistant starch is well recognised. Megazyme amyloglucosidase is essentially devoid of cellulase, whereas other commonly used preparations contain significant contamination with this activity, which leads to solubilisation and underestimation of  $\beta$ -glucan. All Megazyme TDF enzymes are supplied in a ready-to-use, stabilised, liquid form.

## SCOPE:

Applicable to cereal grains, fruit and vegetables, cereal and fruit products and foods.

## ENZYME PURITY AND STANDARDISATION:

The effectiveness and purity of Megazyme  $\alpha$ -amylase, protease and amyloglucosidase have been evaluated using the standards recommended in AOAC Method 985.29 and 991.43, and AACC Method 32-05.01. Megazyme thermostable  $\alpha$ -amylase (**E-BLAAM**)

has an activity of 3,000 U/mL (Ceralpha method); protease is supplied at a concentration of 50 mg/mL (~ 350 tyrosine U/mL); and amyloglucosidase is supplied at a concentration of 200 U/mL (*p*-nitrophenyl  $\beta$ -malto-side substrate) (or 3,300 U/mL on soluble starch). This amyloglucosidase activity is 150% the concentration traditionally used in TDF assays, so that 0.2 mL (instead of 0.3 mL) is used in the assay.

Megazyme amyloglucosidase (**E-AMGDF**) is essentially devoid of cellulase, whereas in other preparations used for TDF determination, the cellulase contamination can be as high as 1% of the amyloglucosidase (on an activity basis). This level of contamination leads to an underestimation of  $\beta$ -glucan by as much as 10-15%.

Methods used for the measurement and standardisation of Megazyme enzymes are available on request.

### **TDF ASSAY KIT:**

Kits with reagents for 100/200 assays are available from Megazyme and contain the full assay method plus:

#### **100 determinations assay kit (cat. no. K-TDFR-100A)**

- Bottle 1:** Thermostable  $\alpha$ -amylase (10 mL, ~ 3,000 U/mL (Ceralpha method); ~ 10,000 U/mL on soluble starch) (Megazyme cat. no. **E-BLAAM**).
- Bottle 2:** Purified protease (10 mL, 50 mg/mL; ~ 350 tyrosine U/mL) (Megazyme cat. no. **E-BSPRT**).
- Bottle 3:** Purified amyloglucosidase (20 mL, 3,300 U/mL on soluble starch) (Megazyme cat. no. **E-AMGDF**).

#### **200 determinations assay kit (cat. no. K-TDFR-200A)**

- Bottle 1:** Thermostable  $\alpha$ -amylase (20 mL, ~ 3,000 U/mL (Ceralpha method); ~ 10,000 U/mL on soluble starch) (Megazyme cat. no. **E-BLAAM**).
- Bottle 2:** Purified protease (20 mL, 50 mg/mL; ~ 350 tyrosine U/mL) (Megazyme cat. no. **E-BSPRT**).
- Bottle 3: (x 2)** Purified amyloglucosidase (20 mL, 3,300 U/mL on soluble starch) (Megazyme cat. no. **E-AMGDF**).

Celite<sup>®</sup>, analytical grade, in 100 g or 500 g packages, is available separately (cat. no. **G-CEL-100G** or cat. no. **G-CEL-500G**).

### **TDF ASSAY CONTROL KIT (K-TDFC):**

This kit is used in conjunction with the TDF Assay Kit to determine enzyme effectiveness and purity. The kit contains one vial of each of the components listed below and a technical data sheet (**K-TDFC; Dietary Fiber Controls**).

The amount of each component included in this kit is adequate for at least 10 assays.

Component	Amount
$\beta$ -Glucan (Barley)	1 g
High Amylose Maize Starch	10 g
Starch (Wheat)	10 g
Casein	5 g
Pectin	1 g
Larch Galactan	1 g

## **METHOD 1: DETERMINATION OF TOTAL, SOLUBLE AND INSOLUBLE DIETARY FIBER**

Based on AOAC Method 991.43 “Total, Soluble, and Insoluble Dietary Fiber in Foods” (First Action 1991) and AACC Method 32-07.01 “Determination of Soluble, Insoluble, and Total Dietary Fiber in Foods and Food Products” (Final Approval 10-16-91).

### **DEFINITION:**

This method is the simplified modification of the AACC total dietary fiber (TDF) method, 32-05.01, and the AACC soluble/insoluble dietary fiber method (for oat products), 32-21.01 (see Note 1, page 9.)

**1. Principle:** Briefly, 1 g dried food samples (duplicate) is subjected to sequential enzymatic digestion by heat-stable  $\alpha$ -amylase, protease and amyloglucosidase.

**2. Soluble/insoluble dietary fiber determination:** Insoluble dietary fiber (IDF) is filtered, and then residue is washed with warm distilled water. Combined solution of filtrate and water washings are precipitated with 4 volumes of 95% ethanol (EtOH) for soluble dietary fiber (SDF) determination. Precipitate is then filtered and dried. Both SDF and IDF residues are corrected for protein, ash and blank, for the final calculation of SDF and IDF values.

**3. Total dietary fiber determination:** SDF is precipitated with EtOH, and residue is then filtered, dried and weighed. Total dietary fiber (TDF) value is corrected for protein and ash content.

## SCOPE:

This method determines soluble, insoluble and total dietary fiber content in processed foods and raw materials, such as cereal products, fruits and vegetables.

## APPARATUS:

1. Beakers, 400 mL and 600 mL tall-form.
2. Fritted crucible, Gooch, fritted disk, Pyrex<sup>®</sup> 50 mL, pore size coarse, ASTM 40-60  $\mu\text{m}$ , Corning<sup>®</sup> No. 32940-50C, or equivalent. Prepare as follows:
  - a. Ash overnight at 525°C in muffle furnace.
  - b. Remove Celite and ash material by using a vacuum.
  - c. Soak in 2% Micro cleaning solution (reagent 7, page 5) at room temperature for 1 h.
  - d. Rinse crucibles with water and deionised water.
  - e. For final rinse, use 15 mL acetone and air dry.
  - f. Add approx. 1.0 g Celite to dried crucibles and dry at 130°C to constant weight.
  - g. Cool crucible in desiccator for approx. 1 h and record weight of crucible containing Celite.
3. Filtering flask, heavy-walled, with 1 L side arm.
4. Rubber ring adaptors for use on filtering flasks.
5. Vacuum source: vacuum pump or aspirator with regulator capable of regulating vacuum.
6. Water bath, shaking, large-capacity (20-24 L) with covers; capable of maintaining temperature of 100°C; equipped with automatic timers for on-off operation.
7. Balance, 0.1 mg accuracy.
8. Ovens, two, mechanical convection, set at 103 $\pm$ 2°C and 130 $\pm$ 3°C.
9. Timer.
10. Desiccator, airtight, with SiO<sub>2</sub> or equivalent desiccant. Desiccant dried biweekly overnight in 130°C oven.
11. pH meter.
12. Pipettors and tips, 50-200  $\mu\text{L}$  and 5 mL capacity.
13. Dispensers
  - a. 15 $\pm$ 0.5 mL for 78% EtOH, 95% EtOH, and acetone.
  - b. 40 $\pm$ 0.5 mL for buffer.

14. Cylinder, 500 mL.
15. Magnetic stirrers and stirring bars.
16. Rubber spatulas.
17. Muffle furnace, 525±5°C.

## REAGENTS:

1. Ethanol, 95% v/v.
2. Ethanol, 78%. Place 821 mL 95% v/v ethanol into a 1 L volumetric flask. Dilute to volume with deionised water. Mix well. Check the level and if necessary add more deionised water to bring it back up to the 1 L mark.
3. Acetone, reagent grade.
4. **Enzymes for TDF assay** (Megazyme). Store at 0-5°C.
  - a.  $\alpha$ -Amylase, heat-stable (**E-BLAAM**); 3,000 Ceralpha Units/mL.
  - b. Protease (**E-BSPRT**); 50 mg/mL; 350 Tyrosine Units/mL.
  - c. Amyloglucosidase (**E-AMGDF**); 200 pNP  $\beta$ -maltoside Units/mL (or 3,300 Units/mL on soluble starch).
5. Deionised water.
6. Celite<sup>®</sup>, analytical grade (Megazyme cat. no. **G-CELITE**).
7. Cleaning solution, Micro (International Products Corp., Trenton, NJ). Make 2% solution with deionised water.
8. MES/TRIS buffer, 0.05 M each, pH 8.2 at 24°C. Dissolve 19.52 g 2(N-morpholino) ethanesulfonic acid (MES) (Megazyme cat. no. **B-MES250**) and 12.2 g tris(hydroxymethyl)aminomethane (TRIS) (Megazyme cat. no. **B-TRIS500**) in 1.7 L deionised water. Adjust pH to 8.2 with 6.0 N NaOH. Dilute to 2 L with water. It is important to adjust pH of buffer to approx. 8.3 at 20°C or approx. 8.1 at 27-28°C.
9. Hydrochloric acid solution, 0.561 N. Add 93.5 mL of 6 N HCl to approx. 700 mL of water in 1 L volumetric flask. Dilute to 1 L with water.
10. pH standards. Buffer solutions at pH 4.0, 7.0 and 10.0.

## ENZYME PURITY:

To ensure presence of appropriate enzyme activity and absence of undesirable enzyme activity, run materials listed below through entire procedure. Each new lot of enzymes should be tested, as should enzymes that have not been tested for previous 6 months.

Alternatively, enzyme activity and purity can be determined using assay procedures as summarised on pages 17 and 18 of this booklet.

Test Sample	Activity Tested	Sample Wt (g)	Expected Recovery (%)
Citrus pectin	Pectinase <sup>a</sup>	0.1	90-95 <sup>c</sup>
$\beta$ -Glucan (barley)	$\beta$ -glucanase (cellulase) <sup>a</sup>	0.1	95-100
Wheat starch	Amylase <sup>b</sup>	1.0	0-1
Casein	Protease <sup>b</sup>	0.3	0-2
High amylose starch	Amylase	1.0	~ 30 <sup>d</sup>

- a This activity should not be present in the tests.
- b This activity should be fully functional in the tests.
- c Values are low due to incomplete precipitation of citrus pectin.
- d This material contains a high level of “enzyme resistant” starch. The exact expected recovery value will be affected by the level of thermostable  $\alpha$ -amylase used in the test.

## PROCEDURE:

### 1. Blanks

With each assay, run two blanks along with samples to measure any contribution from reagents to residue.

### 2. Samples

- a. Weigh duplicate  $1.000 \pm 0.005$  g samples accurately into 400 mL tall-form beakers.
- b. Add 40 mL MES-TRIS blend buffer solution (pH 8.2) to each beaker. Add magnetic stirring bar to each beaker. Stir on magnetic stirrer until sample is completely dispersed in solution (this prevents lump formation, which would make sample inaccessible to enzymes).

### 3. Incubation with heat-stable $\alpha$ -amylase

- a. Add 50  $\mu$ L heat-stable  $\alpha$ -amylase solution, while stirring at low speed.
- b. Cover each beaker with aluminium foil squares.
- c. Place covered samples in shaking water bath at 98-100°C and incubate for 30 min with continuous agitation. Start timing once all beakers are in hot water bath.

### 4. Cool

- a. Remove all sample beakers from hot water bath and cool to 60°C.
- b. Remove foil covers.
- c. Scrape any ring around beaker and gels in bottom of beaker with spatula, if necessary.

- d. Rinse side wall of beaker and spatula with 10 mL distilled water by using pipettor.
- e. Adjust temperature of water bath to 60°C by draining some of hot water from water bath and adding cold water.

## 5. Incubation with protease

- a. Add 100  $\mu$ L protease solution to each sample.
- b. Re-cover with aluminium foil.
- c. Incubate in shaking water bath at  $60 \pm 1^\circ\text{C}$ , with continuous agitation for 30 min. Start timing when temperature of water bath reaches 60°C.

## 6. pH check

- a. Remove sample beakers from shaking water bath.
- b. Remove covers.
- c. Dispense 5 mL of 0.561 N HCl solution into sample while stirring.
- d. Check pH, which should be 4.1-4.8. Adjust pH, if necessary, with additional 5% NaOH solution or 5% HCl solution (see Note 2, page 10).

## 7. Incubation with amyloglucosidase

- a. Add 200  $\mu$ L amyloglucosidase solution while stirring on magnetic stirrer.
- b. Replace aluminium cover.
- c. Incubate in shaking water bath at 60°C for 30 min with constant agitation. Start timing when temperature of water bath reaches 60°C.

## A. INSOLUBLE DIETARY FIBER

### 8. Filtration setup

- a. Tare crucible containing Celite to nearest 0.1 mg.
- b. Wet and redistribute bed of Celite in crucible using approx. 3 mL distilled water.
- c. Apply suction to crucible to draw Celite onto fritted glass as an even mat.

### 9. Filter enzyme mixture from Step 7 through crucible into a filtration flask.

### 10. Wash residue twice with 10 mL distilled water pre-heated to 70°C. Use water to rinse beaker before washing residue in crucible. Save filtrate and water washings for determination of SDF. Transfer solution to a pre-tared 600 mL tall-form beaker (for SDF determination, go to Step 11 of SDF procedure, page 8).

### 11. Wash residue twice with 10 mL of:

- a. 95% EtOH
- b. Acetone



- 12. Dry crucible** containing residue overnight in 103°C oven.
- 13. Cool crucible** in desiccator for approx. 1 h. Weigh crucible containing dietary fiber residue and Celite to nearest 0.1 mg. To obtain residue weight, subtract tare weight, i.e. weight of dried crucible and Celite.
- 14. Protein and ash determination.**

One residue from each type of fiber is analysed for protein and the second residue of the duplicate is analysed for ash.

  - a. Perform protein analysis on residue using Kjeldahl method. Use 6.25 factor for all cases to calculate g of protein.
  - b. For ash analysis, incinerate the second residue for 5 h at 525°C. Cool in desiccator and weigh to nearest 0.1 mg. Subtract crucible and Celite weight to determine ash content (see Note 3, page 10).

## **B. SOLUBLE DIETARY FIBER**

### **1-10. Follow Steps 1-10 of IDF method.**

- 11. Weigh** combined solution of filtrate and water washings in pre-tared beaker from Step 10 of IDF procedure.
- 12. Precipitation of SDF**
  - a. Add 4 vols 95% EtOH pre-heated to 60°C. Use a portion of EtOH to rinse filtering flask from IDF procedure (step 10). Alternatively, adjust weight of combined solution of filtrate and water washings to 80 g and add 320 mL of pre-heated (60°C) 95% EtOH.
  - b. Allow the precipitate to form at room temperature for 60 min.
- 13. Filtration setup**
  - a. Tare crucible containing Celite to nearest 0.1 mg.
  - b. Wet and redistribute the bed of Celite in the crucible, using 15 mL of 78% EtOH from wash bottle.
  - c. Apply suction to crucible to draw Celite onto fritted glass as an even mat.
- 14. Filtration**
  - a. Filter precipitated enzyme digest from SDF Step 12 through crucible.
  - b. Using a wash bottle with 78% EtOH and a rubber spatula, quantitatively transfer all remaining particles to crucible.
- 15. Wash**

Using a vacuum, wash residue successively with two 15 mL portions of the following: (see Note 4, page 10).

  - a. 78% EtOH
  - b. 95% EtOH
  - c. Acetone
- 16. Dry crucible** containing residue overnight in 103°C oven.

17. Proceed with Steps 13 and 14 of IDF method.

## C. TOTAL DIETARY FIBER

1-7. Follow Steps 1-7.

### 8. Precipitation of dietary fiber with EtOH.

- To each sample, add 225 mL 95% EtOH pre-heated to 60°C. Measure volume after heating. Ratio of EtOH volume to sample volume should be 4:1. If 95% EtOH is accidentally over-heated to 65°C, add 228 mL for expanded alcohol volume adjustment.
- Cover all samples with large sheets of aluminium foil.
- Allow precipitate to form at room temperature for 60 min.

9. Proceed with Steps 13-17 of SDF procedure.

## CALCULATIONS:

$$\text{Dietary Fiber (\%)} = \frac{\frac{R_1 + R_2}{2} - p - A - B}{\frac{m_1 + m_2}{2}} \times 100$$

### where:

$R_1$  = residue weight 1 from  $m_1$ ;  $R_2$  = residue weight 2 from  $m_2$

$m_1$  = sample weight 1;  $m_2$  = sample weight 2

A = ash weight from  $R_1$ ; p = protein weight from  $R_2$  and

$$\begin{aligned} B &= \text{blank} \\ &= \frac{BR_1 + BR_2}{2} - BP - BA \end{aligned}$$

### where:

BR = blank residue;

BP = blank protein from  $BR_1$

BA = blank ash from  $BR_2$ .

**NOTE:** These calculations can be simplified by using the Megazyme **Mega-Calc™**, downloadable from where the product appears on the Megazyme web site ([www.megazyme.com](http://www.megazyme.com)).

## NOTES:

- Differences between this method and AACC methods 32-05.01 and 32-21.01 are as follows:
  - 40 mL MES-TRIS buffer, 0.05 M each, pH 8.2 at 24°C is

used instead of 50 mL phosphate buffer, 0.08 M, pH 6.0. The pH of MES-TRIS buffer changes with temperature. The pH of MES-TRIS buffer (i.e. 8.2 at 24°C) reaches 6.9-7.2 at 85-90°C and 7.4-7.6 at 55-60°C. Note that pH optimum of heat-stable  $\alpha$ -amylase moves from pH 6.0 at 60°C toward pH 7.0 at 90°C.

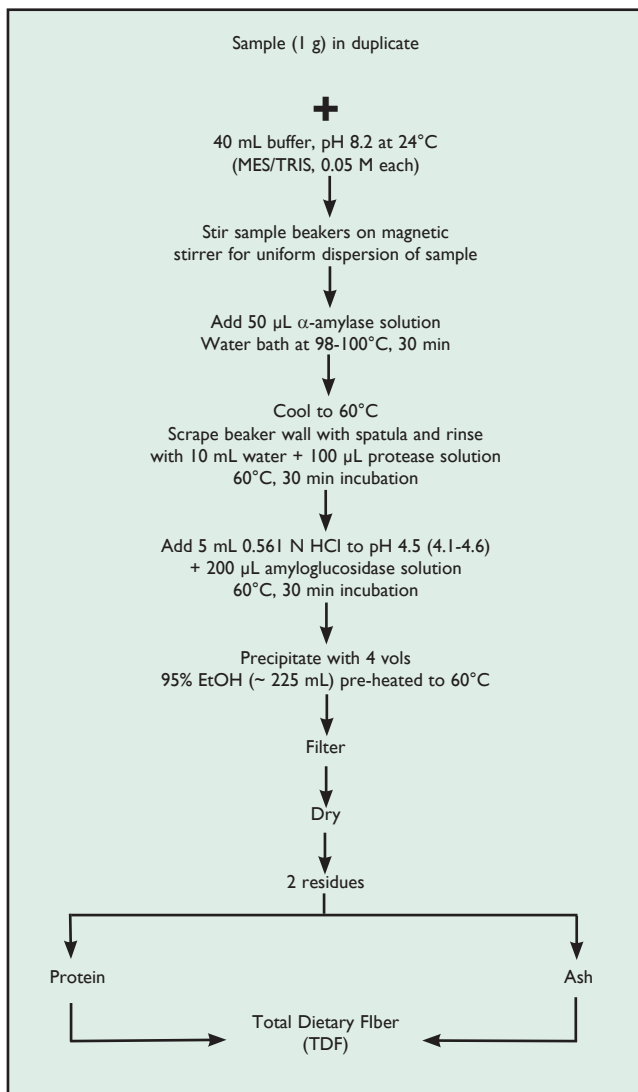
- b. The volume of thermostable  $\alpha$ -amylase used has been reduced from 200  $\mu$ L to 50  $\mu$ L due to the higher activity of the enzyme employed here.
  - c. Any ring left around the beaker after heat-stable  $\alpha$ -amylase incubation is scraped, if necessary. With pipettor, 10 mL of water is added to rinse spatula and side wall of beaker after heat-stable  $\alpha$ -amylase incubation.
  - d. No pH adjustment is needed for protease action, thus no NaOH is added to the incubation mixture.
  - e. For amyloglucosidase action, 5 mL of 0.561 N HCl solution is added.
  - f. For TDF determination, the amount of 95% EtOH added for the precipitation step is 225 mL instead of 280 mL. For SDF/IDF determination, weight of filtrate and washing solution is adjusted to 80 g instead of 100 g. Thus, 320 mL of 95% EtOH at 60°C is added. Alternatively, weigh combined solution of filtrate and washing solution and add 4 vols. 95% EtOH. Total filtration volume is reduced to 375-400 mL with this modification (see Figures 1 and 2, page 11 and 12).
2. It is important to leave the beaker in 60°C water bath until it is ready for pH adjustment, since pH of solution increases at a lower temperature. Normally, additional pH adjustment (with 5% HCl or 5% NaOH) is not required for most oat, barley, wheat and corn products. For such known products, one can skip the pH checking procedure after addition of 5 mL of 0.561 N HCl to sample. Routine checking of pH of blank is recommended as a disaster check. If blank is not within desirable pH range, samples should also be checked.
  3. There is some indication that delay in washing IDF residues with 95% EtOH and acetone may cause inflated IDF values. Thus, it is suggested that IDF residues not be washed toward end of SDF/IDF procedures.
  4. In some samples a gum is formed, trapping liquid. If this occurs, break layer of film with spatula.

## REFERENCES:

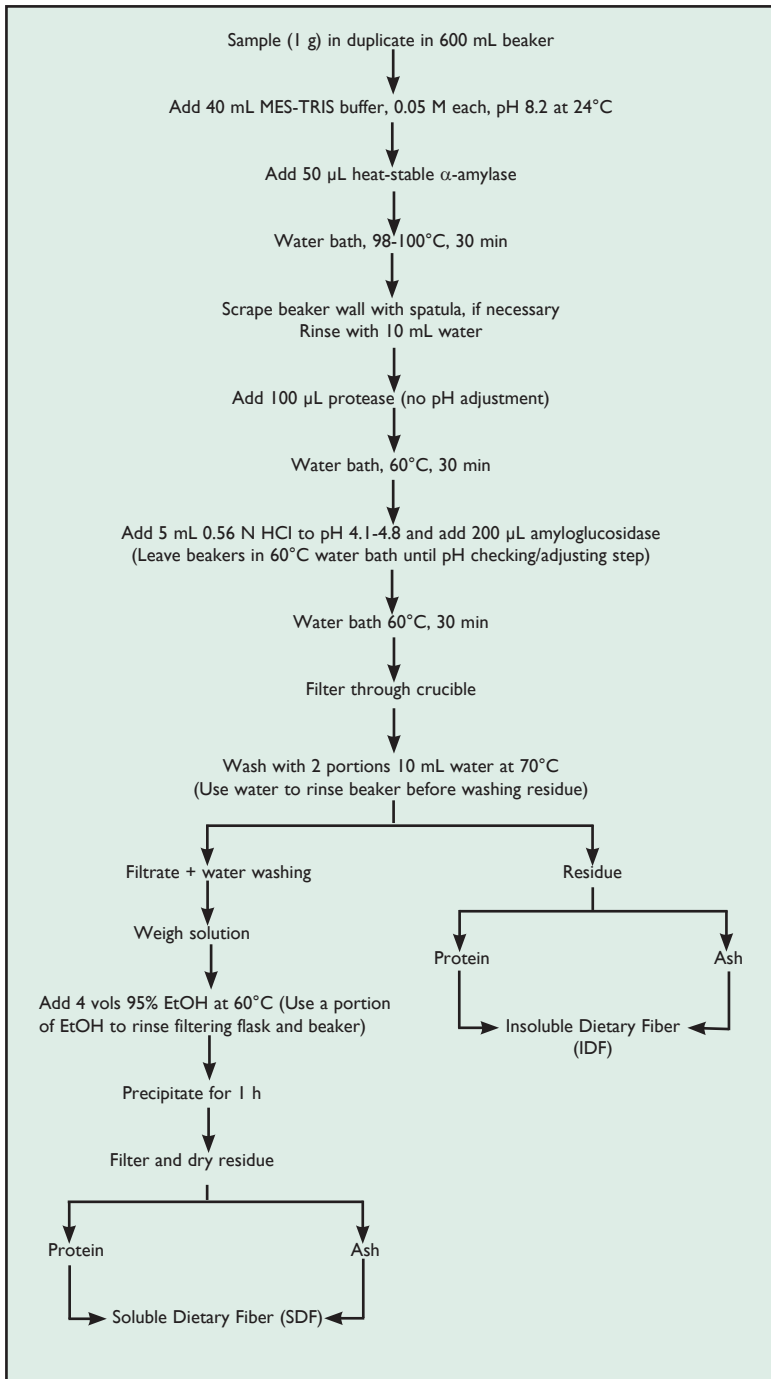
1. Lee, S. C., Prosky, L. & DeVries, J. W. (1992). Determination of total, soluble, and insoluble, dietary fiber in foods - enzymatic-

gravimetric method, MES-TRIS buffer: Collaborative study. *J. Assoc. Off. Anal. Chem.*, **75**, 395-416.

2. Prosky, L., Asp, N. G., Schweizer, T. F., DeVries, J. W. & Furda, I. (1988). Determination of insoluble, soluble, and total dietary fiber in foods and food products: Interlaboratory study. *J. Assoc. Off. Anal. Chem.*, **71**, 1017-1023.
3. Prosky, L., Asp, N. G., Schweizer, T. F., DeVries, J. W. & Furda, I. (1992). Determination of insoluble and soluble dietary fiber in foods and food products: Collaborative study. *J. Assoc. Off. Anal. Chem.*, **75**, 360-367.



**Figure 1.** Analytical scheme for the total dietary fiber determination procedure.



**Figure 2.** Analytical scheme for soluble and insoluble dietary fiber determination procedures.

## METHOD 2: DETERMINATION OF TOTAL DIETARY FIBER

Based on AACC method 32-05.01 and AOAC Method 985.29.

### APPARATUS:

1. Dispensers
  - a.  $280 \pm 2.0$  mL for 95% ethanol.
  - b.  $10 \pm 0.5$  mL for 78% ethanol, 95% ethanol and acetone.
  - c.  $50 \pm 0.5$  mL for buffer.
2. All other equipment is as described on page 4 of this booklet.

### REAGENTS:

1. Phosphate buffer, 0.08 M, pH 6.0. Dissolve 1.400 g disodium phosphate anhydrate ( $\text{Na}_2\text{HPO}_4$ ) (or 1.753 g dihydrate) and 9.68 g disodium phosphate monobasic monohydrate ( $\text{NaH}_2\text{PO}_4$ ) (or 10.94 g dihydrate) in approx. 700 mL distilled water. Dilute to 1 L with water. Check pH with pH meter.
2. Sodium hydroxide solution, 0.275 N. Dissolve 11.00 g ACS grade NaOH in approx. 700 mL distilled water, using appropriate handling precautions, in 1 L volumetric flask. Cool and dilute to volume with water.
3. Hydrochloric acid solution, 0.325 N. Dilute stock solution of known titer (i.e. 325 mL of 1.0 N HCl) to 1 L with water in volumetric flask.

### PROCEDURE:

#### Preparation of sample

Total dietary fiber should be determined on an as-is basis on dried, low-fat or fat-free sample. Homogenise sample and dry overnight in  $70^\circ\text{C}$  vacuum oven. Cool in desiccator, reweigh and record weight loss due to drying. Dry-mill portion of dried sample to 0.3-0.5 mm mesh. If sample cannot be heated, freeze-dry before milling. If high fat content ( $> 10\%$ ) prevents proper milling, defat with petroleum ether three times with 25 mL portions (per g of sample) before milling. When analysing mixed diets, always extract fat before determining total dietary fiber. Record weight loss due to fat. Correct final % dietary fiber determination for both moisture and fat removed. Store dry-milled sample in capped jar in desiccator until analysis is run.

## Method

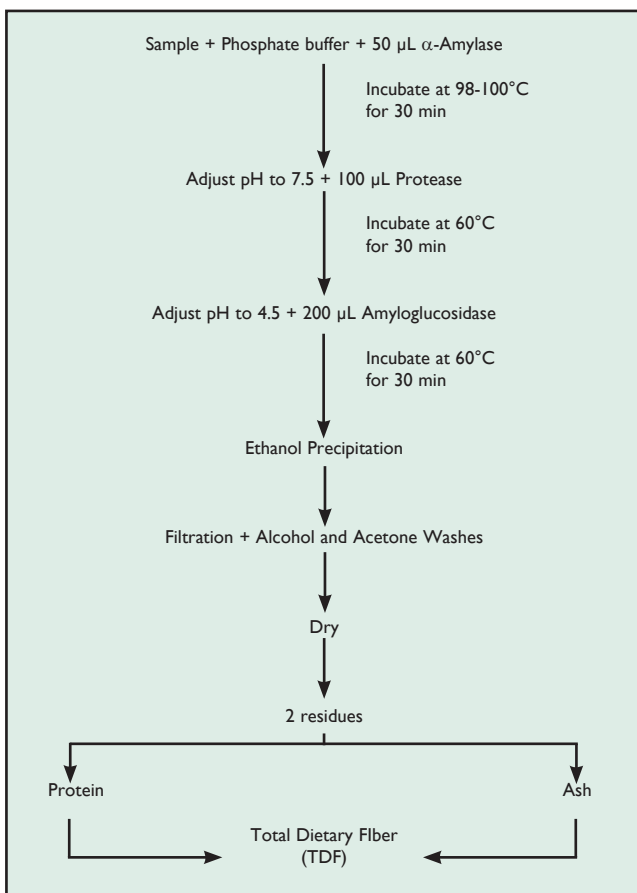
Run blank through entire procedure along with samples to measure any contribution from reagents to residue.

1. Weigh duplicate 1 g samples, accurate to 0.1 mg, into 400 mL tall-form beakers. Sample weights should differ by less than 20 mg from each other. Add 50 mL phosphate buffer (pH 6.0) to each beaker and check pH with pH meter. Adjust if pH does not equal  $6.0 \pm 0.1$ .
2. Add 50  $\mu$ L heat-stable  $\alpha$ -amylase solution.
3. Cover beaker with aluminium foil and place in boiling water bath. Beaker must be incubated at 98-100°C for 15 min. Shake gently at 5 min intervals.

**Note:** Increase incubation time when number of beakers in bath makes it difficult for beaker contents to reach internal temperature of 98-100°C. Use thermometer to indicate that 15 min at 98-100°C is attained. Total of 30 min in boiling water bath should be sufficient.

4. Cool solutions to room temperature.
5. Adjust to pH  $7.5 \pm 0.1$  by adding 10 mL 0.275 N NaOH solution. Check pH with pH meter.
6. Add 100  $\mu$ L of protease solution.
7. Cover beaker with aluminium foil and incubate at 60°C with continuous agitation for 30 min.
8. Cool and add 10 mL 0.325 N HCl solution to adjust pH to  $4.5 \pm 0.2$ . Check pH with pH meter.
9. Add 200  $\mu$ L amyloglucosidase, cover with aluminium foil, and incubate for 30 min at 60°C with continuous agitation.
10. Add 280 mL 95% EtOH pre-heated to 60°C (measure volume before heating). Let precipitate form at room temperature for 60 min.
11. Weigh crucible containing Celite to nearest 0.1 mg, then wet and distribute bed of Celite in crucible by using stream of 78% EtOH from wash bottle.
12. Apply suction to draw Celite onto fritted glass as even mat. Maintain suction and quantitatively transfer precipitate from enzyme digest to crucible.

13. Wash residue successively with three 20 mL portions of 78% EtOH, two 10 mL portions of 95% EtOH, and two 10 mL portions of acetone. In some cases, gums may form during filtration, trapping liquid in residue. If so, break surface film with spatula to improve filtration. Long filtration times can be avoided by careful intermittent suction throughout filtration.
14. Dry crucible containing residue overnight in 70°C vacuum oven or 105°C air oven.
15. Cool in desiccator and weigh to nearest 0.1 mg. Subtract crucible and Celite weights to determine weight of residue.
16. Analyse residue from one sample of set of duplicates for protein by AACC Method 46-13, using  $N \times 6.25$  as conversion factor.
17. Incinerate second residue sample of duplicate for 5 h at 525°C. Cool in desiccator and weigh to 0.1 mg. Subtract crucible and Celite weights to determine ash.



**Figure 3.** Analytical scheme for total dietary fiber procedure.



## CALCULATIONS:

Uncorr'd av blank residue (UABR)	=	Av blank residue of duplicate blanks (from step 15) in mg
Blank protein residue (BPR)	=	$UABR \times \% \text{ protein in blank (step 16)}/100$
Blank ash residue (BAR)	=	$UABR \times \% \text{ ash in blank (step 17)}/100$
Corrected blank (CB)	=	$UABR - BPR - BAR$
Uncorr'd av sample residue (USAR)	=	Av sample residue of duplicate samples (from step 15) in mg
Sample protein residue (SPR)	=	$USAR \times \% \text{ protein in sample (step 16)}/100$
Sample ash residue (SAR)	=	$USAR \times \% \text{ ash in sample (step 17)}/100$
Corrected sample residue (CSR)	=	$USAR - SPR - SAR - CB$
% TDF	=	$100 \times CSR/\text{mg sample}$

Correct final % TDF for fat and/or water if defatting or drying of sample was done during sample preparation step.

**NOTE:** These calculations can be simplified by using the Megazyme **Mega-Calc**<sup>™</sup>, downloadable from where the product appears on the Megazyme web site ([www.megazyme.com](http://www.megazyme.com)). Also see page 9 of this booklet.

## REFERENCES:

1. Association of Official Analytical Chemists. (1985). *Official Methods of Analysis*, 14th ed., 1st suppl. Secs. **43**, A14-43, A20, p.399.
2. Association of Official Analytical Chemists (1986). *Changes in methods*. *J. Assoc. Off. Anal. Chem.*, **69**, 370.
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4. Prosky, L., Asp, N. G., Furda, I., DeVries, J. W., Schweizer, T. F. & Harland, B. F. (1985). Determination of total dietary fibre in foods and food products: Collaborative study. *J. Assoc. Off. Anal. Chem.*, **68**, 677.
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# MEASUREMENT OF THE ACTIVITY AND PURITY OF ENZYMES USED IN TOTAL DIETARY FIBER MEASUREMENTS

For successful application of the AACC/AOAC INTERNATIONAL dietary fiber assay procedures, the enzymes used must have required activity and be devoid of important contaminating activities. Some problems which have been experienced to date are:

- a. contamination of amyloglucosidase with cellulase, xylanase and pectinase (which results in degradation and underestimation of  $\beta$ -glucan, arabinoxylan and pectin).
- b. varying concentrations of thermostable  $\alpha$ -amylase (which affects resistant starch measurements) (see reference 1).
- c. contamination of protease with 1,3:1,4- $\beta$ -glucanase (which results in an underestimation of  $\beta$ -glucan).

## Standardisation of Required Activities:

Based on the history of use of  $\alpha$ -amylase, amyloglucosidase and protease in AACC/AOAC INTERNATIONAL dietary fiber determinations, the required activities for accurate and reliable measurements are:

### **Amyloglucosidase (*A. niger*); 0.2 mL per assay.**

**3,300 U/mL** (on soluble starch with reducing sugar assay) or **200 U/mL** (AMG Assay Reagent; *p*NP- $\beta$ -maltoside in the presence of excess  $\beta$ -glucosidase).

### **Thermostable $\alpha$ -Amylase (*B. licheniformis*); 0.05 mL per assay.**

**10,000 U/mL** (on soluble starch with reducing sugar assay) or **3,000 U/mL** (Ceralpha Reagent; blocked *p*-nitrophenyl maltoheptaoside in the presence of thermostable  $\alpha$ -glucosidase).

### **Protease (*B. licheniformis*; Subtilisin A); 0.10 mL per assay.**

**350 Tyrosine Units/mL.** (50 mg/mL; 7 Tyrosine Units/mg) (assayed on casein with tyrosine standard).

Alternatively, protease can be conveniently assayed using Azo-Casein (Megazyme cat. no. **S-AZCAS**).

#### **Further Details:**

McCleary, B. V. (1999). Enzyme Purity and Activity in Fiber Determinations. *Cereal Foods World*, **44(8)**, 590-596.

## Important Contaminating Activities:

### 1. In Amyloglucosidase Preparations:

An evaluation of amyloglucosidase preparations used in dietary fiber determinations showed that many preparations (except very high purity and expensive preparations) contain significant levels of contaminating  $\beta$ -glucanase (cellulase). In some preparations, this contamination was as high as 1% (on an activity basis) and resulted in an underestimation of  $\beta$ -glucan by as much as 10-15%. Cellulase contamination in amyloglucosidase can best be demonstrated and estimated by viscometric studies using barley  $\beta$ -glucan as substrate or by using the Megazyme Beta-Gluczyme test tablets (for the measurement of  $\beta$ -glucanase and cellulase).

The effect of cellulase contamination of amyloglucosidase on the viscosity (i.e. molecular size) of  $\beta$ -glucan is demonstrated in Figure 4. Barley  $\beta$ -glucan (10 mL, 10 mg/mL) in sodium acetate buffer (50 mM, pH 4.5) was incubated with amyloglucosidase (0.2 mL of preparation as used in the TDF assay) at 40°C in a Type C, U-tube viscometer. Viscosity measurements were taken at various time intervals, and the specific viscosity was calculated as  $(t-t_0)/t_0$ , where  $t_0$  is the time of flow of the solvent and  $t$  is the time of flow of the digest.

Two enzyme preparations were compared:

- A. Megazyme amyloglucosidase (**E-AMGDF**) (at the same AMG concentration as other commercial preparations used for TDF determinations).
- B. Another commercially available amyloglucosidase preparation recommended for use in dietary fiber determinations.

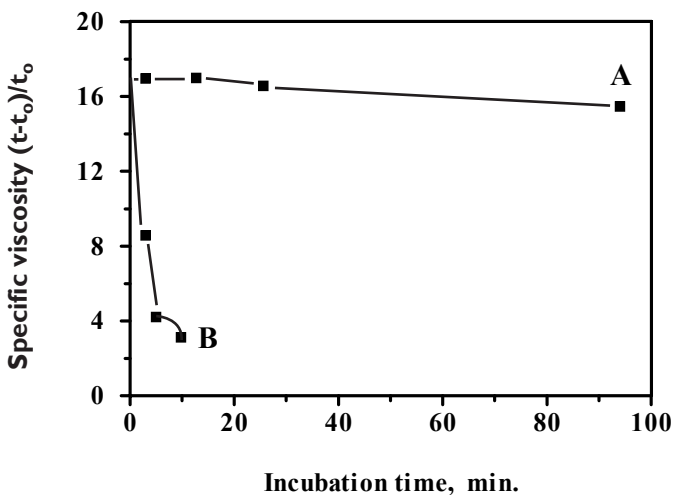
It is evident that Megazyme amyloglucosidase (**E-AMGDF**) is essentially devoid of contaminating  $\beta$ -glucanase, whereas the other preparation contains significant levels of this contaminant.

### 2. In Protease Preparations:

The degree of contamination of protease with (1,3)(1,4)- $\beta$ -glucanase can be determined using the procedure described for the measurement of cellulase in amyloglucosidase, with the modification that the assay pH is 7.5 (sodium phosphate buffer, 50 mM).

### 3. In Alpha-Amylase Preparations:

No contaminating enzyme activities were detectable in thermostable  $\alpha$ -amylase.



**Figure 4.** Assay for cellulase contamination in amyloglucosidase preparations using a viscometric assay with barley  $\beta$ -glucan as substrate (refer to text).

**A.** Megazyme amyloglucosidase (**E-AMGDF**).

**B.** Another amyloglucosidase preparation which has been used in dietary fiber determinations.



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