

Low-Temperature Anaerobic Extraction of Free and Bound Lipid from Wheat Flour¹

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ABSTRACT

Flour was milled from normal and degermed wheat of three Canadian varieties, Thatcher, Lemhi, and Mindum. These flours were extracted serially with hexane and water-saturated n-butanol (WSB) to yield "free" and "bound" lipid, and with WSB directly to yield total lipid. All extractions were carried out in the cold (1°-5°C.), under nitrogen. Two methods of removing nonlipid material were compared. The yield of lipid, its atomic nitrogen/phosphorus ratio, and its chromatographic purity were determined. The present procedure was compared with other methods for removal of lipid from wheat flour.

The possible relation between lipids and the baking quality of wheat flour has inspired considerable research. The role of "free" and "bound" lipid in wheat flour (1, 2, 3) and the possible contribution of germ lipid to endosperm lipid (4) have been the subject of speculation. Improved methods of extraction (5, 6, 7) and lipid separation (8, 9) provide a background for thoughts on the binding of lipid to protein (10, 11) and on the structure of the wheat lipid-protein complex (12, 13, 14). The present work was carried out as a preliminary to a study of the physical and chemical properties of lipid-free flours. In this paper, "free" and "bound" lipid in a straight-run flour prepared from whole and degermed wheat and an anaerobic extraction method are described. Details of the lipid composition of these flours have been reported (15, 16).

MATERIALS AND METHODS

The wheats used were Thatcher (1959), Lemhi (1958), and Mindum (1958), representing hard red spring, soft white spring, and amber durum, respectively. All were grown on similar soil near C.D.A. Research Station, Lethbridge, Alberta. A 7-kg. sample of each wheat was mixed thoroughly, tempered to 15.5% moisture, and divided into two portions. One half of each sample was milled directly in a Buhler mill to 65-70% extraction and the other half was first degermed by the method of Johnston and Stern (17) and then milled. The flour was placed in metal cans, the air space flooded with oxygen-free nitrogen, and the can sealed and stored at 2°C. The following code was used to identify the flour samples: NT, DT, NL, DL, NM, DM, N, and D refer to flour milled from normal and degermed wheat respectively; T, L, and M designate Thatcher, Lemhi, and Mindum.

Preparation of Lipid-Free Flour

Lipid was extracted from each of the six flour types in the following manner: "free" lipid by extraction with hexane (H) and total lipid by extraction with n-butanol saturated with water at +1°C. (WSB). The lipid material removed by WSB from hexane-extracted flour was called "bound" lipid.

¹Contribution No. 95 of the Food Research Institute.

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To avoid oxidative deterioration and dehydration during extraction, the following general precautions were taken. Reagent-grade n-butanol and diethyl ether were saturated with water at 1°C. Phillips technical-grade n-hexane (95 mole %) was distilled before use. Nitrogen was bubbled through each batch of solvent for at least 20 min. before use to displace dissolved oxygen. Lipids were extracted from the flour at 1°C. by shaking a 1:9 (w./v.) slurry of flour and solvent in stoppered 250-ml. centrifuge bottles on a Northcott shaker at 150 strokes per min. for 1 hr. The air above the solvent was displaced by nitrogen before shaking was begun. The flour slurries were then centrifuged at $900 \times g$ for 1 hr. at 0°C. Residual solvent was removed from the slurries in a rotating evaporator under nitrogen atmosphere and at a water-bath temperature of 35°C.

A. *Hexane Extraction.* Flour, in 20-g. portions, was weighed into 250-ml. centrifuge bottles, and 180 ml. of hexane was added to each bottle. After shaking and centrifugation, the bottles were placed in an ice bath and the supernatant liquid, containing the "free" lipid, was withdrawn and stored under nitrogen at -18°C. The packed flour was broken up and washed twice with 50 ml. cold hexane (shaken at 1°C. for 20 min. each time) and centrifuged for 30 min. and the supernatant liquid was discarded. The washed flour was transferred to Petri dishes, dried under a stream of dry nitrogen in a desiccator, then ground in a mortar and finally dried for at least 15 min. under the same conditions. When the odor of hexane had completely disappeared, the flour was stored under nitrogen in jars at 1°C.

B. *Butanol Extraction.* Extraction was performed as in Part A with WSB (180 ml.) instead of hexane. The total lipid thus extracted was stored under nitrogen at -18°C. WSB remaining in the flour was then removed in the following manner: The hard-packed flour in each bottle was broken up into small pieces and transferred to a metal Waring Blendor jar fitted with an outside cooling coil to keep the temperature around 0°C. To each batch of flour was added 150 ml. of cold-water-saturated diethyl ether, and the mixture was blended for 2 min. with continuous flow of nitrogen through the blender jar. The flour-ether slurries were returned to their original 250-ml. centrifuge bottles; the blender was rinsed each time with 40 ml. of cold water-saturated ether, which was added to the original ether extract.

The bottles were then centrifuged for 30 min. and the supernatant liquid discarded. The packed flour in each bottle was again broken up and shaken in a further 100 ml. of water-saturated ether for 20 min. and centrifuged, and the ether was discarded. This was followed by two similar washings with 100 ml. of cold hexane. The flour obtained in this manner was dried, pooled, and stored as in part A.

C. *Hexane Followed by Butanol Extraction.* The hexane extraction was carried out as described in part A with only one washing after the initial centrifugation. The hexane was removed as before and the hexane-extracted flour returned to the original 250-ml. centrifuge bottles. The flour was then extracted with WSB as in part B. The hexane extract containing "free" lipid and the butanol extract containing the "bound" lipid were stored under nitrogen at -18°C.

Quantitative Extraction of Lipids

Quantitative extractions were also made of each of the six flours to determine total extractable matter (TEM). The total hexane-extractable material (containing "free" lipid) was obtained by extraction as in part A, except that all the hexane washes were combined. The solvent was removed under nitrogen and the TEM weighed. A similar procedure was followed to obtain the total WSB-extractable material from hexane-extracted flour ("bound" lipid), and also to obtain the total WSB-extractable (total lipid) content. The TEM was treated by two methods to obtain true lipid material (TLM). In the first method the TEM was shaken with hexane to form a dilute solution of approximately 1 mg./ml. and left overnight under nitrogen at +5°C. The insoluble nonlipid material (NLM) was removed by filtration under nitrogen through a 100-m μ Millipore® filter.

In the second method NLM was removed from TEM by passage through a column 12 cm. \times 0.9 cm. i.d. containing 4 g. Sephadex G-25 prepared according to Wells and Dittmer (18). Chloroform:methanol:water (60:30:-4.5) (40 ml.) containing the sample was passed through the column at a flow rate of not more than 0.4 ml. per min. This was followed by 40 ml. chloroform:methanol (2:1) at the same flow rate (18). The time required for passage of the lipid solution and eluting solvent through the column was of great importance, but the concentration of lipid in the solution was not a factor. Eluates from the Sephadex column were filtered through a 1.5- μ type OH Millipore® filter.

Other Determinations

For thin-layer chromatography, Silica gel G in a layer 0.25 mm. thick was used as the adsorbant on plates 20 \times 20 cm. Polar lipids were separated by two solvent systems, (a) diisobutyl ketone:acetic acid:water (40:25:5) (19) and (b) chloroform:methanol:water (65:25:4). Amino acids were compared with a known mixture by development with the upper phase of equilibrated butanol:acetic acid:water (4:1:5). The following detection reagents were used as sprays: (a) perchloric acid, 20% aqueous to distinguish different types of polar lipids (20); (b) molybdenum reagent of Dittmer and Lester (21) to detect phosphorus-containing spots; (c) ninhydrin to detect amino acid-containing spots; (d) periodate-benzidine to detect sugars (22); and (e) Dragendorff reagent to detect choline (23). Known lipids were used as reference standards on each plate.

Atomic nitrogen/phosphorus ratios were calculated for various lipid extracts from values obtained by conventional semimicro Kjeldahl and micro phosphorus analyses. Flour moisture contents were determined by oven drying at atmospheric pressure. Complete removal of solvent from the flour was checked by leaving the flour in tightly sealed screw-capped jars at room temperature for 1 week and then quickly smelling the head space of the jar.

RESULTS

Extraction

The extraction with WSB and removal of butanol with water-saturated

diethyl ether essentially maintained the flour moisture at the initial level: initial flour moisture, 14%; after extraction, 12%.

The total amounts of hexane-extractable (free) lipid from the three wheats are listed in the table below. With each variety, slightly more lipid

<i>Flour Type</i>	<i>Free Lipid</i> weight %	<i>Bound Lipid</i> weight %
NT	0.89	0.88
DT	0.79	0.62
NL	1.14	0.84
DL	0.98	0.58
NM	0.97	0.60
DM	0.84	0.66

was extracted from the flour from normal wheat than from the degermed wheat. Lemhi contained most free lipid, and Thatcher least. The amounts of lipid removed by WSB from hexane-extracted flour (bound lipid) are also shown in this table. Less bound than free lipid was found in all varieties. The flour from normal Thatcher and Lemhi contained more bound lipid than that from the corresponding degermed wheats. The reverse was true for Mindum. Thatcher contained most bound lipid and Mindum least.

Purification of Extracted Lipid

The lipid obtained by a single extraction with WSB (total lipid) was used in a comparison of purification methods. The amounts of purified lipid (TLM) obtained from TEM after (a) equilibration in dilute solution in cold hexane and (b) passage through a Sephadex column are listed in the table below.

<i>Flour Type</i>	<i>Hexane</i> %	<i>Sephadex</i> %
NT	71.4	82.4
DT	91.2	87.8
NL	75.2	79.9
DL	84.9	81.0
NM	79.6	91.0
DM	69.9	87.9

Nitrogen and phosphorus percentages and nitrogen/phosphorus atomic ratios are given in Table I. The recovery of a mixture of purified lipids from Sephadex was 99.6%. The newer Sephadex method of Siakotos and Rouser (24) was compared later and the results obtained confirmed those obtained earlier with the Wells and Dittmer procedure.

The extent of separation of lipid from nonlipid was determined by TLC. In the nonlipid fractions from Sephadex and the hexane-insoluble residue, no positive spots appeared with the Dittmer molybdate spray (21). Ninhydrin-positive spots of low R_f were present. An intense spot at the R_f of glucose and galactose appeared with periodate-benzidine (22). In the hexane-insoluble material there was also a much less intense sugar spot at a higher R_f , possibly digalactosyl diglyceride.

The hexane-soluble lipid (TEM-NLM) and the lipid obtained by Sephadex chromatography were compared by TLC (Fig. 1). Both contained

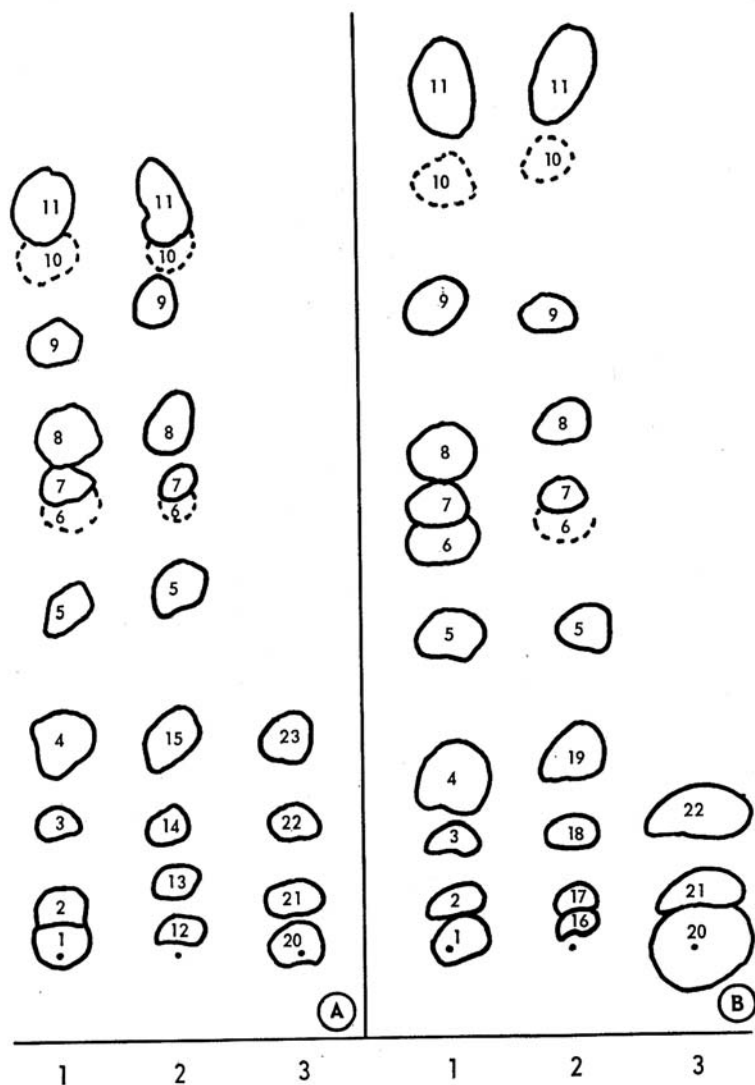


Fig. 1. Thin-layer chromatographic comparison of hexane purification and Sephadex purification. A, hexane-soluble; B, Sephadex-purified. 1, Total extract; 2, lipid; 3, non-lipid. Developing solvent, diisobutylketone-acetic acid-water (8:5:1).

Ninhydrin +ve (amino), 1, 2, 3, 5, 12, 13, 20, 21, 22 (5 = phosphatidyl ethanolamine).

Periodate-benzidine +ve (sugars), 1, 2, 20, 21; 12 less intense.

Periodate-benzidine +ve on standing, 4, 15, 19, 23 digalactosyl diglyceride.

3, 14, 18, phosphatidyl choline; 12 (in part), 16, lyso phosphatidyl choline; 13, 17 phosphatidyl inositol.

7, steryl glycoside; 9, esterified steryl glycoside; 8, monogalactosyl diglyceride; 11, nonpolar lipids.

TABLE I
NITROGEN, PHOSPHORUS, N/P ATOMIC RATIO IN TOTAL LIPID^a

FLOUR TYPE	NITROGEN			PHOSPHORUS			N/P ATOMIC RATIO		
	TEM	TLM-H	TLM-S	TEM	TLM-H	TLM-S	TEM	TLM-H	TLM-S
	%	%	%	%	%	%			
NT	0.61	0.92	0.55	0.57	0.60	0.72	2.4	3.4	1.7
DT	0.88	1.03	0.49	0.58	0.61	0.73	3.4	3.7	1.5
NL	0.41	0.59	0.46	0.44	0.50	0.49	2.1	2.6	2.1
DL	0.64	0.59	0.44	0.48	0.51	0.57	2.2	2.6	1.7
NM	0.68	0.90	0.64	0.47	0.58	0.62	3.2	3.4	2.3
DM	0.65	0.86	0.48	0.34	0.57	0.62	4.2	3.3	1.7

^aTEM = total extractable matter; TLM-H = true lipid material obtained by hexane equilibration; TLM-S = true lipid material obtained by Sephadex purification.

phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol as shown by molybdate-positive spots at the same R_f values as corresponding reference spots on the same plate. The trace of lyso phosphatidyl ethanolamine found in more detailed studies (16) did not show under these conditions. Periodate-benzidine showed a hexose spot in the untreated extract (TEM) and also in the hexane-soluble lipid (TLM). Whereas hexose spots appeared immediately when the plate was sprayed with periodate-benzidine, the digalactosyl diglyceride spot appeared only after a period of time had elapsed. Ninhydrin-positive spots of lower R_f than phosphatidyl choline (as shown by Dragendorff reagent) appeared in the untreated material, and much less intense corresponding spots appeared in the hexane-soluble (TLM). Neither periodate-benzidine- nor ninhydrin-positive spots appeared during the chromatography of the Sephadex-purified lipid. TLC thus confirms nitrogen and phosphorus analysis in showing a greater degree of purification by Sephadex chromatography than by hexane equilibration.

DISCUSSION

Extraction of sugars and nitrogen containing material by WSB at 1°C. (19.2% water) confirms Bloksma's observation (25) that these substances were extracted with butanol containing more than 10% water. The object of protecting the flour from dehydration has, however, been achieved with WSB. Moreover, the sugars removed by this method are readily replaced to restore the baking quality.

The quality of the lipid extract by the present WSB method has been compared with that obtained by other methods. The nitrogen and phosphorus content of the lipid obtained by various extraction methods has been listed by Tsen *et al.* (7) and the present authors, and nitrogen/phosphorus ratios were calculated from these data. Table II shows the nitrogen and phosphorus contents and nitrogen/phosphorus atomic ratios for the extracts listed by Tsen *et al.* and the average values for the WSB-extracted, hexane-

TABLE II
COMPARISON OF WSB EXTRACTION WITH OTHER METHODS^a

METHOD	NITROGEN	PHOSPHORUS	N/P ATOMIC RATIO
	%	%	
Rapid method (5)	0.74	0.68	2.4
Petroleum ether	0.71	0.24	6.6
Ethanol	0.94	0.74	2.8
Acid hydrolysis	0.07	0.06	2.6
chloroform-purified	0.02	0.06	0.7
WSB (hexane)	0.81	0.56	3.2
WSB (Sephadex)	0.51	0.63	1.8

^a Percent N and percent P compiled by Tsen *et al.* (7); N/P calculated from Tsen *et al.* WSB average figures calculated from Table I.

purified and WSB-extracted, Sephadex-purified material listed in Table I. As pointed out by Tsen *et al.*, the low N % and P % found in the acid-hydrolyzed and chloroform-purified extracts are due to hydrolysis of phospholipids and loss of the water-soluble nitrogen- and phosphorus-containing fragments.

The presence of cerebroside, sulphatide, phytosphingosine, or hexosamine units would raise the nitrogen/phosphorus ratio, whereas such nitrogen-free lipids as phosphatidyl glycerol, phosphatidic acid, and phosphatidyl inositol would lower it. Galactolipids, high in wheat flour (16), would have no effect. Thus, a nitrogen/phosphorus atomic ratio approaching 1 suggests reasonable success in purification. The WSB-extracted, Sephadex-purified lipid approaches this figure more closely than most of the other methods listed. Successful separation of lipid from nonlipid by Sephadex was confirmed by TLC. The WSB extraction at low temperatures was chosen to prevent damage to the protein and also to extract lipid. For this double purpose, it has proved more satisfactory than other systems.

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[Received August 2, 1967. Accepted January 15, 1968]