CHEMICAL COMPOSITION OF THE WATER-SOLUBLE CONSTITUENTS OF BLEACHED CAKE FLOUR[1]

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ABSTRACT

The water-soluble constituents of bleached cake flour have been separated by means of curtain electrophoresis at pH 2.27 into four proteinaceous and one carbohydrate fraction. The fractions have been analyzed for ash, moisture, nitrogen, and carbohydrate content. Three of the proteinaceous fractions were found to contain carbohydrate, the amount of which could be decreased by re-electrophoresis in the presence of hydrogen bond-breaking agents. The amino acid compositions of these fractions were determined and revealed the relation of these proteins to the albumins and gliadin isolated and characterized by other authors. Horizontal paper chromatography indicated heterogeneity of all proteinaceous fractions isolated. The carbohydrate fraction was separated into materials of low and high molecular weight by means of dialysis and preparative paper chromatography. The low-molecular-weight materials amounted to 77.4% of the total carbohydrate fraction and consisted of fructose, glucose, galactose, sucrose, maltose, and oligosaccharides. The nondialyzable oligosaccharides (6% of the fraction) and polysaccharides (16.6%) contained xylose, arabinose, galactose, and smaller amounts of glucose. The most cationic fractions, P_1 and P_2, contained 39 and 8% of ash, respectively. The ash contained a spectrum of 16 different metal ions. The most anionic fraction, P_4, contained 49% ash, 60% of which consisted of phosphate. An analytical recovery of 93.5% of the water-soluble constituents was achieved.

The use of curtain electrophoresis for separating the water-soluble constituents of bleached cake flour is described in the accompanying paper (16). The application of curtain electrophoresis for the separation of the water-soluble constituents, in contrast to the former salt-fractionation techniques which are generally based on the procedures of Ritthausen (13) and Osborne and Voorhees (11), permits a rapid separation of the various materials and is not encumbered with the problem of removal of the added salts required for precipitation of the various fractions. Since volatile electrolytes can be used for the electrophoretic process, direct analytical determinations on the various fractions are possible after lyophilization.

The present paper describes the techniques used for separation and identification of the mineral, carbohydrate, and proteinaceous constituents of the flour water-solubles.

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[1]Manuscript received September 28, 1962. Contribution from the Miami Valley Laboratories, The Procter & Gamble Co., P.O. Box 39175, Cincinnati 39, Ohio.

Materials and Methods

The procedures for isolation, separation, and qualitative identification of the water-soluble constituents of bleached cake flour are described in the accompanying paper (16). Moisture, protein, ash, and phosphate contents were determined by the methods of the AACC.

Determination of Metal Ions. Semiquantitative determination of the metal ions was carried out by means of emission spectroscopy.

Separation of Metal Ions from Proteinaceous Materials. The freeze-dried fractions obtained by curtain electrophoresis at pH 2.27 were subjected to further curtain electrophoresis in acetic acid-pyridine-water (10:3:750), pH 4.11. Under these conditions the proteinaceous materials migrated more slowly than the metal ions and satisfactory separation was achieved.

Dialysis of the Carbohydrate Fraction. The carbohydrate fraction obtained by curtain electrophoresis was subjected to dialysis in cellulose dialyzing tubing (No. 4465-A2, diameter 27/32 in., Arthur Thomas, Philadelphia). Dialysis was carried out for a total of 66 hr. at 4°C. against distilled water. Changes of the distilled water were made after 16 and 48 hr. The dialyzable fractions were obtained by freeze-drying.

Horizontal Paper Chromatography. Paper chromatography was carried out by the horizontal arrangement described by Strobel (15). The solvents used for the separation of proteinaceous materials were butanol-acetic acid-water[2], 4:1:5 (12), and n-butanol-pyridine-acetic acid-water, 4:1:1:5 (15).

The solvents used for separation of the mono- and disaccharides were ethyl acetate-pyridine-water, 2:1:2 (6), 40:11:6 (2), 2.5:1.0:3.5 (7); and n-butanol-pyridine-water, 6:4:3 (4).

Preparative chromatography of the nondialyzable carbohydrate fraction was carried out with ethyl acetate-pyridine-water, 2:1:2. Approximately 50 mg. of material was dissolved in 800 λ of pyridine-water (1:1, v/v). The solution was evenly applied on a line (28 cm. long) on 30-cm. horizontal chromatographic paper (S&S 2043b). The applied solution was dried in a *cold* air stream and chromatographed at 24°C. In order to achieve good separation the material was chromatographed a total of three times. After chromatography a strip 2 cm. wide was cut off from each side of the sheet and stained with diphenylamine-aniline-phosphoric acid. The various fractions thus visualized were cut out and eluted from the paper with a mixture of pyridine-water (1:1, v/v) by means of the siphon technique. The eluates were freeze-dried and the yields determined by weighing.

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[2] This solvent was also used for carbohydrates.

Hydrolysis of Oligo- and Polysaccharides. Three milligrams of material were dissolved in 3 ml. of 1*N* sulfuric acid, hydrolyzed in a sealed tube at 100°C. for 4 hr., and subsequently deionized with Amberlite IR 45. The eluate was freeze-dried. Five-tenths milligram of the hydrolysate was used for chromatographic identification of sugars.

Borate Electrophoresis. Borate electrophoresis on the polysaccharide fraction was carried out according to Consden and Stanier (1), using electrolytes of pH 9.2 and 9.8.

Quantitative Carbohydrate Determination. For quantitative determination of reducing mono- and disaccharides on paper chromatograms, the method of Wallenfels *et al.* (18) as improved by Fischer and Dörfel (2) was used. The sugars, separated with n-butanol:pyridine:water, 6:4:3 (three times rechromatographed), were stained with triphenyl tetrazolium chloride (2% dissolved in 0.5*N* sodium hydroxide containing methanol) and heated for 30 min. at 70°C. in a moist atmosphere. The red spots are cut out and eluted for 10 min. with a solution of 10 vol. % methanol and 1 vol. % glacial acetic acid. The absorbance of the eluted material was measured at 482 mμ.

The determinations of hexoses and pentoses in the collecting tubes from the curtain electrophoresis were carried out by the anthrone (10) and orcinol (9) methods.

Electrophoresis for Removal of Carbohydrates from Proteinaceous Material. To remove carbohydrates which may be bound to the proteins by hydrogen bonding, curtain electrophoresis was carried out in the presence of phenol (3). Crystalline phenol was added to 1% solutions of the various proteinaceous fractions in electrolyte of pH 2.27 to give a final phenol concentration of 3%.

Protein Hydrolysis and Quantitative Amino Acid Determinations. To prevent the formation of humin during hydrolysis of the proteinaceous materials (glycoproteins), the hydrolysis procedure of Hirs *et al.* (5) was changed by decreasing the concentration of the solution by a factor of 20 and using oxygen-free 5.7*N* hydrochloric acid[3]. Oxygen-free hydrochloric acid can be obtained by scrubbing hydrochloric acid with oxygen-free nitrogen for 30 min. Oxygen-free nitrogen was prepared from prepurified nitrogen according to the method of Meites and Meites (8). Amino acids were determined with the use of the Phoenix automatic amino acid analyzer.

Results and Discussion

General Composition of the Various Fractions of the Flour Water-Solubles. The electrophoretic separation pattern of the water-solubles

[3] A more detailed report on the hydrolysis procedure will appear elsewhere.

of bleached cake flour (TWS) at pH 2.27 is shown in Fig. 3 of the accompanying paper (16). Values, from a composite sample of five electrophoretic separations, of the total analysis of the fractions P_1 through P_4 for ash, moisture, nitrogen, and carbohydrates are presented in Table I.

TABLE I

COMPOSITIONS OF THE POOLED FRACTIONS OF THE TOTAL WATER-SOLUBLES OF BLEACHED CAKE FLOUR AFTER PREPARATIVE ELECTROPHORESIS AT pH 2.27

	YIELD					
	P_1 (4.4%)	P_2 (9.0%)	P_3 (45.7%)	Carbohydrate (36.5%)	P_4 (4.4%)	Total (100%)
	%	%	%	%	%	%
Ash	39.0	8.0	1.2	0.6	49.0	5.3
Moisture	9.0	9.1	2.8	5.2	7.4	4.7
Protein	18.6	62.8	75.0	1.2	19.5	41.6
Carbohydrate	6.5	17.4	13.8	93.0	0.0	41.9
Total	73.1	97.3	92.8	100.0	75.9	93.5

Mineral Analyses. As shown in Table I, the ash content drops sharply from the most cationic fraction P_1 to P_3, reaches a minimum in the carbohydrate fraction, and increases markedly again in the most anionic fraction. Semiquantitative determination of metal ions by means of emission spectroscopy on P_1 and P_2 revealed a spectrum of the 16 different metal ions listed in Table II.

TABLE II

METAL IONS IN P_1 AND P_2 FRACTIONS[a]

METAL IONS	P_1	P_2	METAL IONS	P_1	P_2	METAL IONS	P_1	P_2
Na	VS	S	Cu	M	M	B	Tr	Tr
K	S	Tr	Zn	M	M	Ag	Tr	Tr
Mg	S	S	Fe	M	M	Ni	Tr	Tr
Al	M	S	Pb	W	W	Cr	Tr	Tr
Ca	M	S	Sn	W	W	Ba	Tr	Tr
						Mn	Tr	Tr

[a] VS = very strong; S = strong; M = medium; W = weak; Tr = trace.

Analyses for P_2O_5 in the ash of the most anionic fraction P_4, the carbohydrate fraction, and P_3 yielded values of 60, 13, and 17%, respectively.

The Carbohydrate Fraction. Quantitative analysis for mono- and disaccharides on the pooled carbohydrate fraction present in the electrophoretic collecting tubes 27 to 31 (see Fig. 3 of the accompanying paper) yielded the results given below.

	%
Maltose	25.8
Sucrose	38.5
Galactose	(<0.5)
Glucose	3.0
Fructose	5.8
Total	73.1

The mono-and disaccharides (as well as dialyzable oligosaccharides) were separated from the polysaccharides by means of dialysis. Figure 1 shows that the composition of the fractions, in terms of the monosaccharides they contain, obtained after 16, 48, and 66 hours of dialysis, is the same. It was also seen from relative stain intensities on the original chromatograms, when ethyl acetate:pyridine:water (2:1:2) was used, that the amount of dialyzable oligosaccharide is greater in the 48- and 66-hour dialysis fraction than in the fraction collected after 16 hours of dialysis.

Figure 2 shows the weight of the dialyzable material plotted against the time of dialysis. Graphical extrapolation of the dialysis curve indicates that the dialysis after 66 hr. was approximately 80 to 90%

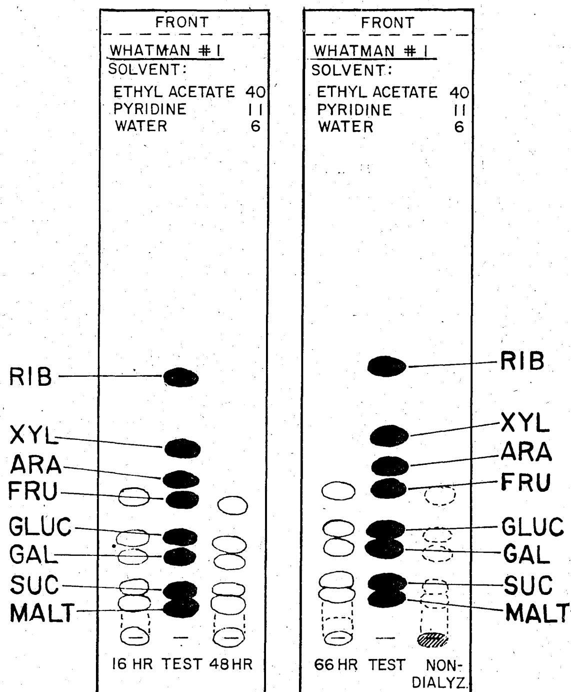

Fig. 1. Horizontal chromatography of the dialyzable fractions (collected after 16, 48, and 66 hr.) and the nondialyzable fraction of the carbohydrate fraction.

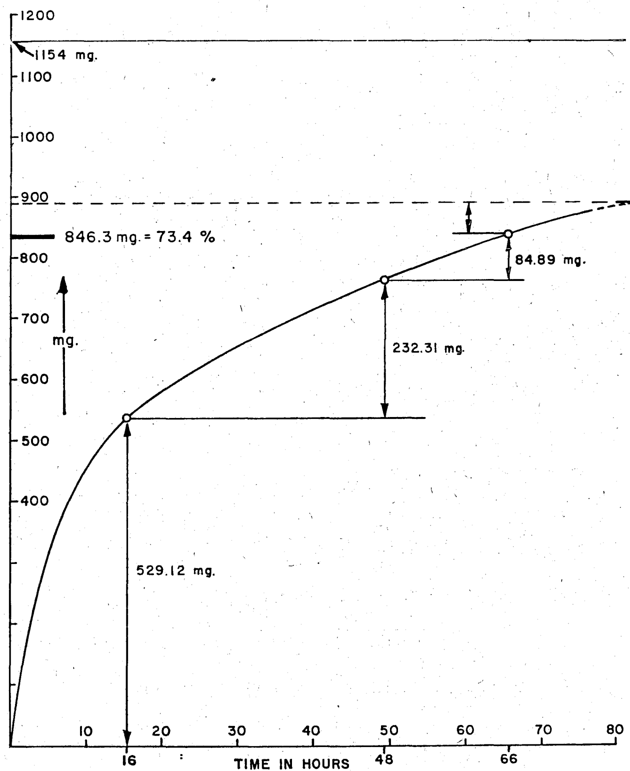

Fig. 2. Dialysis of the carbohydrate fraction. Starting material, 1,154 mg. Dialyzable material collected during 66 hr. of dialysis, 846.3 mg. (73.4%). Graphical extrapolation of the end point of dialysis is indicated by the broken line.

complete. This finding was experimentally confirmed by means of preparative horizontal chromatography. In a typical experiment 49.17 mg. of the "nondialyzable" material was chromatographed with ethyl acetate:pyridine:water (2:1:2). The results of quantitative determination on the various fractions, as shown in Fig. 3, revealed that the "nondialyzable" fraction contained 15% mono- and disaccharides, 10.4 and 12.3% short-chain and long-chain oligosaccharides (arbitrarily subdivided), and 62.3% polysaccharides.

Calculation of the total composition of the carbohydrate fraction reveals that 77.4% are dialyzable mono-, di-, and oligosaccharides. Since 73.1% mono- and disaccharides were determined directly, the difference of ~4% accounts for dialyzable oligosaccharides. The nondialyzable oligosaccharides amount to 6% and, finally, 16.6% is accounted for as polysaccharides.

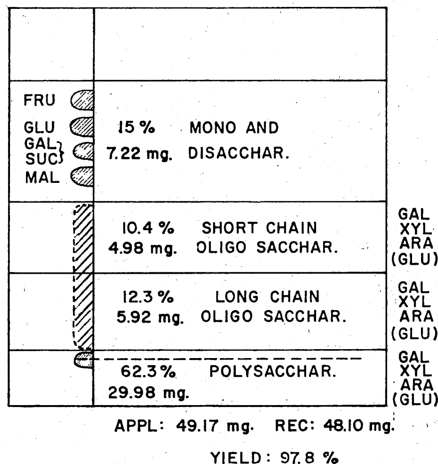

Fig. 3. Preparative horizontal chromatography of the "nondialyzable" fraction of the carbohydrate fraction. Paper: S&S 2043b. Solvent: ethyl acetate:pyridine:water (2:1:2). Three times rechromatographed.

Identification of the sugars contained in the nondialyzable oligo- and polysaccharides revealed the compositions shown below. (Crosses indicate the estimated relative amounts.)

Sugar	*Short-Chain Oligosaccharides*	*Long-Chain Oligosaccharides*	*Polysaccharides*
Glucose	+	(+)	(+)
Galactose	+++	+++	+++
Arabinose	+++	+++	+++
Xylose	+++	+++	+++

Since it is not quite clear whether the polysaccharide fraction consists of a copolymer composed of hexoses and pentoses or whether it is a mixture of pentosan and hexosan, attempts were made to fractionate the polysaccharide fraction by means of chromatography and borate electrophoresis. Borate electrophoresis (1) was tested at two different pH values (9.2 and 9.8). The results indicated some heterogeneity of the polysaccharide fraction, but the resolution achieved was insufficient to be suitable for preparative separation. A separation into one main fraction and three minor fractions was recognized by horizontal paper chromatography using n-butanol:pyridine:water (6:4:3). The R_f values and the estimated amounts of these fractions are listed below. The main fraction (96%) with an R_f value of 0.09 showed an even coloration throughout the spot, suggesting that this fraction may be a uniform compound. Determination of the sugar composition of

Staining Behavior with Diphenylamine-Aniline-Phosphoric Acid	R_f	*Estimated Amount*
		%
Gray-green	0.09	96
Faint yellow	0.45	3
Blue	0.85	1
Faint yellow	0.95	trace

the main fraction did not reveal any differences from the composition of the polysaccharide before chromatographic separation. Additional work is necessary to clarify whether the polysaccharide is a copolymer, a mixture of pentosans and hexosans, or a mixture of copolymers.

Figure 4 summarizes the separation procedure of the carbohydrate fraction.

The Proteinaceous Fractions. Figure 5 shows chromatographic separations of the total water-solubles (TWS) and of the proteinaceous fractions P_1 through P_3. The patterns not only indicate heterogeneity in each fraction, but also show that effective enrichment of certain sub-

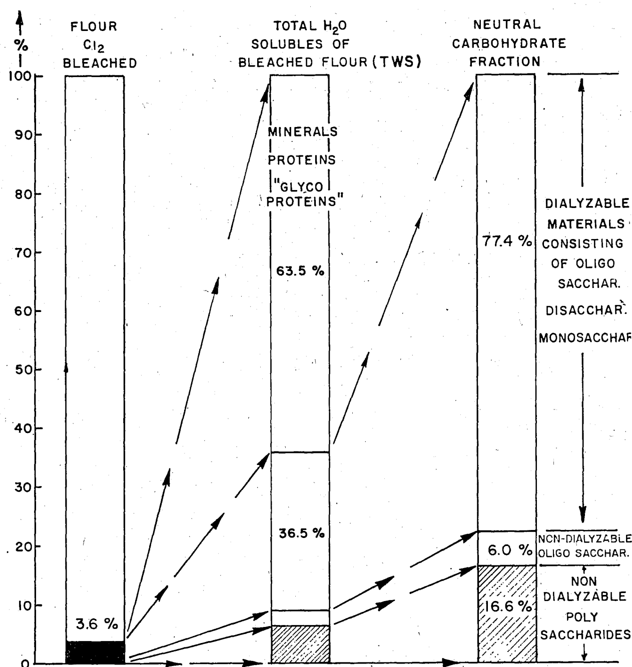

Fig. 4. Composition of the carbohydrate fraction. The first column indicates the amount of water-soluble (TWS) material obtained from bleached cake flour. The second column indicates the composition of TWS from preparative electrophoresis. The third column indicates the total composition of the carbohydrate fraction as elucidated by dialysis and preparative horizontal chromatography.

fractions in P_1 through P_3 has been achieved by means of curtain electrophoresis.

Direct determination of the amino acid compositions of P_1 and P_2 was impossible, because of the presence of high amounts of metal

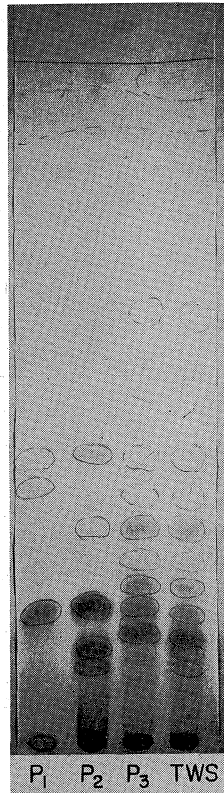

Fig. 5. Horizontal chromatographic separation of the protein fractions, P_1, P_2, P_3, and TWS. Solvent: n-butanol:acetic acid:water (4:1:5). Paper: S&S 2043b.

ions. Large amounts of ammonia in the protein hydrolysate indicated that the amino acids had undergone a Strecker degradation during hydrolysis. No difficulties were encountered in the analysis of P_3. Attempts to separate the metal ions from P_1 and P_2 by means of curtain electrophoresis at pH 4.11 were successful to a degree that enabled quantitative determination of the amino acids in these fractions. Table IV gives the amino acid compositions.

The occurrence of a levulinic acid peak (14) (degradation product of hexoses) in each fraction P_1 through P_3 indicated the presence of

carbohydrates (hexoses). It was of interest to test whether these carbohydrates were removable by electrophoresis in the presence of a hydrogen bond-breaking agent, phenol. The determination of nitrogen in the fractions P_1 through P_3 before and after use of phenol during electrophoresis revealed the differences listed in Table III. It is evident from the results shown in this table that considerable amounts of

TABLE III

NITROGEN CONTENTS OF THE PROTEINACEOUS FRACTIONS P_1 THROUGH P_3 BEFORE AND AFTER RE-ELECTROPHORESIS WITH PHENOL

Fraction	Nitrogen		Difference in Protein[a] Content before and after Use of Phenol
	Without Phenol	After Re-electrophoresis with Phenol	
	%	%	%
P_1	3.25	4.37	6.5
P_2	10.92	13.97	17.4
P_3	12.72	15.06	13.8

[a] The N conversion factors were 5.72 for the P_1 and P_2 fractions, and 5.89 for the P_3 fraction.

carbohydrates are bound to the various proteinaceous fractions by means of hydrogen bonds. However, the detection of levulinic acid remaining in the various fractions even after use of phenol suggests the presence of covalently bound carbohydrates, and is consistent with the recent findings of Waldschmidt-Leitz and Hochstrasser (17). No change in the relative amino acid composition of the proteinaceous fractions was observed after removal of the more loosely bonded carbohydrates.

Comparison of the quantitative amino acid contents of the fractions P_1 and P_2 with that obtained for total albumin by Waldschmidt-Leitz and Hochstrasser (17) and of P_3 with that reported for gliadin by Woychik and co-workers (19) reveals similarities for characteristic amino acids such as glutamic acid, aspartic acid, proline, cystine, alanine, and arginine (see Table IV).

These results support the conclusion that P_1 and P_2 consist mainly of albumins, whereas P_3 is made up of a mixture of gliadin and albumins.

In summary, then, it can be seen in Table I that the total recovery of analyzed materials accounts for 93.5% of the starting material. The missing 6.5% probably consists of carbohydrates which were not quantitatively analyzable by the methods used in the present study, and smaller amounts of lipids which are present in the P_3 fraction as revealed by qualitative staining.

The present outline on the separation and identification of flour

TABLE IV
AMINO ACID COMPOSITIONS OF FLOUR PROTEIN FRACTIONS[a]

Amino Acids	Fraction			Literature Values	
	P_1	P_2	P_3	Albumin (17)[b]	Gliadin (19)
Glucosamine	0.07	0.42	0.19	...	...[c]
Lysine	4.48	4.07	1.34	9.8	.8
Histidine	2.32	2.36	2.10	3.8	2.4
Ammonia	3.00	2.51	4.31	.3	4.8
(Unknown)	2.66	2.34	0.00	...	...
Arginine	8.97	8.72	3.67	6.7	2.9
Cysteic acid[d]	3.68	7.42	0.05	...	...
Methionine sulfoxide[d]	1.33	1.29	0.16	...	...
Aspartic acid	8.51	7.20	3.71	7.0	1.7
Methionine sulfone[d]	0.00	0.49	0.00	...	...
Threonine	3.38	3.42	2.32	2.6	2.5
Serine	4.86	4.85	4.30	4.2	4.5
Glutamic acid	13.48	13.73	33.60	15.9	37.8
Proline	8.32	7.39	11.83	7.5	16.1
Glycine	5.20	5.02	2.42	2.8	4.0
Alanine	5.02	4.45	2.56	5.0	3.5
Cystine/2	0.00	0.00	2.88	6.0	1.8
Valine	6.92	6.37	4.57	7.3	3.9
Methionine	0.19	1.11	1.56	...	1.5
Isoleucine	3.08	3.07	4.07	3.7	4.0
Leucine	7.79	7.32	6.87	9.6	6.8
Tyrosine	4.48	4.47	3.26	3.0	2.6
Phenylalanine	4.56	2.37	2.09	4.5	4.3
Tryptophan		...	...	...	1.1

[a] Values given in % based on weight of total protein.
[b] Recalculated on basis of 100% protein.
[c] Not reported.
[d] Oxidation products due to the presence of metal ions during hydrolysis.

water-solubles appears to show the suitability of these techniques for determination and identification of flour constituents. As we have seen, most of the fractions are very complex and much needs to be done to investigate their detailed compositions.

Acknowledgments

The authors wish to acknowledge the valuable assistance of Mrs. B. Jackson and Mr. D. Etson with the experimental portion of this work.

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