Chapter 14

DETERMINATION OF TOTAL DIETARY FIBER BY DIFFERENCE AND OF PECTIN BY COLORIMETRY OR COPPER TITRATION

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I. INTRODUCTION

We are interested in the role that dietary factors can play in the primary prevention of atherosclerosis. As it has been claimed [1] that removal of fiber from the diet might raise serum cholesterol levels, we have recently investigated whether a high-fiber diet can lower serum cholesterol levels in healty volunteers under controlled conditions [2]. For such studies data are needed on the content and composition of dietary fiber in foodstuffs. Of the components of dietary fiber, pectic compounds have been reported to be particularly effective in lowering serum cholesterol [3,4]. We have therefore selected from the literature analytical methods for total dietary fiber and for pectin, modified them as needed, and applied them to Dutch foodstuffs.

This paper describes these methods and the results obtained for samples distributed under the EEC/IARC collaborative study and for some ordinary foodstuffs.

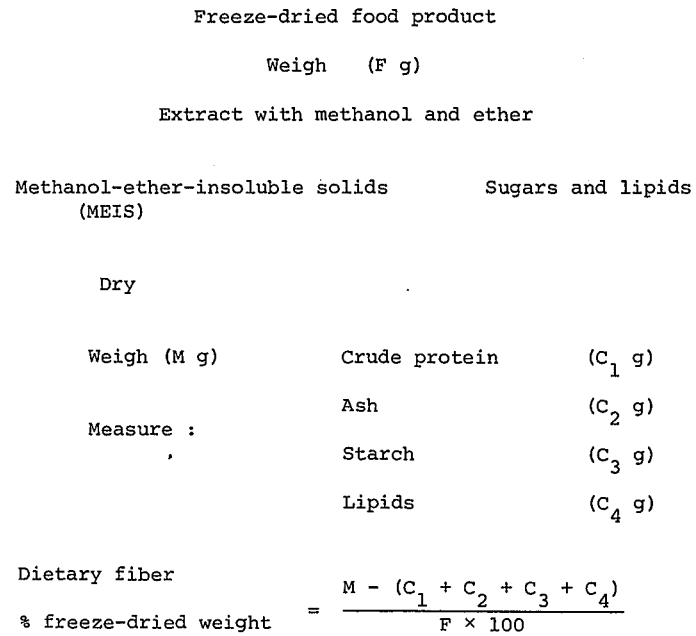

Figure 1. Simplified flow chart for the analysis of unavailable carbohydrates plus lignin by difference.

II. GENERAL DESCRIPTION OF METHODS

A. *Determination of Total Dietary Fiber by Difference*

A flow chart of the method (originally described by McCance et al. [5]) is presented in Figure 1. The principle of the method is as follows:

The sample is extracted with methanol and ether as described by Southgate [6] so as to remove sugars, pigments, and lipids. The residue, called the methanol-ether insoluble solids (MEIS), is weighed, and all nonfiber components such as ash, moisture, protein, starch, and fat are determined. Crude protein is measured in almost all products but starch determinations are limited to cereal products, potatoes, and legumes. In bread and some other cereal products, residual lipid material is determined as well.

The amount of dietary fiber is then calculated by subtracting the amounts of nonfiber material (ash, protein, starch, and lipids)

from the weight of the MEIS. (This difference in weight was originally taken to be the value for unavailable carbohydrates by McCance et al. [5].) By taking into account the percentage of MEIS in the product itself, the dietary fiber content of the whole product can now be calculated using the formula shown in Figure 1.

Some of the details of this general scheme are given below and in the Appendixes.

Preparation of Methanol-Ether Insoluble Solids (MEIS)

Between 4 and 5 g freeze-dried and ground material was extracted with 25 ml of a methanol-water mixture (85:15, vol/vol) on a combined magnetic stirrer and hotplate. The solids were collected by centrifugation followed by filtration and extracted three more times with hot 85% methanol. The hotplate was allowed to cool down and the extraction was repeated three times with diethyl ether. The solids were then dried overnight in a vacuum oven at 70°C.

Determination of Protein, Fat, and Starch

Crude protein was determined in a Tecator Kjeltec System I. For each sample 7 mg Se was used as catalyst. Factors for converting N to protein are given by Southgate [7]. Fat was measured by acid hydrolysis according to Weibul modified as in the officially accepted method [8]. Starch was liberated from the MEIS by extraction with 1.6 M HCl in dimethyl sulfoxide (Me_2SO) and then hydrolyzed to glucose using amyloglucosidase. Glucose was measured using hexokinase plus glucose-6-phosphate dehydrogenase. The starch determination is described in detail in Appendix 1.

B. Determination of Polygalacturonic Acid by Colorimetry and by a Copper Binding Assay

Pectic substances consist of a polygalacturonic acid backbone with side chains of neutral sugars [9]. In addition, rhamnose units may occur in the main polygalacturonic acid chain [10]. We have compared two methods for the determination of polygalacturonic acid.

In the first method, pectin and other fiber components were

broken down by a commercial enzyme preparation containing pectic, hemicellulytic, and cellulytic enzymes [12], and uronic acids were quantified by a modified carbazole reaction [11]. Details are presented in Appendix 2.

The second method, which is described in Appendix 3, was developed by Keybets and Pilnik [12]. It is based upon the high affinity of Cu^{2+} ions for the acidic groups of polygalacturonic acid. After a wash with 70% isopropanol, the intact sample was incubated with aqueous $CuSO_4$ at low pH. Nonbound Cu^{2+} was washed out with H_2O and bound Cu^{2+} eluted with HCl and quantified.

A variable proportion of the acidic groups in pectic substances is blocked by methyl esterification. These groups must be exposed by alkaline hydrolysis prior to the determination of total polygalacturonic acid by this method. By performing a Cu^{2+} binding assay before and after hydrolysis, values are obtained for both the proportion of esterified residues and the total polygalacturonic acid content.

III. RESULTS

A. Enzymatic Determination of Starch

The pretreatment with 1.6 M HCl in Me_2SO for 30 min at 60°C is vigorous. However, as shown in Table 1, treatment of a variety of starchy high-fiber foodstuffs with acid Me_2SO yielded a negligible amount of glucose. Appreciable quantities of glucose were produced only after enzymic treatment. Thus, only polymers hydrolyzable by amyloglucosidase, i.e., α-1,4, α-1,6 glucans, contributed to the figure for starch, and little or no glucose was nonspecifically split from either starch or the nonstarchy fiber components during the acid pretreatment.

B. Suppression of Glucose Interference in the Carbazole Test by Addition of Sulfamate

The colorimetric carbazole assay as originally described [13,14] is not specific for uronic acids; neutral sugars also contribute to the result. Galambos [11] presented evidence that the addition

Table 1 Liberation of Glucose from Foodstuffs by Pretreatment with Acid Dimethyl Sulfoxide With or Without Amyloglucosidase Incubation[a]

Sample	Anhydroglucose released, g/100 g dry weight	
	Acid Me_2SO pretreatment	After enzymatic hydrolysis
Starch (Merck 1252)	0.3	96.0
Whole wheat meal, raw	0.4	56.1
Potatoes, boiled	0.0	66.7
Garden peas, bottled	1.3	38.6
Brown marrowfat peas, canned	0.4	43.2
French beans, frozen	0.3	19.5

[a]Foods were first extracted with methanol and ether as described in the methods section. Between 0.1 and 1 g MEIS sample was then incubated in 20 ml Me_2SO plus 5 ml 8 M HCl for 30 min at 60°C. Enzymatic degradation of starch was performed with 400 mg of amyloglucosidase (Sigma) at 45°C for 30 min. Glucose was measured enzymatically, using hexokinase plus glucose-6-phosphate dehydrogenase. Values are given as grams of anhydroglucose (equivalent to polymeric starch) per 100 g dry product. For solutions of pure glucose taken through the complete procedure recoveries varied between 97 and 100%.

of $NH_4NH_2SO_3$, ammonium sulfamate, suppresses this interference. The results shown in Figure 2 support this finding: in the presence of sulfamate (plus borate), 1 g of glucose gave only 1.5% as much absorbance as 1 g of galacturonic acid, while in its absence interference was 19%.

C. *Comparison of the Carbazole Method and the Copper Binding Test*

Both the colorimetric and the copper binding method were applied to a number of Dutch food products. Figure 3 shows that for vegetables and fruits the methods give similar results: the line obtained by linear regression of the results for the Cu^{2+} binding method on those for the carbazole method deviates only slightly from $y = x$. If appreciable amounts of Cu^{2+} had been bound by structures other than polygalacturonic acid one would have expected a systematic difference between the copper and the carbazole methods, but this does not seem to be the case.

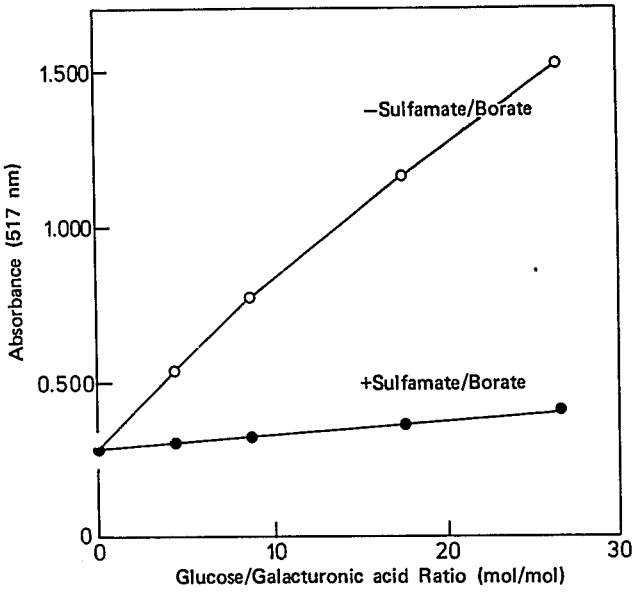

Figure 2. Suppression of glucose interference in the carbazole test for galacturonic acid, by addition of sulfamate (and borate) to the assay. o, *without sulfamate;* ●, *with sulfamate. Solutions containing 0.113 mmol/l galacturonic acid (24 mg/l galacturonic acid monohydrate) and between 0.5 and 3.0 mmol/l glucose (90-540 mg/l) were assayed in the presence of ammonium sulfamate plus potassium borate as described in Appendix 2. For the controls, water was added instead of sulfamate and borate.*

D. Results with the EEC samples

Results are displayed in Table 2. The agreement between the polygalacturonic acid values according to the carbazole method and the copper binding method is rather poor for wheat bran and rye flour. The apparently low degree of esterification for potato powder is probably not due to binding of Cu^{2+} ions by phosphate groups in potato starch (a problem described by Keybets and Pilnik [121]) because all starchy samples were subjected to enzymatic hydrolysis of starch prior to the copper binding test.

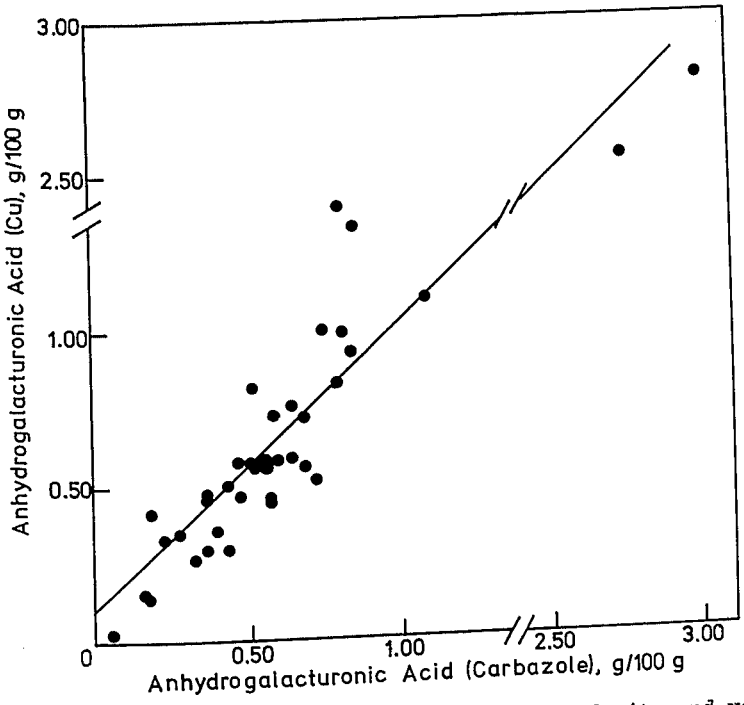

Figure 3. Polygalacturonic acid content of fruits and vegetables measured by enzymatic digestion plus carbazole colorimetry (horizontal axis) and by copper binding (vertical axis), g/100 g edible product. Figures are presented for 39 samples. The regression equation calculated by the method of least squares was: [Copper] = 0.93 x[Carbazole] + 10; r = 0.94 (95% confidence limits for r, 0.88 - 0.97); standard error of estimate $S_{y \cdot x}$ = 0.19 g/100 g. The point placed at the intercept of the vertical axis has x = 0.80, y - 1.73.

Table 2 Results for Samples of EEC/IARC 1978 Collaborative Study on Analysis of Dietary Fiber[a]

A. Amount and Composition of Methanol-Ether-Insoluble Solids

Sample	Methanol-ether-insoluble solids, g/100 g dry matter	Ash	Crude protein	Starch g/100 g MEIS	Fat	Total dietary fiber
1. Wheat bran	86.1	5.6	17.0	29.0	3.8	44.6
2. Rye flour A	90.7	1.4	8.6	68.8	2.2	19.0
3. Rye biscuit A	90.7	1.1	9.4	67.0	2.2	20.3
4. Rye flour B	90.2	1.4	9.0	68.3	2.0	19.3
5. Rye biscuit B	90.8	1.1	10.5	66.2	2.3	19.9
6. Apple pulp	17.1	2.0	5.2	-	-	92.8
7. Citrus pectin	102.1	3.2	1.3	-	-	95.5
8. Potato powder	93.9	2.2	5.5	80.4	-	11.9
9. Soya flour	60.1	5.7	61.6	1.5	3.1	28.1

B. Dietary Fiber and Polygalacturonic Acid Content of Samples

	Sample	Total solids g/100 g as received		Total dietary fiber by difference, g/100 g dry matter	Polygalacturonic acid[c]		
		By our standard method	By Southgate method[b]		Carbazole method, g/100 g dry matter	Cu binding method, g/100 g dry matter	Degree of esterification, mol/100 mol
1.	Wheat bran	92.6	90.8	38.4	0.21	1.40	-
2.	Rye flour A	90.8	88.5	17.2	0.16	0.49	-
3.	Rye biscuit A	95.3	95.9	18.4	0.46	0.59	-
4.	Rye flour B	91.1	88.9	17.4	0.16	0.47	-
5.	Rye biscuit B	98.1	96.0	18.1	0.48	0.59	-
6.	Apple pulp	95.4	96.7	15.9	3.72	3.22	77
7.	Citrus pectin	99.2	94.9	97.5	80.00	73.00	68
8.	Potato powder	94.5	93.1	11.2	0.80	0.95	7
9.	Soya flour	96.8	95.6	16.9	0.44	-	-

[a] Products analyzed without further homogenization.

[b] These are the figures used for further calculations.

[c] Equivalent to anhydrogalacturonic acid.

IV. DISCUSSION

A. *Total Dietary Fiber*

The principle of the subtraction method of McCance et al. [5] as described here differs from that of other gravimetric methods such as those of Hellendoorn [15] and Van Soest (Chapter 8). In the latter methods, nonfiber components are carefully removed by enzymatic hydrolysis or chemical extraction. The dietary fiber remains and is quantified by weighing. This demands a good method for removing the starch because it must be completely effective and must not at the same time degrade any of the dietary fiber components themselves.

In the difference method used by us, however, only glucose liberated from components of fiber during the starch determination could bias the dietary fiber value. As shown in Table 1, no such interference was found for a number of starchy foodstuffs: glucose was produced from them only after addition of a starch-splitting enzyme.

An obvious disadvantage of the difference method described in this chapter is that it does not yield figures for the different types of polymers which together constitute total dietary fiber. The residue from the starch determination could be used for analyses of the components as described by Southgate (Chapter 1). However, one would first need to ascertain whether the acid-Me_2SO-amyloglucosidase treatment degraded any fiber components to yield monomers other than glucose before one could be sure that the method was appropriate.

B. *Polygalacturonic Acid (Pectin)*

The determination of pectin in foodstuffs is complicated by a number of factors:

1. Concentration of pectin is often low.
2. Pectic substances are often heterogeneous and poorly delineated from other components of fiber.
3. A variable part of the pectin dissolves in water.

As yet, no definitive method for the determination of pectic substances in foodstuffs is available (cf. Refs. 16, 17). The widely used carbazole method is subject to interference by neutral sugars, as mentioned above. In our hands it also gave problems with colored foodstuffs such as red beets where high blanks caused inaccurate results. Problems with the copper binding method arose with cereal products because only a small fraction of their alcohol-insoluble residue is pectin, and also because Cu^{2+} was bound non-specifically. Low values for the degree of esterification also raised our suspicions: The amount of Cu^{2+} bound after alkaline hydrolysis of the methyl esters was often lower than before hydrolysis, probably because interfering material had been removed by alkali. It is unlikely that starch constituted this interfering material because starch was removed by enzymatic hydrolysis prior to the copper binding assay.

It may therefore be wise to apply routinely two independent methods for the determination of pectic substances in foodstuffs until a definitive method becomes available. One advantage of using the Cu^{2+} binding assay is that it yields a value for the degree of esterification and this information could be helpful in research into the effects of pectic compounds on serum cholesterol concentrations [18].

For products such as bread, macaroni, and breakfast cereals the copper binding test unfortunately does not yield reliable results. An accurate determination of uronic acids in cereal products is of interest because even low levels of pectic substances in cereals will contribute significantly to the total daily pectin intake of Europeans. For example, if bread contains only 1% of pectin on a dry weight basis, then four slices of bread would contribute about the same amount of pectin as a medium-size apple [16, 17]. We are therefore attempting to find the appropriate conditions so that the copper binding method can be applied with confidence to grain products.

ACKNOWLEDGMENTS

The authors are grateful to Prof. Dr. W. Pilnik and his co-workers from the Dept. of Food Technology, Agricultural University, Wageningen for valuable help and advice and to Mrs. J. Andriessen-Bos and Mrs. C. Germing-Nouwen for performing many of the analyses. Part of this research was supported by the Netherlands Heart Foundation, Grant No. 24.004.

APPENDIX 1

Determination of Starch in the Methanol-Ether Insoluble Solids (MEIS) of Food Products

Principle

Starch is liberated from the MEIS by an acid-dimethyl sulfoxide (Me_2SO) extraction and then hydrolyzed to glucose using amyloglucosidase (AGS).

$$\text{MEIS sample} + \text{acid-}Me_2SO \rightarrow \text{dissolved starch}$$

$$\text{Starch} + H_2O \xrightarrow{AGS} \text{glucose}$$

The glucose is then reacted with hexokinase and glucose-6-phosphate dehydrogenase, the formation of NADPH indicating the amount of glucose present in the sample.

Glassware and Equipment Needed

Wide-necked Erlenmeyers flasks, 100 ml; centrifuge tubes, 10 ml; polystyrene cuvettes, 1-cm light path, with stirring rods; shaking waterbath at 60°C; pH meter; magnetic stirrer; burette, 50 ml; centrifuge; Eppendorf pipettes of 0.020, 0.1000, 0.200, and 1.0 ml; spectrophotometer reading at 340 nm.

Reagents and their Preparation

1. Citrate buffer, pH 4.6. Dissolve 440 mg citric acid, monohydrate and 680 mg trisodium citrate, $2H_2O$ in water. Add water to 100 ml, mix, and if necessary add acid or alkali to bring the pH to 4.6.
2. Amyloglucosidase (Sigma A-7255). Dissolve 400 mg in 20 ml

citrate buffer while shaking and filter off the insoluble matter. Prepare freshly each time.

3. Triethanolamine buffer, pH 7.6. Dissolve 14.0 g triethanolamine hydrochloride and 0.25 g magnesium sulfate, $7H_2O$ in about 80 ml H_2O. Add 5 M sodium hydroxide to give a pH of 7.6, and water to 100 ml. The solution will keep for 4 weeks at 4°C.
4. NADP (Boehringer 127353). Dissolve 60 mg in 6 ml water. This solution will keep at 4°C for 4 weeks.
5. ATP (Boehringer 126888). Dissolve 300 mg ATP and 300 mg sodium bicarbonate in 6 ml water. The solution will keep for 4 weeks at 4°C.
6. Hexokinase/glucose-6-phosphate dehydrogenase mixture (Boehringer 127183). Use undiluted.
7. Hydrochloric acid, 8 M. To 322 ml 37% HCl add water to 500 ml.
8. Dimethyl sulfoxide, Me_2SO, 100%.

Preparation of Samples

Grind the samples to a particle size of about 0.3 mm diameter in a mortar, Braun minimixer, or Culatti mill.

Extraction

Weigh 0.1- to 1-g sample into an Erlenmeyer of 100 ml, depending on starch content (see below). In each series include one Erlenmeyer containing reagents only and no sample as a blank.

Add 20 ml Me_2SO and 5 ml 8 M HCl.

Incubate at 60°C for 30 min in a shaking waterbath.

Add about 50 ml water and let the Erlenmeyer cool down to room temperature.

Put the Erlenmeyer on a magnetic stirrer and slowly add 5% sodium hydroxide from a burette to bring the pH to 2-3. Then add 1 M sodium hydroxide from a Pasteur pipette to bring the solution to pH 4-5.

Transfer the contents of the Erlenmeyer to a measuring flask of 200 ml.

Enzymatic Hydrolysis

To each flask add 20 ml filtered amyloglucosidase (AGS) enzyme solution. Put the flasks in a 45°C shaking waterbath for 30 min. Then let them cool down and add water to the 200-ml mark. Filter through a fluted filter and save the filtrate.

Determination of Glucose in the Extract After Hydrolysis

Mark polystyrene cuvettes: one for each sample, one for a blank containing amyloglucosidase and hexokinase/glucose-6-phosphate dehydrogenase (AGS + HK), and one for a blank with the hexokinase/glucose-6-phosphate dehydrogenase (HK) solution only.

Depending on the amount of glucose in the filtrate, pipette into the sample cuvette 0.1-1.0 ml of filtrate corresponding to 10-50 µg glucose.

Pipette 1 ml blank filtrate into the blank AGS+HK cuvette.

Pipette 1 ml water into the blank HK cuvette.

To all cuvettes add the following with Eppendorf pipettes in succession: 1.0 ml triethanolamine buffer, 0.10 ml NADP solution, and 0.10 ml ATP solution.

With a measuring pipette, add to the sample cuvette water to a final volume of 3.20 ml; to the blank cuvettes AGS + HK and HK add 1.0 ml water. Mix well with a plastic stirring rod and read the extinction at 340 nm after 3 min (E_1) with water as a blank. Use one plastic stirring rod for each cuvette. Finally add 0.02 ml HK/G-6-PDH enzyme mixture (shake before use) to each cuvette and mix with the same plastic stirring rod.

Read the extinction at 340 nm against water after 10 and after 15 min (E_2). If there is a difference between the extinction at 10 and at 15 min then continue measuring every 5 min until the extinction is constant or until the change every 5 min remains constant. In the latter case E_2 is found by extrapolation to time 0.

Calculation

For the blank containing AGS + HK calculate the change in extinction:

$$\Delta E_{AGS+HK} = (E_2 - E_1)_{AGS+HK}$$

Similarly calculate ΔE_{HK} for the blank containing the HK/G-6-PDH mixture. Now calculate $\Delta E_{AGS} = \Delta E_{AGS+HK} - \Delta E_{HK}$.

This value of ΔE_{AGS} applies to the volumes and dilutions of the blank. Recalculate it as a blank for each sample by adjusting for the volume of sample used.

For example: A blank from 400 mg AGS in a 200-ml flask of which 1 ml ends up in the cuvette contains 2 mg AGS per cuvette whereas a sample is derived from the same 400 mg AGS in the 200 ml but 0.2 ml might go into the cuvette which then contains 0.4 mg AGS. Therefore the net extinction of the blank ΔE_{AGS} is divided by 5 to give the ΔE_{AGS} for this sample.

Calculate the net extinction for each sample:

$$\Delta E_S = (E_2 - E_1)_S - \Delta E_{AGS} - \Delta E_{HK}$$

Calculate the glucose concentration, C, in mg/ml in the filtrate:

$$C = \frac{V \times 162.1}{\varepsilon \times v \times 1000} \times \Delta E_S$$

and the concentration of starch in the MEIS sample:

$$\% \text{ starch} = C \times f \times \frac{100}{m} \qquad (g/100\ g)$$

where

V = volume in cuvette (3.22 ml)

v = sample volume (0.1-1 ml)

ε = extinction coefficient of NADPH at 340 nm = 6.3 $(\text{mmol/liter})^{-1} \times \text{cm}^{-1}$

f = volume of hydrolyzate (200 ml)

m = sample weight (0.1-1.0 g)

Commentary on Method

The amount of dried sample used depends on its starch content and the amount of MEIS available for the determination. The minimum is 0.1 g and the maximum 1 g. If NADPH is measured at 340 nm the amount of starch in the cuvette should correspond to 3-50 µg. To reach this range the volume of filtrate pipetted into the cuvette may be increased to a maximum of 1 ml. Starting with 250 mg MEIS sample and the minimum dilution (to 200 ml) the limit of detection (with 1 ml filtrate in the cuvette) is about 0.2% starch.

The use of blanks allows for any glucose in the reagents, enzymes, etc. The velocity of enzyme reactions is temperature-sensitive. The reaction times suggested are based on a temperature of 20-25°C. Therefore the triethanolamine buffer as well as the NADP and the ATP solutions should be warmed to room temperature.

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APPENDIX 2

Analysis of Pectic Substances after Enzymatic Hydrolysis

Principle

Pectic substances in food products are hydrolyzed to water-soluble galacturonic acid using the enzyme preparation Ultrazym 100 [12]. After dilution the amount of galacturonic acid is determined by a color reaction with carbazole. Interference by monosaccharides is suppressed by the addition of ammonium sulfamate [11].

Reagents

1. Citric acid, 1 M. Dissolve 19.2 g citric acid in 100 ml water.
2. Ultrazym 100, 0.2% (Ciba-Geigy, Basel, Schweiz). Dissolve 200 mg Ultrazym 100 in 100 ml water. Prepare freshly each day.
3. Sodium hydroxide, 1 M (4 g in 100 ml water).
4. Ammonium sulfamate, 4 M. Dissolve 45.6 g in water to 100 ml.
5. Borate, 1 M. Dissolve 6.18 g boric acid in 70 ml warm water. Allow to cool. Bring the pH to 8.4 using 1 M potassium hydroxide and add water to 100 ml.
6. Sulfuric acid, 96%.
7. Carbazole solution, 0.2%. Dissolve 200 mg carbazole in 100 ml purified ethanol. This solution is stable in the dark at 4°C for 6 months.
8. Purified ethanol. Reflux 1 liter ethanol with 4 g zinc powder and 2 ml 96% sulfuric acid for about 6 hr. Distill and discard the first and the last 100 ml. Redistill with 4 g zinc powder and 4 g KOH.
9. Standard galacturonic acid solutions. Transfer 120 mg galacturonic acid.H_2O (Merck 4081) quantitatively to a 1 liter measuring flask, with water. Add about 500 ml water and 0.5 ml 1 M sodium hydroxide solution. Shake until dissolved and add water to 1 liter. Leave to dissolve overnight. Dilutions: Pipette 10, 20, and 30 ml, respectively, into three measuring flasks of 100 ml and add water to 100 ml. These flasks will now contain 0.996, 1.993, and 2.989 mg anhydrogalacturonic acid *per 100 ml*.

Enzymatic Hydrolysis

Weigh 250 mg (freeze dried and ground) sample into an Erlenmeyer of 100 ml. In each series include one Erlenmeyer containing reagents only and no sample as an enzyme blank.

Add 40 ml water and then bring to pH 4 with 1 M citric acid.

Add 5 ml freshly prepared 0.2% Ultrazym 100, mix well, and flush particles adhering to the glass wall down into the solution with a little bit of water.

Incubate the Erlenmeyer for one night at room temperature (without shaking).

Transfer the contents of the Erlenmeyer into a 100-ml measuring flask with water and add water to 100 ml. Then mix and filter off the solids with a fluted filter and save the filtrate. Dilute part of the filtrate to give a concentration of anhydrogalacturonic acid of about 2 mg/100 ml.

The Carbazole Color Reaction

Pipette in duplicate 1 ml of each standard solution into reagent tubes (20 × 200 mm). Pipette in triplicate 1 ml of the diluted sample extract into reagent tubes; mark one tube as a blank. Pipette in triplicate 1 ml of the enzyme blank into reagent tubes; mark one tube blank. Add to all tubes 100 µl ammonium sulfamate, 100 µl borate and mix. Put the tubes in ice water and then add 5 ml 96% sulfuric acid with a dispenser; mix and put the tubes in ice water again. Heat the tubes for 6.5 minutes in a boiling water bath and then cool them in ice water. While the tubes are still in ice add 200 µl ethanol to the blank tubes and 200 µl 0.2% carbazole solution to the standard and sample tubes. Mix and put the tubes for 10 min in a boiling water bath. Allow the tubes to cool and measure the extinction against 96% sulfuric acid at 517 nm. The color is stable for several hours.

Calculation

From the sample extinction subtract the sample blank and the enzyme blank. Calculate the linear regression line from the extinctions of the standards and their concentrations in milligrams anhydrogalacturonic acid per 100 ml. Calculate the contents of pectic substances in the freeze-dried products expressed as anhydrogalacturonic acid, taking into account the dilution of the filtrate, if any, and the sample weight.

Reference

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APPENDIX 3

Determination of Pectic Substances by Copper Ion Exchange

Principle

This method is based on the high affinity of Cu^{2+} for pectic carboxylic acid groups and can be used for the simultaneous determination of the degree of esterification of pectic carboxylic acid groups and the polygalacturonic acid content of a mixture of polysaccharides [12].

Reagents

1. 2-Propanol, 70% (vol/vol). Dilute 700 ml 2-propanol in a volumetric flask with water to 1 liter.
2. Copper sulfate, 40 mM. Dissolve 10 g $CuSO_4 \cdot 5H_2O$ in 1 liter water.
3. Hydrochloric acid, 0.6 M, in 70% 2-propanol. Transfer to a 1-liter volumetric flask 700 ml 2-propanol and 50 ml 37% hydrochloric acid, dilute with water to 1 liter and mix.
4. Sodium hydroxide, 0.1 M, in 70% 2-propanol. Transfer to a 1-liter volumetric flask 700 ml 2-propanol and 100 ml 4% sodium hydroxide solution. Dilute with water to 1 liter and mix.
5. Acetic acid, 1 M, in 70% 2-propanol. Transfer to a 1-liter volumetric flask 700 ml 2-propanol, add 57 ml 100% acetic acid. Dilute with water to 1 liter and mix.
6. Copper indicator sticks merckoquant (Merck 10003).
7. Copper standard solutions.
   a. Stock I (1 g/liter). Dilute Titrisol (Merck 9987, containing 1.0 g of Cu as $CuCl_2$) to 1 liter with water.
   b. Stock II (0.4 g/liter). Pipette 40 ml of stock I into a 100-ml volumetric flask and adjust the volume to 100 ml with water.
   c. Stock III (0.15 g/liter in 70% 2-propanol). Pipette 15 ml of stock I into a 100-ml volumetric flask and adjust the volume to 100 ml with water.

d. Standard 1. This can be used for measuring the absorbance at 217.9 nm. Pipette 2 ml of stock I into a 100-ml volumetric flask; add 70 ml 2-propanol and adjust the volume to 100 ml with water. Do the same with 3, 4, 5, and 6 ml of stock I solution. These standards than contain 1.2, 1.8, 2.4, 3.0, and 3.6 mg Cu *per 60 ml* 70% 2-propanol.

e. Standard 2, for measuring at 327.4 nm. Dilute stock II in the same way as described for standard 1. The solutions will then contain 0.48, 0.72, 0.96, 1.20, and 1.44 mg Cu *per 60 ml*.

f. Standard 3, for measuring at 324.7 nm. Dilute stock III in the same way as described for standard 1. This gives 0.18, 0.27, 0.36, 0.45, and 0.54 mg Cu *per 60 ml*, respectively.

Procedure

Propanol-insoluble solids. Weigh out duplicate portions of 100-250 mg freeze-dried sample containing 5-10 mg anhydrogalacturonic acid and transfer to a G3 Schott glass filter funnel with a stopcock. Place the funnel on a suction flask and close the stopcock. Add about 20 ml 70% 2-propanol and stir with a glass rod. After 10 min open the stopcock and suck the filter dry. Repeat this process with two further portions of 20 ml 70% 2-propanol.

Determination of unesterified pectin. Add about 20 ml 40 mM aqueous copper sulfate solution. Stir with a glass rod and suck the filter dry after 10 min. Again add 20 ml 40 mM copper sulfate solution; close the stopcock and let it stand for 30 min; stir from time to time. After that suck the filter dry. Add about 20 ml water, stir and suck dry again. Repeat this washing until the filtrate does not react for Cu on testing with Merckoquant sticks. Six washings are generally enough to remove the free copper. Place the glass filter funnel on a dry and clean suction flask (250 ml). Add exactly 20 ml 0.6 M Hydrochloric acid dissolved in 70% 2-propanol and stir with a glass rod. After 10 min suck the

filter dry. Repeat this process with two further 20-ml portions of 0.6 M propanolic hydrochloric acid. Mix the filtrates and transfer 10 ml to a plastic stoppered test tube.

Determination of total pectin. Add to the residue on the filter about 20 ml 0.1 M sodium hydroxide in 70% 2-propanol to saponify the methyl esters. Let it stand for 60 min; stir from time to time. After that open the stopcock and suck the filter dry. Add about 20 ml 1 M acetic acid in 70% 2-propanol. Stir again and suck the filter dry. Then repeat the steps from the beginning of the procedure for the determination of unesterified pectin but start by adding 2 volumes of 20 ml 70% 2-propanol, stirring the solution in the closed Schott filter funnel with a glass rod.

Determination of the amount of copper. Measure the absorbance of copper on an atomic absorption spectrophotometer. Use calibration standards 1, 2, or 3 and the appropriate wavelengths depending on the amount of copper in the sample solution.

Calculation. Calculate the linear regression line from the absorbances of the standards and their concentrations in mg per 60 ml. Calculate the amount of copper bound by the weighed sample before and after saponification. Calculate the degree of esterification, DE, as follows:

$$DE = [1 - (\text{mg Cu bound before saponification}/\text{mg Cu bound after saponification})] \times 100\%$$

Finally, calculate the percentage of pectic substances, % AG, expressed as anhydrogalacturonic acid in the freeze-dried product:

$$\% \text{ AG} = \frac{\text{mg Cu bound after saponification} \times 2 \times 176 \times 100}{63.6 \times \text{mg weighed sample}}$$

Note that if the sample contains starch it is essential to remove it by enzymatic hydrolysis. After this hydrolysis some materials are extremely difficult to filter but this can be helped by adding a filter aid such as acid-washed Celite 545.

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