Extractable and "Bound" Fatty Acids in Wheat and Wheat Products[1]

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ABSTRACT

To provide quantitative data on the fatty acids in a broadly representative group of samples of wheat and wheat products, total lipid was obtained by extraction with chloroform-ethanol-water and subsequent hot acid hydrolysis of the extracted residue to obtain "bound" lipid. The chloroform-ethanol-water (CEW) solvent was equally as effective an extractant as water-saturated n-butanol (WSB); however, both solvent systems extracted substantial amounts of non-lipid (hexane-insoluble) substance, and neither effected complete removal of lipid material from the sample as shown by subsequent acid hydrolysis of the extracted residue. The extractable lipid from wheat and wheat flour contained much more stearate, much less palmitate and usually more linoleate than the "bound" lipid removable only after acid hydrolysis. One hundred fifty-six samples of wheat, wheat flour and wheat consumer products were analyzed for total and individual fatty acids. The data indicate that individual wheat products have nearly the same fatty acid composition in all regions of the country.

The lipids which wheat contribute to the dietary have received relatively little attention, perhaps because they constitute a small percentage of the total kernel. The exact magnitude of this percentage has not been established for any of today's important food wheats, although approximate values have been obtained by extraction with solvents. The subject was reviewed recently (1).

In this paper we present evidence that neither water-saturated butanol (WSB) nor chloroform-ethanol-water (CEW), which is easier to use, removes all of the lipid, and that one must resort to hydrolysis to obtain "bound" lipid. Data on total lipid and fatty acid composition are presented for a number of wheats and wheat products.

MATERIALS AND METHODS

Samples included five hard wheats, two durum wheats and four soft wheats, and flours and some baked products made therefrom as well as 100 samples of wheat flours and wheat products obtained from five regions in the United States. (See Tables III and IV under "Results and Discussion".) Detailed information on the source of the samples is described elsewhere (2). The samples were collected, ground, blended, sealed in cans under nitrogen and delivered to this laboratory packed in dry ice. They were stored in the original cans at −20°C. until analyzed. When analysis was to begin, each sample was subdivided at 0°C. into two portions which were placed in dried, precooled plastic bottles; one was used for the lipid analysis, the other for moisture determination (3, p. 191). All samples were brought to room temperature before being opened for analysis. All solvents used were of analytical grade; each was shown to be free of nonvolatile residue before use.

Extraction (Chloroform-Ethanol-Water)

The sample (25 g., or 10 g. for high-fat material) was placed in a 1-liter

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round-bottomed flask, 29/42; 300 ml. of warm (45° C.) CEW (chloroform-ethanol-water, 200:95:5, v./v./v.) was added, and the flask was stoppered and shaken vigorously at 28° C. for 16 hr. The slurry was poured into a percolation column (4.5 cm. i.d., 40 cm. long) in which a glass-wool plug was covered with 2 to 3 cm. of diatomaceous earth (Supercel). After washing with 300 ml. of fresh CEW, a 1-liter flask containing the clear percolate was connected to a rotary evaporator and the solvent removed at 50°C. The dry residue was extracted with 40 ml. of hexane (Phillips, normal) in several portions which were transferred to a 50-ml. centrifuge tube. After centrifuging 15 min. at 3,000 r.p.m., the clear extract was decanted. Residue, when present, was washed twice with 30 ml. of hexane to assure complete extraction. The residues from the centrifuge tube and the evaporation flask were recombined with that in the percolation tube for acid hydrolysis treatment. The lipid extract was reduced in volume on a rotary evaporator, transferred to a tared 50-ml. beaker, and taken to dryness under a stream of nitrogen. The beaker was then placed in a vacuum oven at 80°C. for 1 hr., allowed to cool, and weighed. The lipid extract was immediately transferred by dropper to a 10-ml. screw-cap vial which contained 0.05 mg. of hydroquinone, the container flushed with nitrogen, capped and stored at −20°C. Analysis (GLC) was begun within 1 week after storage.

Acid Hydrolysis of Residue

To the combined residues 50 ml. of alcohol and 250 ml. of 6N HCl (3, p. 194) were added and the contents were then boiled for 15 to 30 min. to effect solution. The cooled hydrolysate was transferred to a 1-liter separatory funnel and extracted twice with 200 ml. of hexane. The combined extracts were washed with two 100-ml. portions of water. The lipid extract was then dried by passing it through a column of anhydrous sodium sulfate, the solvent removed under reduced pressure and the lipid transferred to a tared 30-ml. beaker with hexane. Drying, weighing and storage were then performed as described above for the CEW extract. Thin-layer chromatography (4) and charring of the chromatogram with phosphomolybdic acid showed this acid hydrolysis fraction to consist almost wholly of free fatty acids.

Preparation of Methyl Esters

The CEW extract (about 100 mg.) in a test tube was treated with 5 ml. of 10% methanolic KOH under reflux on a sand bath for 45 min. The reaction mixture was washed into a 125-ml. separatory funnel with 25 ml. of water and acidified to pH 1.0 with 6N HCl. The crude fatty acids were then extracted with 25 ml. of ether, the ether extract was washed with two 25-ml. portions of water and dried over sodium sulfate, then evaporated to dryness on a rotary evaporator. The lipid residue was transferred with 8 ml. of ether-methanol (9:1, v./v.) to a centrifuge tube and treated with freshly distilled diazomethane (5). Samples of free fatty acids from the acid hydrolysis procedure were treated similarly. After removal of the solvent in a stream of nitrogen, the methyl ester samples were dissolved in hexane and portions were used for gas-liquid chromatography (GLC) analysis.

Gas-Liquid Chromatography

The instrument (Barber-Colman, model 10) was equipped with an argon ionization detector. Glass columns, 300 cm. long, 3 mm. i.d., packed with 15%

diethyleneglycol adipate polyester on 60/80 mesh Chromosorb W were maintained at 185°C.; flash heater at 275°C.; detector 205°C.; argon flow-rate 50 to 60 ml. per min. Disc integrator tracings on chromatograms provided the basis for calculation of percentage of individual fatty acids. Linear detector responses to fatty acid methyl esters over the range of molecular weights encountered were established by using fatty acids standards A-D from the U.S. Public Health Service, Metabolic Study Section, National Institutes of Health. Individual fatty acid standards used for reference compounds were obtained from the Hormel Institute, Austin, Minnesota.

Determination of Total Fatty Acids

The CEW extract (0.5 g.) was dissolved in 50 ml. of hot ethanol in a 250-ml. r.b. flask, 2 ml. of a 50% KOH solution was added, and the mixture was heated on a steam bath for 45 min. Solvent evaporation during the heating reduced the volume to 10 ml. The mixture was transferred to a 500-ml. separatory funnel with 150 ml. of hot water and 175 ml. of hexane, and the contents were shaken. After phases had separated, the hexane was removed by siphon and the aqueous layer re-extracted with 175 ml. of hexane; the aqueous phase was then transferred to a second 500-ml. funnel and acidified to pH 1 with 10 ml. of 6N HCl. The free fatty acids were extracted with two 100-ml. portions of ether, the extract was dried over sodium sulfate, and the solvent removed on a rotary evaporator. The fatty acid residue was transferred to a tared beaker with a small amount of hexane which was then evaporated under a stream of nitrogen. The beaker and its contents were dried at 80°C. in a vacuum oven, cooled, and the residue was weighed. Since many of the 25-g. samples did not yield sufficient lipid for this determination, it was necessary to make up composite samples of similar origin to obtain values for total fatty acids.

Analytical Variation and Tolerances

Each sample was analyzed for CEW extract and acid hydrolysis lipid independently by two analysts. If CEW values did not agree within ± 2.5%, based on the higher value, or if the total CEW extract plus acid hydrolysis lipid did not agree within these limits, replicate analyses were made until such agreement was reached or until the supervisor concluded that the sample was not homogeneous. This conclusion was reached for some of the doughnut samples in which case four or more values were averaged. For total fatty acids, agreement within ± 0.5% for duplicates or ± 1.25% for four or more replicates was required. In the GLC analyses chromatograms showed only trace amounts of fatty acids shorter than C_{12}. Chromatographic responses for esters from 12:0 through 18:2 were converted without correction of integrator values. For esters 18:3 through 22:0, 0.5% was added after calculation from integrator trace values. These corrections were based on detector responses in increments in reference samples. Precision of GLC analyses was ascertained by injection of five portions of each of four known mixtures of esters of the major fatty acids which had been prepared to approximate the proportions present in wheat. Acids which constituted 25% or more of the mixture showed average coefficients of variation below 2, while acids which constituted less than 3% of the mixture showed average coefficients of 7 to 10. Sensitivity to small quantities varied with chain length; recorder responses were obtained with as little as 0.1% of C_{16} and 0.5% of C_{20} acids in a mixture. No significant differences

($P<0.01$) were observed between analyses either on the same day or on different days. For CEW extract esters, if values for 16:0, 18:1, or 18:2 differed by more than ± 5% based on the higher value, another extract was prepared from the original sample. For acid hydrolysis esters, the tolerance was ± 10% instead of 5%. Thus, if duplicate values for 16:0 were 22.5 and 20.0, then $2.5 \div 22.5 \times 100 = 11.1\%$, and the entire analysis was repeated. For the total lipid and individual fatty acid summary data (see Tables III and IV under "Results and Discussion") weighted averages of CEW values and acid hydrolysis lipid values were calculated.

RESULTS AND DISCUSSION

Removal of Total Lipid

The objective of reporting distribution of total fatty acids in a sample dictates the necessity of ascertaining that removal of lipid from the sample is complete. Water-saturated butanol (WSB) has been regarded as a suitable solvent for such complete removal (6,7). However, its volatility and hence poor removal characteristics, as well as its unpleasant odor, caused us to seek another solvent for routine analysis of the large number of samples. Acetone-hexane and chloroform-methanol were tested with and without hydration. An air-dry wheat flour sample was freeze-dried to 2% moisture as the basis for hydration studies. When portions of this sample were extracted with acetone-hexane (80:20, v./v.) and with chloroform-methanol (2:1, v./v.) each with increments of water added (5 to 25%), it was found that 15% of water yielded the highest percentage of hexane-soluble extract. The chloroform-methanol had a slight advantage in yield. However, this solvent combination removed a large amount of nonlipid material, which made subsequent extraction with hexane somewhat difficult. Mixtures of chloroform with other alcohols were then tried. With air-dry samples of flour (about 10% moisture), chloroform-ethanol-water (200:95:5) gave the highest yield of lipid and extracted a smaller amount of nonlipid material than chloroform-methanol. Complete removal of solvent was also more rapid than for the chloroform mixtures with propanol or butanol.

Acid hydrolysis of the extracted residues revealed that some lipid still remained following either CEW or WSB extraction (Table I). The CEW removed as high a percentage of the total lipid as any solvent tried, however, and since its use facilitated laboratory manipulations as compared to other solvents, it was adopted as the extractant for all samples. Nevertheless, to obtain total lipid an acid hydrolysis step to free the "bound" fatty acids by liquefaction of the sample was required.

These bound fatty acids clearly represent a different family of lipid-bearing compounds than those contained in the CEW extract, as is evident from the fatty acid distribution data (Table II). Hydrolysis of the bound lipid fraction with alkali instead of acid yielded similar amounts of lipid which showed the same fatty acid distribution on GLC analysis. In the acid hydrolysis lipid from wheats and wheat flours, 16:0 was 130 to 165%, 18:1 was 60 to 70%, and 18:2 was 50 to 98% of the comparable fatty acid values in the CEW extract. With one exception, these differences were highly significant, statistically.

The relationship of these observations to previous studies is of interest. Observations that hydrolysis can release lipids not extractable with fat solvents led to adoption of such methods many years ago (3, p. 194; 8). However, the fat

TABLE I. A COMPARISON OF PROCEDURES FOR REMOVAL OF LIPID FROM WHEAT FLO

Solvent[a]	Flour	No. of Replicates	Average Lipid Obtained		
			Solvent extraction %	Acid hydrolysis %	Total %
Ether-WSB (8)	A	4	1.44	0.28	1.72
Ether-WSB	B	4	0.96	0.15	1.11
CEW	B	4	1.01	0.18	1.19
CEW	C	4	1.02	0.28	1.27

[a]WSB, water-saturated n-butanol; C-E-W, chloroform-ethanol-water (200:95:5).

solvents used were relatively nonpolar. Schoch (9) observed that polar solvents such as methanol, 80% dioxane, and methyl cellosolve removed most of the lipid from corn starch in a Soxhlet extractor but that subsequent acid hydrolysis by the AOAC procedure (3, p. 194) gave 0.06% to 0.26% additional lipid. Mecham and Mohammad (6) adapted water-saturated butanol to the extraction of wheat products for the purpose of obtaining a gluten low in lipid. They observed that this solvent extracted more chloroform-soluble substance than did ethanol. Subsequent alkaline hydrolysis of the flour residue led to recovery of "very little fatty acid" and to the deduction that essentially complete extraction had been obtained. This deduction is open to question since the alcohol-extracted flour also yielded very little fatty acid under the conditions of hydrolysis employed.

Demonstration that the weight of total lipid extractable by various solvents is equal to that obtainable by direct hydrolysis does not constitute evidence for complete extraction of total lipid by such solvents. Even the least polar of the common solvents, petroleum ether, will extract lipoprotein from wheat flour (10). Silicic acid chromatography of WSB extracts from wheat shows that they contain large amounts of phospholipids and glycolipids (11) and alkaline hydrolysis shows that fatty acids constitute only half of their weight (7). Herd and Amos (8) observed that acid hydrolysis lipid was much lower in nitrogen and phosphorus content than lipid obtained by extraction with either ether or petroleum ether.

TABLE II. AMOUNTS OF THREE FATTY ACIDS IN C-E-W EXTRACT AND ACID HYDROLYSIS LIPID, EXPRESSED AS MEAN PERCENTAGES OF TOTAL FATTY ACIDS

Kind of Sample[a]	C-E-W Extract			Acid Hydrolysis Lipid		
	16:0 %	18:1 %	18:2 %	16:0 %	18:1 %	18:2 %
H-W	18.07	18.05	57.99	29.89	10.34	54.50
H-F	21.06	13.37	61.37	32.48	9.21	54.25
H-B1	22.13	38.10	21.91	30.11	17.94	43.43
H-B2	11.90	38.02	39.01	26.17	14.20	47.62
S-W	18.35	16.24	59.75	30.55	9.93	54.24
S-F1	26.81	12.20	56.44	35.14	8.94	50.18
S-F2	20.31	13.41	61.00[b]	33.78	9.08	51.40[b]
S-F3	25.13	14.65	54.70[b]	34.03	8.60	53.58[b]
S-F4	26.08	12.58	57.03	36.95	7.58	61.58
All purpose flour	21.03	14.10	60.90	32.23	9.10	54.54
Whole wheat cereal	17.62	19.10	57.65	32.20	9.71	54.09
Shredded wheat	16.61	20.15	57.12	25.84	11.50	57.75
Wheat flake cereal	16.60	18.90	59.20	26.20	12.30	56.95

[a]Abbreviations: H, hard; S, soft; W, wheat; F, flour; B, bread.

[b]No significant difference between data, pairs; all other differed at P = <1%.

Since the weight of the fatty acid-bearing components before hydrolysis can be substantially greater than that of the acids after cleavage from the "bound" state, one should expect that the solvent-extractable lipid substance may weigh more than the fatty acids obtainable after destruction by hydrolysis, even though extraction by the solvent is incomplete. The data on consumer products (see Table V below) confirm this. Fatty acids constituted 70.4% of the hexane-soluble CEW extract from wheat flour and 75 to 92% of that from the consumer products made from wheat flour.

Fatty Acids of Wheats and Flours

The durum wheats and flours (semolina) made from them were appreciably higher in total lipids than the hard red winter wheats and the soft red and soft white wheats and their comparable flours (Table III). Lipid content of the flour fell with increased exclusion of fractions during milling (samples S-F_1 to S-F_4). Significant differences in fatty acid distribution were also absent except for 18:2, which was significantly different ($P<0.05$) in both wheats and flours. Breads, cakes, and crackers made from these wheats differed sharply in lipid composition, undoubtedly because of added fat in the formulation.

Consumer Product Samples

These samples were broadly representative of products marketed throughout the country. For example, each of the ten different conventional white bread samples represented a composite of three to nine commercial products.

In analyzing the data, the linear model assumed for the consumer samples was as follows:

$$X_{ijk} = \mu + R_i + W_{(i)j} + E_{(ij)k}$$

$$\begin{aligned} i &= 1.....5 \text{ (fixed} \\ j &= 1,2 \quad \text{(random)} \\ k &= 1,2 \quad \text{(random)} \end{aligned}$$

The term μ = the overall mean effect

$$\begin{aligned} R_i &= \text{regional effects} \\ W_{(i)\,j} &= \text{effects within regions} \\ E_{(ij)k} &= \text{effects due to samples} \end{aligned}$$

Analysis of the data revealed no significant differences due to geographical region for any of the products except doughnuts. It is not certain that this difference is meaningful because the doughnut samples were difficult to mix and may have been somewhat heterogeneous owing to their high fat content. Some sporadic differences of total lipid within regions were significant, statistically; however, no pattern was observed. Gas liquid chromatography of the methyl esters from these lipids revealed, besides the five major fatty acids (Table IV), traces of others which are reported collectively. The unsaturated fatty acids were identified only as to number of double bonds rather than position or configuration. Since added fats in some of the products undoubtedly were partially hydrogenated, the

TABLE III. MEAN PERCENTAGES OF TOTAL LIPID AND ITS COMPONENT FATTY ACIDS IN THREE TYPES OF WHEAT, THEIR FLOURS, AND PRODUCTS MADE FROM FLOURS. ALL PERCENTAGES ON THE AIR DRY BASIS

Kind of Sample[a]	No. of Samples	Total Lipid	Fatty Acids 16:0	18:0	18:1	18:2	18:3	Other
H-W	5	2.550±0.01[b]	18.01±0.14	0.92±0.04	17.74±0.16	57.22±0.28	4.84±0.08	1.27
H-F	5	1.215±0.01	21.23±0.19	0.99±0.08	13.02±0.25	60.47±0.28	3.49±0.14	0.80
H-B1	5	4.578±0.01	22.01±0.14	12.38±0.17	37.03±0.15	22.19±0.17	1.25±0.07	5.14
H-B2	5	4.355±0.03	12.10±0.16	8.45±0.10	36.83±0.17	38.61±0.09	2.68±0.04	1.33
D-W	2	3.195±0.01	17.87±0.20	0.95±0.03	20.78±0.20	54.78±0.32	4.32±0.15	1.30
D-F	2	1.620±0.01	20.12±0.32	0.98±0.02	16.45±0.66	57.45±0.27	3.85±0.15	1.15
D-M	2	1.478±0.01	19.65±0.25	1.05±0.05	17.93±0.12	56.27±0.12	3.70±0.16	1.40
S-W	4	2.569±0.02	18.33±0.09	0.89±0.08	15.93±0.19	58.87±0.39	4.83±0.13	1.15
S-F1	4	1.194±0.01	26.78±0.08	1.66±0.10	11.99±0.23	55.68±0.30	3.04±0.08	0.85
S-F2	4	1.498±0.01	20.82±0.24	1.13±0.13	13.07±0.15	59.85±0.49	4.14±0.18	0.99
S-F3	2	1.458±0.01	25.12±0.13	1.68±0.22	14.35±0.38	54.00±0.43	3.68±0.13	1.17
S-F4	2	1.330±0.03	25.98±0.10	1.15±0.07	12.20±0.00	56.07±0.33	3.33±0.21	1.27
S-Ca	6	16.821±0.02	14.31±0.09	13.82±0.13	54.98±0.30	15.46±0.13	0.35±0.01	1.08
S-Cr	7	11.681±0.01	23.24±0.16	12.42±0.11	41.06±0.12	15.41±0.16	0.96±0.05	6.91

[a]Abbreviations: H, hard; D, durum; S, soft; W, wheat; F, flour; B, bread; M, macaroni; Ca, cake; Cr, cracker.

[b]Standard error of the mean.

TABLE IV. MEAN PERCENTAGES OF TOTAL LIPID AND ITS COMPONENT FATTY ACIDS IN CONSUMER PRODUCTS DERIVED MAINLY FROM WHEAT. ALL PERCENTAGES ON THE AIR DRY BASIS

Kind of Sample[a]	No. of Samples[a]	Total Lipid	Fatty Acids 16:0	18:0	18:1	18:2	18:3	Other[b]
All purpose flour	10	1.310±0.03[c]	22.49±0.16	1.01±0.20	13.16±0.17	59.24±0.24	3.35±0.19	0.75
Biscuit mix	10	12.794±0.14	21.16±0.55	16.10±0.50	43.46±1.33	12.86±0.39	0.83±0.07	5.59
Whole wheat cereal	10	3.155±0.06	18.31±0.13	0.76±0.01	18.23±0.36	56.65±0.41	4.90±0.15	1.15
Shredded wheat cereal	10	2.335±0.08	18.24±0.20	0.79±0.06	18.22±0.28	56.44±0.44	4.89±0.14	1.42
Wheat flake cereal	10	2.310±0.04	18.09±0.17	0.80±0.03	17.55±0.36	58.20±0.72	4.59±0.18	0.77
White bread (conventional)	10	5.298±0.21	19.94±0.37	11.40±0.27	36.92±0.69	25.46±0.47	1.55±0.05	4.73
White bread (continuous)	10	4.867±0.04	20.32±0.53	13.88±0.44	33.70±0.58	25.51±1.07	1.83±0.18	4.76
Whole wheat bread	10	6.655±0.23	17.57±0.59	8.86±0.16	36.30±0.77	31.35±1.06	2.09±0.10	3.83
Hamburger rolls	10	7.546±0.22	20.61±0.59	12.44±0.55	40.35±0.95	20.56±0.76	1.26±0.09	4.78
Doughnuts	10	25.676±0.41	19.70±0.83	15.46±0.59	43.69±1.46	15.23±0.49	1.36±0.11	4.56

[a]Ten samples of each product, two from each of five regions: Northeast, Southeast, Midwest, Northwest and Southwest.

[b]All products contained traces of 10:0, 12:0, 14:0, 15:0, 16:1, 17:0, 20:0 and 20:1. Breads, biscuit mixes and doughnuts contained traces of 14:1, up to 3% of 14:0 and 16:0, and up to 1% of 20:0 and 20:1.

[c]Standard error of the mean.

TABLE V. TOTAL LIPID AND FATTY ACID COMPOSITION OF WHEAT CONSUMER PRODUCTS EXPRESSED AS GRAMS PER 100 g. OF MOISTURE-FREE SAMPLE

Kind of Sample	Total Lipid g.	Total Fatty Acid g.	16:0 g.	18:0 g.	18:1 g.	18:2 g.	18:3 g.
All purpose flour	1.50	1.13	0.25	0.01	0.14	0.67	0.04
Biscuit mix	14.19	12.22	2.59	1.96	5.32	1.57	0.10
Whole wheat cereal	3.48	2.74	0.50	0.02	0.50	1.55	0.14
Shredded wheat cereal	2.54	2.17	0.40	0.02	0.40	1.22	0.11
Wheat flake cereal	2.43	1.92	0.35	0.01	0.34	1.12	0.09
White bread (conventional)	5.53	4.66	0.93	0.54	1.72	1.18	0.07
White bread (continuous)	5.08	4.42	0.90	0.61	1.49	1.12	0.08
Whole wheat bread	6.94	6.33	1.11	0.56	2.31	1.98	0.13
Hamburger rolls	7.85	6.32	1.30	0.78	2.56	1.30	0.08
Doughnuts	26.57	24.62	4.87	3.84	10.71	3.74	0.33

presence in the 18:2 fraction, for instance, of isomers other than linoleic acid is to be expected. The close similarity of fatty acid distribution in the whole wheat cereal, shredded wheat cereal, and wheat flake cereal probably indicates that little or no added fat is present in these products.

Those who have need for dietary information on individual fatty acids in consumer products are referred to Table V which presents the quantity of each of the five major acids per 100 g. of sample. These data are expressed on the moisture-free basis because this laboratory received the products as blended and partially dried samples rather than as purchased.

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