Combination of Thin Layer Chromatography and Gas Chromatography in the Analysis on a Microgram Scale of Lipids From Wheat Flour and Wheat Flour Doughs

A. GRAVELAND, Institute for Cereals, Flour and Bread TNO, Wageningen, The Netherlands

Abstract

Lipids were extracted on a microgram scale from 3 to 6 mg flour or from freeze-dried dough by percolation. The impure extract was separated into 12 nonpolar and 14 polar lipids by TLC. Then the fatty acid composition of most of these lipids was determined by gas chromatography. The microscale method renders possible qualitative and quantitative determination of the composition of the lipids in minute quantities of flour and dough. The selectivity of different extraction solvents was studied. In both flour and dough the fatty acid composition of a given component is identical in the free and bound lipid fractions. In dough, the quantity of free lipids is less than in flour; the extent of the decrease depends on the atmosphere in which it is mixed. During mixing, the carotenoid pigments, α-monoglycerides and the free linoleic acid and linolenic acid are oxidized. Oxidation is apparently a function of the concentration of oxygen and of lipoxidase.

Introduction

WHEAT FLOUR CONTAINS only a small amount of lipids, viz. 1.5% to 3% of its weight. Despite this low percentage, a great deal of attention has been devoted to these substances in recent years. On the one hand, lipids are important because of their nutritional value and as a source of fat-soluble vitamins; on the other hand, lipids play an important part in the aging of flour, in the mixing behavior of dough and in bread baking. Particularly the latter two items are of interest to the cereal chemist. The recent results of studies on the role of lipids in baking are highly contradictory.

Research in the field of wheat lipids has been chiefly limited hitherto to extraction and reconstitution tests. Different solvents were used to extract the lipids, and the changes in baking quality were determined (extraction tests). Thereupon the extract was added again to the flour from which it had been extracted and the effect investigated (reconstitution tests). The results obtained varied considerably, depending on the flour as well as on the solvent used.

Moreover owing to lack of good analytical techniques, no one has checked thoroughly which lipids are extracted by any given solvent or which influence the solvent has on the nonlipid flour components. To obtain more insight in this matter and to get to know which reactions are taking place, the different components must be studied separately.

Using modern chromatographic techniques we developed a micro method which consists of a combination of thin-layer chromatography and gas chromatography. This method was used to determine which lipids and how much of each of them were extracted by a given solvent and also whether the fatty acid composition exerted any influence upon the degree to which lipids could be extracted. We also studied the change occurring during mixing.

Experimental Procedures

Preparatory Work

The quantities of lipids that can be extracted depend on the solvent, the extraction method and the physical nature of the sample. The following extraction methods were tested on samples of 5 g of material: Soxhlet extraction, stirring with a blender (Braun), shaking in a shaking machine, and percolation. Judging from the results obtained, percolation proved to be the best method, at least for samples of a maximum of 5 g. This method has the greatest yield of lipids, gives relatively few impurities, and is very well suitable from the technical viewpoint for extracting minute quantities of material. Because of this experimental result, we selected percolation as the method of extraction.

Materials

Flour. A commercial, unbleached, improver-free bread flour, having a protein content (N × 5.7) of 10.1% (14% moisture basis).

Doughs. Doughs were prepared from 100 g flour, 54 ml distilled water and 1.0 g sodium chloride (dissolved in part of the water). These ingredients were mixed in a GRL mixer (1) at 66 rpm in nitrogen, air, or oxygen, at 30 C for various times. The doughs were prefrozen in a stream of air of −40 C moving at 2 m/sec. Then they were freeze-dried, pulverized in a mortar and, when necessary, stored at 4 C.

Methods

Lipid Extraction. The material to be tested (3, 6 or 9 mg) was put on top of and around a cotton wick fixed in the narrowed outlet of a Pasteur pipet. A small piece of the wick protruded from the capillary (Fig. 1). Some powdered silicon carbide was deposited on the material to be tested to avoid drifting during percolation. Prior to use, the silicon carbide was purified by Sohxlet extraction with methanol. The solvent (1 ml) was put on the silicon carbide and slowly percolated through the material to the bottom. The extract was removed along the cotton wick and, without previous purification, applied immediately upon a TLC plate.

Qualitative TLC. Glass slides (19 × 5 cm) were coated with a layer of silica gel DO (Fluka's, Switzerland) by immersing the slides in a suspension of silica gel in chloroform (2). Neither the silica gel nor the chloroform was purified beforehand; however, any impurities present were removed by heating the coated slides on a thermostatically controlled hot plate at a temperature of 400 C for ½ hr. During this heat treatment all disturbing impurities were removed, and the slides were well activated. After cooling the plates, the extract was applied in the way described and the plate was subjected to chro-

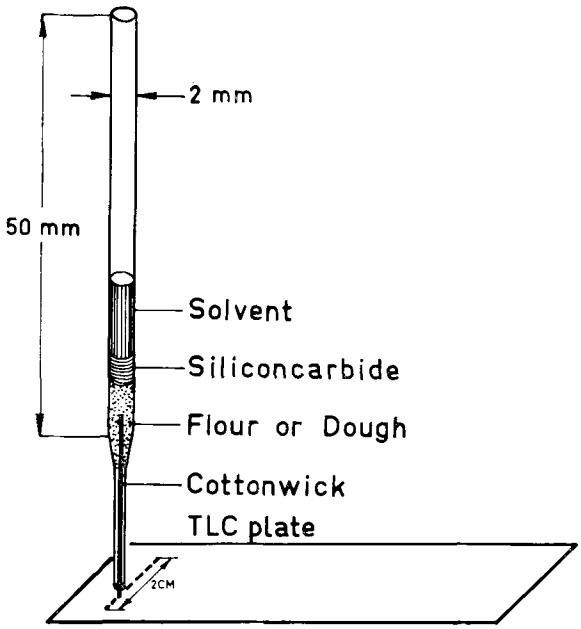

FIG. 1. Micro-extraction apparatus.

matography as soon as possible to prevent redeposition of atmospheric impurities on the silica gel layer. The extract was applied direct from the Pasteur pipet in a 2.0 cm long band at a distance of 1.5 cm above the lower edge of the layer. Different solvent mixtures were used to separate the lipids. The plates were developed in tanks saturated with vapor of the same solvent mixtures.

Separation of Nonpolar Lipids. Freeman and West's (3) system was used for the separation of the nonpolar lipids. First, the slides were developed in solvent system I: diethylether-benzene-ethanol-acetic acid (40:50:2:0.2). The solvent front was allowed to run a distance of 9 cm from the origin. After drying for 10 min, the slides were developed in the same direction in solvent system II: diethyl-ether-hexane (6:94). This solvent was allowed to run 13 cm from the origin. The time taken for total development in these two systems was 50 min. Separations were carried out at room temperature. When this procedure was used, the polar lipids remained at the origin.

Separation of Polar and Nonpolar Lipids. In order to separate polar and nonpolar lipids, the slides were first developed in system III, the development time being 30 min at 4 C and the running distance 10 cm. System III consists of a mixture of chloroform-methanol-water (65:25:4) (4). The polar lipids were separated, whereas the nonpolar lipids were found at the front of the solvent in the form of two bands. Next the nonpolar lipids were separated by developing the slides with solvent system I in the same direction. This solvent was run 14 cm from the origin.

For further fractionation of certain polar lipid components that had not been separated with the chloroform system mentioned above, the following systems were used. System IV: chloroform-methanol-water-glacial acetic acid (25:15:2:4) (5) and system V: chloroform-methanol-water-ammonia 25% (60:25:5:3) (6). All solvents were of analytical grade, redistilled from glass.

The spots were located and made visible in one of the following manners: (a) by heating the plates at 200 C for 10 min after spraying with 50% volume of aqueous sulfuric acid, for all components, (b) by spraying with a 0.2% solution of ninhydrin in butanol-water (7), a spray specific for lipids that contain a free amino acid group: peaks 1, 2, 7, 8 and the origin; (c) by spraying with Dragendorff reagent (8), for choline phosphatides and glycolipids: peaks 1, 4, 8, 9, 10, 13; (d) by spraying with molybdenum spray (9) for detection of phospholipids: peaks 1, 2, 4, 6, 7, 12; (e) by partial hydrolysis (10), for sphingomyelin and cerebrosides: peaks 3, 11.

The different components on the TLC plate were identified by comparing the Rf values with those taken from literature, by using specific sprays and by comparing with pure components. The standard substances used were linoleic acid, monoglyceride, cholesterol, lecithin, lysolecithin, phosphatidyl-ethanolamine, cerebrosides and monogalactosyl glyceride, all obtained from the Applied Science Laboratory, State College, Pa., U.S.A.

Quantitative TLC. The extract obtained from an accurately weighed sample of flour or dough (3 or 6 mg) was applied direct from the Pasteur pipet in a series of single spots so close to each other as to form a narrow band on the TLC plate. Good homogeneity was obtained by superimposing the extract in a number of layers. The first drops must be very small since they are relatively rich in lipids. The width of the origin must be exactly 2 cm.

After chromatography, the solvent was evaporated from the plate in a vacuum desiccator. Then the TLC plate was sprayed with 50% volume of aqueous sulfuric acid (11) and heated at 200 C for 10 min; this makes the lipid bands char. Next the plate was scanned with a densitometer (Vitatron UTD 500, filter 363 nm). The transmitted light was recorded on a linear extinction scale. The peaks were symmetrical.

The described procedure does not separate all components from each other. Complete fractionation can be obtained, after weak staining in iodine vapor, by scraping off a band (that contains several components) and rechromatographing in a different solvent system. For reliable quantitative measurements, two determinations are required, one with a small quantity of flour or dough (3 mg) for major components, and one with twice that quantity (6 mg) for minor components.

Preparative TLC. Preparative TLC was used to obtain pure components of lipids from the flour and dough sample examined in order to determine their fatty acid composition. In order to have sufficient lipid material available for determining the fatty acid composition of a given component, 90 mg of flour or dough were used (approx. 1 mg of lipids). After chromatography, the separate bands were rendered visible with a very light spray of dichlorofluorescein, viewed under ultraviolet light, and the lipid areas were scraped off the plate directly into 5 ml test tubes.

GLC. Fatty acid methyl esters were routinely separated by GLC. A glass column (2 m × 3 mm) containing 12% ethylene glycol adipate on chromosorb W was used at 195 C in a Fractovap model GV gas chromatograph (Carlo Erba Co.) operating with a flame ionization detector. The carrier gas was nitrogen. The total lipid extracts of the separate components isolated from a TLC plate were saponified with 0.5 N KOH in methanol at 65 C during 15 min. Then the fatty acids were esterified (5 min.

at 65 C) with BF_3-methanol (the methanol contained 14% BF_3, from British Drug Houses, Ltd.). Saponification and esterification of the individual components was achieved without removal of the silicic acid. The methyl esters were dissolved in heptane and analyzed (12). Standard methyl esters mixtures (Applied Science Laboratory, State College, Pa.) were used for identification of the fatty acids. Further confirmation of identity was obtained by hydrogenation of the esters followed by gas-chromatography. The retention time of the methyl esters was also compared on 15% Apiezon-L on Gas-Chrom. P.

Results and Discussion

Methods

There are different methods for quantitatively measuring the components separated on a TLC plate: gravimetrically, colorimetrically and densitometrically. In our tests we used densitometry. Peifer (2) and Blank et al. (13) demonstrated their ingenious use of the charring procedure for quantitative TLC by densitometry. The degree of charring depends on the composition of the lipid, in particular on its carbon percentage. In order to determine the absolute quantities from the densitogram, one should multiply the densitometric units of each component with a specific factor (14). We did not need these factors in our experiments as we wished to compare the various components of flour and dough. Therefore, the quantities in Tables I, II and III are expressed in cm^2 peak surface. In approximation, 1 cm^2 corresponds to 3×10^{-4} mg of lipids.

The method of extraction described in the preceding paragraphs makes it possible to apply the extract in a narrow, homogeneous band without damaging the silica gel layer. When silica gel DO of Fluka's is used, there is no deformation or any widening of the bands, nor tailing. The method is highly sensitive, as small a quantity as 0.1 μg lipids can be detected. Moreover, the method is reproducible, provided working conditions are standardized. Because the analysis is performed rapidly (extraction takes 2 min and separation 50 min) and no purification process is involved, the possibility of changes occurring as a result of oxidation or hydrolysis is small. Therefore, this method offers the possibility of determining which lipids play a role in some dough reactions and what changes they undergo during these reactions.

Extraction and Fractionation of Lipids From Flour

The flour lipids were separated by means of the solvent systems III and I; and, since the extracts contained relatively large amounts of lipids, the nonpolar lipids were additionally separated on another TLC plate by using solvent systems I and II. In the latter case the α- and β-monoglycerides were separated. An example of a separation of polar and nonpolar lipids of a benzene-ethanol-water extract from flour is shown in Fig. 2.

In Table I the peak surfaces of densitograms of various extraction solvents are listed. By means of comparison with pure reference materials, specific staining reactions and Rf-values from literature most components have been identified (Column 2). Peaks consisting of several components were fractionated further by scraping off and rechromatographing with solvent systems IV and V.

In the first instance, this micro-method has been used for studying the selectivity of a series of solvents with increasing polarity. Free lipids are defined as the lipids extracted with ether; residual lipids which can be extracted with a mixed solvent system containing ethanol or methanol are considered to be the bound lipids (15). In these extraction

TABLE I

Composition of Wheat Flour Lipids Fractionated by TLC After Extraction by Various Solvent Systems

TLC no.	Lipid class	Extraction solvents: Hexane	Ether	Chloroform	Ethanol-ether 2:1	Chloroform-methanol 2:1	Ethanol-ether-water (2:2:1)	Benzene-ethanol-water (10:10:1)
	Polar lipids							
1	Lysolecithin	0.6[a]	0.4	1.1	2.4	2.5	2.5	2.6
2	Lysophosphatidyl-ethanolamine	0.7	1.0	1.2	1.6	1.2	3.6	3.0
3	Sphingomyelin							
4	Lecithin	1.8	2.2	3.8	4.5	5.0	4.8	5.0
5	Unknown	0.9	0.9	1.1	2.2	2.4	2.2	2.2
6	Phosphatidyl-inositols	0.9	1.0	1.0	1.2	1.3	1.4	1.4
7	Phosphatidyl-serine + unknown	6.0	5.9	6.9	11.0	11.4	11.4	11.2
8	Digalactosyl-glyceride + phosphatidyl-ethanolamine	15.0	19.6	31.2	38.6	40.8	41.0	40.4
9	Unknown + sterolglycoside	9.0	10.0	13.9				18.0
10					25.0	25.2	25.2	
11	Cerebrosides	2.6	7.5	7.6				9.2
12	Phosphatidic acid	0.9	1.2	1.2	1.0	1.0	1.4	1.4
13	Monogalactosyl-glyceride	5.0	10.2	10.4	11.4	11.2	11.6	11.6
14	Unknown	0.6	0.8	0.8	1.8	0.6	1.0	1.0
		44.0	50.7	80.2	100.7	102.6	106.1	107.0
	Nonpolar lipids							
15	Unknown	1.2	2.2	2.4	2.5	2.4	2.6	2.6
16	α-Monoglycerides	1.6	1.5	2.0	3.5	3.5	3.5	3.4
17	β-Monoglycerides	3.6	5.2	6.0	5.8	6.1	6.0	6.0
18	Carotenoids	0.2	0.2	0.4	1.0	0.8	1.0	1.0
19	Oxidation products	0.1	0.1	0.1	0.3	0.2	0.2	0.2
20	Sterol	1.6	1.6	1.5	2.1	1.8	2.0	2.0
21	Free fatty acids	20.8	21.8	22.6	25.0	26.4	26.0	26.6
22	1,2-Diglycerides	8.0	8.0	8.2	8.4	8.6	8.6	8.8
23	1,3-Diglycerides	5.0	5.0	5.0	5.1	5.2	5.3	5.2
24	Unknown	1.3	1.4	1.4	1.8	1.8	1.8	1.8
25	Triglycerides	57.2	58.3	58.4	56.0	59.0	56.6	60.0
26	Sterolesters	9.7	9.8	10.0	8.8	10.4	9.0	10.2
		110.3	115.1	118.0	117.5	126.2	122.6	127.8
	Total lipids	154.3	165.8	198.2	218.2	228.8	228.7	234.8

[a] These figures relate to quantities of 6 mg of flour; they represent the number of cm^2 peak surface (1 cm^2 represents 3.10^{-4} mg lipids).

TABLE II

Total Amounts of Polar and Nonpolar Lipids of Dough

	Flour	Dough mixing in nitrogen	Dough mixing in nitrogen + iodate[a]	Dough mixing in air	Dough mixing in oxygen
Polar lipids					
free	50.7(47%)	43.6(41%)	43.6(41%)	46.3(43%)	53.3(50%)
bound	55.9(53%)	62.7(59%)	63.0(59%)	60.3(57%)	53.1(50%)
total	106.6	106.3	106.6	106.6	106.4
Nonpolar lipids					
free	115.1(89%)	94.5(74%)	94.5(74%)	87.0(80%)	96.9(89%)
bound	13.3(11%)	33.9(26%)	32.8(26%)	22.0(20%)	12.7(11%)
total	128.4	128.4	127.3	109.0	109.6

[a] Added 1.83 moles of iodate per gram of flour.

experiments the basic quantity was always 6 mg of flour.

A mixture of benzene-ethanol-water extracts both free and bound lipids. Extraction with this solvent can be considered to be complete since it has not been possible to demonstrate, after acid hydrolysis, the presence of lipid in the residual material. By means of ether, the free lipids are extracted. Table I shows that this fraction contains 90% of the total amount of the nonpolar and 47% of the polar lipids.

From Table I we can easily read that the amount of lipid extracted from flour is enhanced by increasing polarity of the solvent. If a solvent contains ethanol or methanol, its effectiveness is greatly enhanced, especially for polar lipids. The presence of water slightly increases extractability.

Table I, moreover, shows the quantity of each individual lipid extracted of a given solvent. For instance, hexane extracts 78% of the free fatty acids (peak 21) as compared to the amount extractable with benzene-ethanol-water and only 37% of the digalactolipid (peak 8), whereas chloroform extracts 85% of the free fatty acids and 76% of the digalactolipid, and so on. Diglycerides (peaks 22 and 23), triglycerides (peak 25) and sterolesters (peak 26) can be easily extracted with a nonpolar solvent.

From literature it is known that flour particles consist of a network of proteins, with the starch granules imbedded in between; the lipids are spread out over the entire substance in the shape of single molecules or as micelles. An apolar solvent probably extracts the lipids located on the external surface of the flour particles as well as those lipids which easily diffuse from the inside to the exterior, such as triglycerides and sterolesters. When a polar solvent is used, the tertiary structure of the protein is disrupted, thus rendering the lipids more accessible to the solvent.

Extraction and Fractionation of Lipids From Dough

We investigated the lipid composition of dough and the changes of all components during mixing under various conditions. On the ground of the results obtained in extracting flour, we used only two solvents for extracting the lipids from freeze-dried doughs: ether for the free lipids and a mixture of benzene-ethanol-water for the bound lipids.

Table II shows the sum of polar and nonpolar lipids; these are listed extensively in Table III. It is evident that when the dough is mixed under nitrogen an increase in lipid binding occurs as compared with flour: 59% of the polar lipids and 26% of the nonpolar lipids are bound. The changes are more obvious in the nonpolar lipids than in the polar ones. When potassium iodate or potassium bromate is present in the dough, mixing under nitrogen has no further influence upon the binding of the lipids.

Mixing in air results in a similar effect, though in a lesser degree. If, in contrast, mixing is done in oxygen, the proportional composition of the lipids is approximately the same as in flour.

Furthermore, it is a striking fact that after mixing in air or oxygen the total amount of nonpolar lipids in dough is smaller than after mixing in nitrogen. The explanation of this will be offered later. The larger the oxygen concentration, the smaller the quantity of bound lipids. This conclusion is in accordance with the findings of Daniels et al. (17), who found even greater differences, which can probably be ascribed to the fact that they used a rapid mixer and worked with a differently composed dough; they also added fats.

Figures 3 and 4 show the separation of the nonpolar lipids from doughs mixed under nitrogen and oxygen, respectively. On comparing these figures, it is evident that when a dough is mixed under oxygen peaks 16 (α-monoglycerides) and 21 (free fatty acids) decrease. Besides, in Fig. 4 a new peak 19 appears; this one could be identified as an oxidation product of linoleic acid, viz. an alcohol of linoleic acid having conjugated double bonds. The yellow color of the band (peak 18) containing carotenoid pigments disappears.

The quantities of the individual components are listed in Table III. As to the polar lipids, lysolecithin (peak 1), lecithin (peak 4) and saccharide-containing lipids (peaks 8 to 11 inclusive) are preferentially bound. As to the nonpolar lipids, when mixing occurs in air or oxygen, practically half of the α-monoglycerides (peak 16) and three quarters of the free fatty acids (peak 21) disappear. During mixing in a nitrogen atmosphere, addition of 30.0 mg potassium bromate or 40.0 mg potassium iodate per kilogram of flour did not bring about further changes; not even when excessive amounts of iodate (400 mg per kg flour) were added. The bromate and iodate do not, therefore, react with the lipids, whereas they probably react with thiol groups of protein (see, for instance, 26).

Fatty Acid Composition of Flour and Dough Lipids

Next, we estimated the fatty acid composition of each lipid component from flour and dough by using a combination of preparative TLC and gas chromatography. The fatty acid composition of a certain component in the free lipid fraction (extractable with ether) proved to be identical to that in the bound lipid fraction (extractable with benzene-ethanol-water after an ether extraction). This holds both for flour and dough. Moreover, the fatty acid composition has no influence upon the extractability of the lipids from flour or freeze-dried dough. Therefore, we present in Table IV only the fatty acid composition of the components from the free lipid fraction.

TABLE III

Composition of Dough Lipids Fractionated by TLC

Mixing conditions	Flour[a]		Dough mixing in nitrogen		Dough mixing in air		Dough mixing in oxygen	
Extraction solvent	A[b]	B[c]	A	B	A	B	A	B
TLC no.								
1	0.4	2.1	0.5	2.1	0.8	1.7	1.0	1.5
2	1.0	2.0	0.9	2.0	1.0	2.2	1.7	1.4
3								
4	2.2	2.7	0.9	4.3	1.4	3.7	1.9	3.0
5	0.9	1.4	1.3	0.7	1.5	0.6	1.9	0.3
6	1.0	0.4	0.7	0.9	0.8	0.6	1.0	0.5
7	5.9	5.3	5.6	5.5	6.1	5.3	6.6	4.4
8	19.6	20.7	15.1	25.6	15.5	25.2	17.8	22.9
9	10.0	7.5	10.7	16.5	11.2	16.0	12.2	14.9
10								
11	7.5	1.8						
12	1.2	0.3	0.4	0.7	0.5	0.7	0.6	0.6
13	10.2	1.3	7.4	4.0	7.4	3.8	8.4	3.2
14	0.8	0.4	0.1	0.4	0.1	0.5	0.2	0.4
	50.7	55.9	43.6	62.7	46.3	60.3	53.3	53.1
15	2.2	0.4	2.0	0.5	2.1	0.4	2.0	0.6
16	1.5	1.7	2.5	1.2	1.2	0.8	1.3	0.7
17	5.2	0.8	3.8	2.3	4.7	1.5	5.1	0.7
18	0.2	0.7	0.1	0.4	0.1	0.2	0.1	0.3
19	0.1	0.2	0.2	0.4	0.2	1.8	0.7	1.5
20	1.6	0.4	18.6	11.0	6.8	3.8	8.5	1.8
21	21.8	5.0						
22	8.0	0.9	6.6	1.8	6.8	2.0	7.9	0.6
23	5.0	0.2	3.4	1.9	4.4	0.7	4.4	0.6
24	1.4	0.5	1.4	0.5	1.0	0.7	1.4	0.6
25	58.3	2.1	47.0	12.7	50.5	9.0	55.9	4.6
	9.8	0.4	8.9	1.2	9.2	1.1	9.6	0.7
	115.1	13.3	94.5	33.9	87.0	22.0	96.9	12.7
Total lipids	165.8	69.2	138.1	96.6	133.3	82.3	150.2	65.8

[a] Flour, see Table I.
[b] Extracted with ether (free lipids).
[c] Extracted with benzene-ethanol-water after an ether extraction (bound lipids).

The atmosphere in which the dough is mixed does not affect fatty acid composition, except for the α-monoglycerides (peak 16) and the free fatty acids (peak 21). The composition of these two components changes only if the dough is mixed in air or oxygen. This is attributable to the fact that during mixing in air or oxygen the free linoleic and linolenic acids and the α- monoglycerides, which also contain linoleic

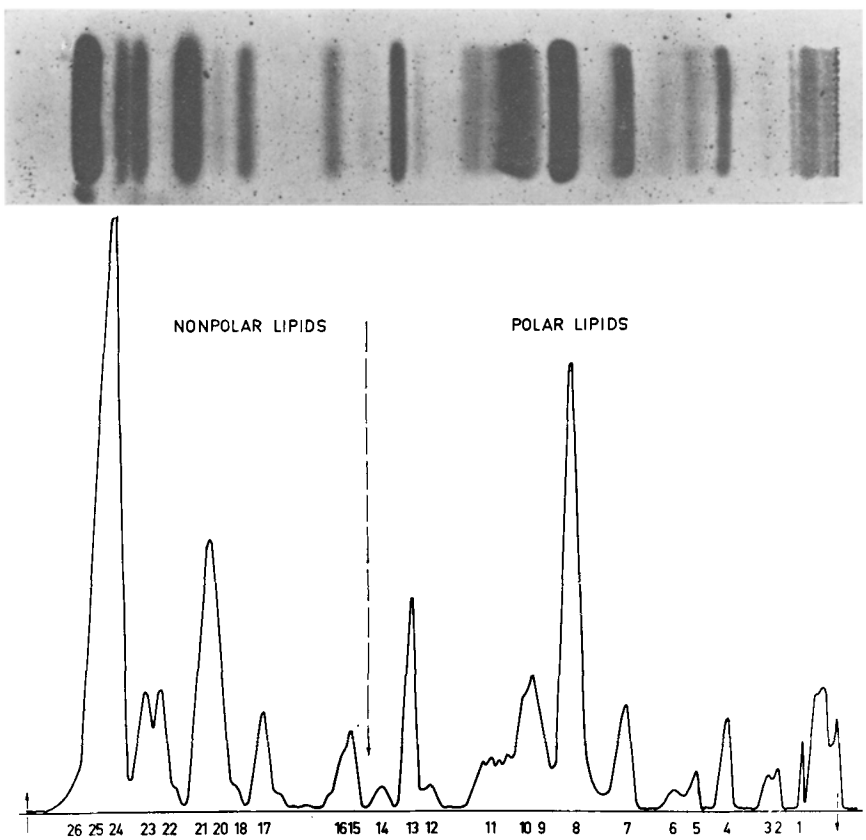

FIG. 2. TLC separation of polar and nonpolar lipids from 6 mg flour developed in systems III and I, charred with 50% sulfuric acid and scanned with a densitometer.

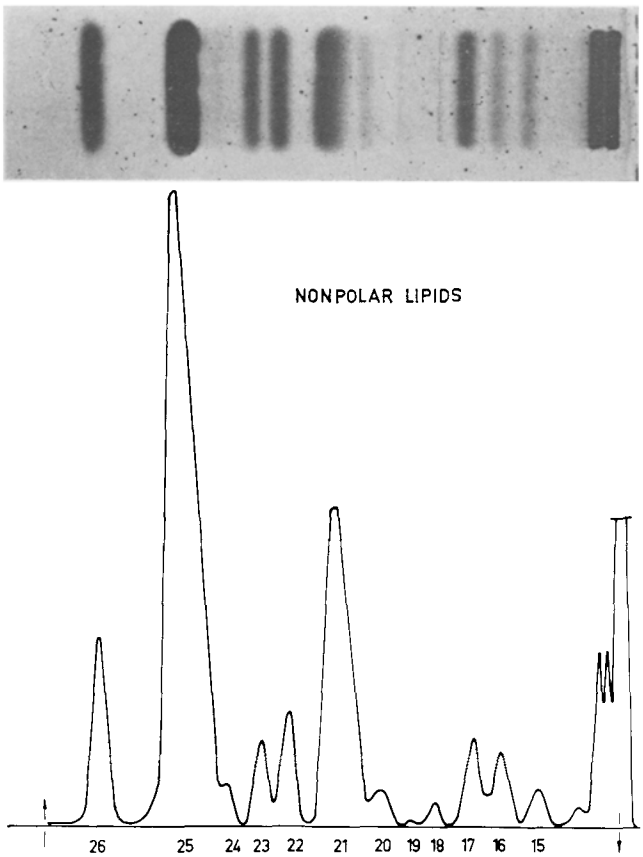

FIG. 3. TLC separation of nonpolar lipids from dough mixed in nitrogen, developed with systems I and II, charred with 50% sulfuric acid and scanned with a densitometer.

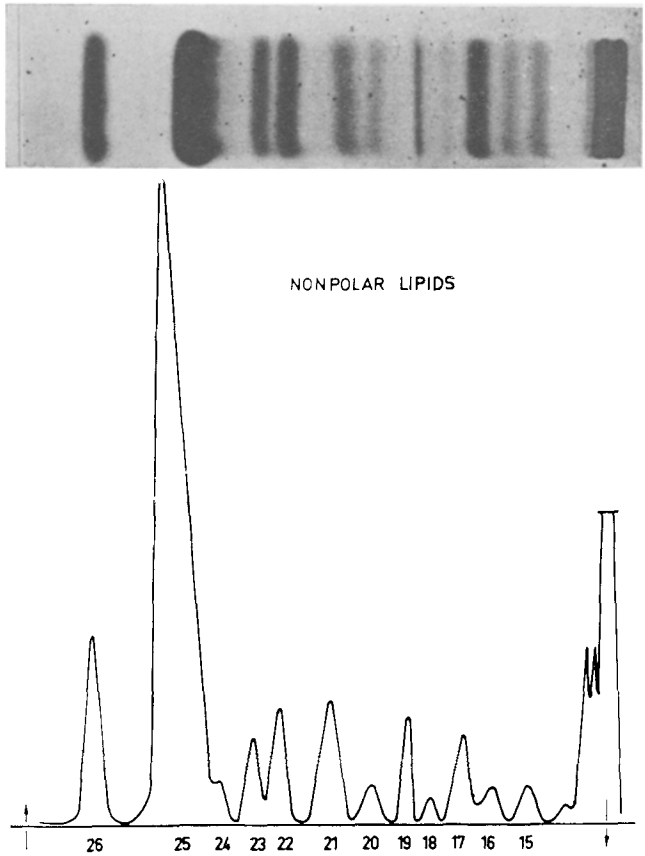

FIG. 4. TLC separation of nonpolar lipids from dough mixed in oxygen, developed with systems I and II, charred with 50% sulfuric acid and scanned with a densitometer.

and linolenic acid, are oxidized. Minor changes were observed in sterolesters (peak 26). In dough this component has less C16:0 and more C18:1 and C18:2 than in flour.

Daniels et al. (18) found, in contrast to our results, slight differences in the fatty acid distribution of the lipid components from the free and bound lipid fractions. This is probably to be ascribed to the fact that they used doughs of an entirely different composition from ours, and also due to incomplete extraction. Our results show furthermore that the linoleic acid containing lipids were not preferentially bound during the mixing of the dough, which contradicts the results of a number of researchers (19,20).

As shown in Table IV, the major fatty acids (above 0.4%) are in decreasing amounts—18:2, 16:0, 18:1, 18:3, 18:0 and 16:1. The minor fatty acids (less than 0.2%) are 14:2, 15:0, 17:0, 20:0, 20:4 and 22:0. These minor fatty acids occur in such minute quantities that we did not pay further attention to them.

Oxidation of Lipids

We carefully checked, with the aid of preparative

TABLE IV

Fatty Acid Distribution of Polar and Nonpolar Lipids from Flour and Dough

Peak (TLC no.)	1	4	7	8	9/11	13	15	16	17	21	22	23	25	26
						Flour:Ether Extract								
16:0[a]	37.0	26.2	26.1	12.2	22.0	4.5	39.4	19.0	19.4	15.5	14.6	16.0	16.1	54.1
16:1	2.0	1.6	2.4	0.5	0.8	0.4	2.2	1.7	0.8	0.4	0.6	0.7	0.6	2.8
18:0	5.0	2.5	4.0	1.3	1.3	0.9	4.7	2.2	1.7	1.2	1.5	1.2	1.0	2.0
18:1	12.8	14.6	10.8	7.4	9.4	9.8	12.0	14.6	10.0	13.2	16.8	16.4	16.7	8.9
18:2	41.0	54.0	54.3	74.1	63.2	80.6	40.0	60.1	64.9	65.8	63.2	62.8	61.4	30.2
18:3	1.6	1.4	1.8	4.5	3.1	3.7	1.8	2.4	3.2	2.9	3.1	2.9	3.6	1.5
						Dough mixed[b] in nitrogen: ether extract								
16:0	36.5	26.8	26.6	11.3	21.0	4.5	40.2	19.6	19.4	16.0	14.5	16.9	16.6	50.0
16:1	2.1	2.0	2.0	0.7	1.2	0.9	2.4	1.6	0.8	1.1	0.7	0.7	0.4	3.2
18:0	4.7	3.0	4.6	1.6	1.7	1.0	4.8	2.2	1.7	2.0	1.4	1.2	1.0	2.5
18:1	13.5	14.6	11.0	8.7	9.5	9.8	11.0	14.6	10.0	13.7	16.1	15.6	16.3	10.0
18:2	41.1	52.0	54.0	73.1	63.0	80.4	39.8	59.2	64.9	64.0	64.3	62.5	62.1	31.8
18:3	1.9	1.6	2.0	4.1	2.8	3.4	1.6	2.6	3.1	3.0	3.0	3.1	3.6	1.5
						Dough mixed[b] in oxygen: ether extract								
16:0	37.0	26.9	26.2	11.6	21.6	4.6	40.0	33.9	19.5	46.7	14.0	16.6	16.4	49.9
16:1	2.1	1.9	2.6	0.8	1.1	0.3	2.6	2.2	0.7	1.5	0.8	0.5	1.7	3.0
18:0	4.7	2.6	4.1	1.6	1.7	0.9	4.2	4.3	1.8	6.1	1.6	1.3	1.0	2.5
18:1	13.0	14.0	10.6	8.9	9.5	9.8	11.8	21.9	10.2	37.8	16.5	16.1	15.7	10.2
18:2	41.0	52.9	54.3	73.2	63.2	80.2	39.7	34.2	65.3	7.6	63.7	61.8	62.6	32.4
18:3	2.2	1.6	2.2	3.8	2.8	3.8	1.7	3.4	2.8	0.1	3.2	3.0	3.6	1.6

[a] Carbon number: number of double bonds.
[b] Doughs were mixed for 20 min in a GRL mixer at a speed of 66.5 rpm.

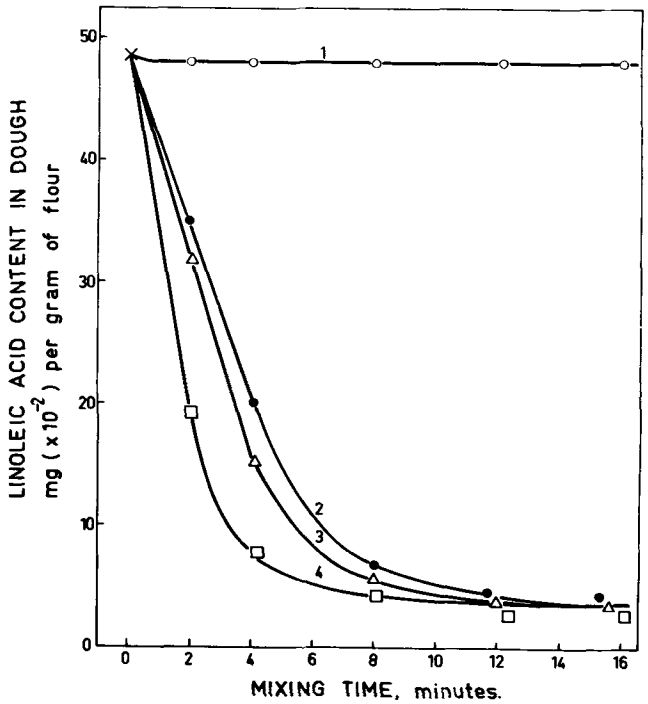

FIG. 5. Rates of oxidation of linoleic acid in dough. 1. Mixing in nitrogen. 2. Mixing in air. 3. Mixing in air with lipoxidase. 4. Mixing in oxygen.

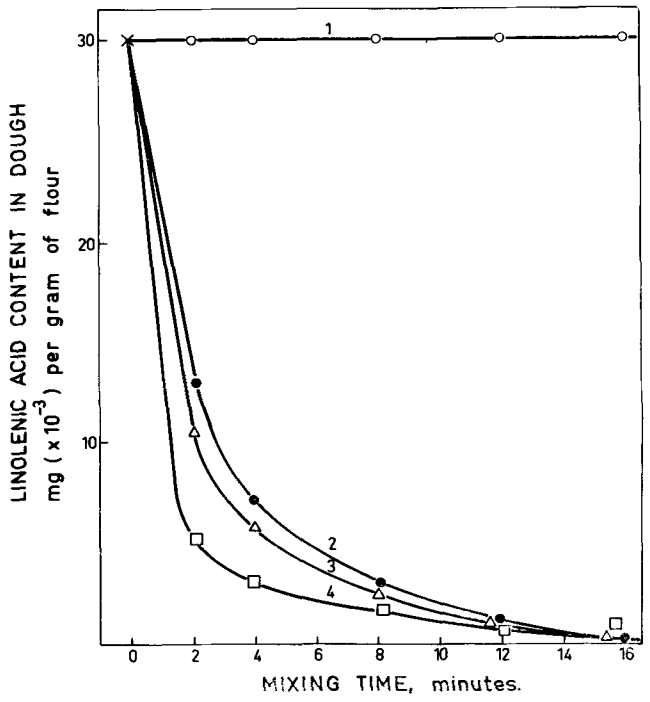

FIG. 6. Rates of oxidation of linolenic acid in dough. 1. Mixing in nitrogen. 2. Mixing in air. 3. Mixing in air with lipoxidase. 4. Mixing in oxygen.

TLC and gas-chromatography, the rate at which the two polyunsaturated free fatty acids decreased under several mixing conditions. The free fatty acids were extracted with benzene-ethanol-water (Fig. 5 and 6).

Mixing in nitrogen has practically no influence on the concentration of these acids. When mixing was effected in air or oxygen, a rapid decrease in both linoleic and linolenic acid took place, the rate of disappearance being more rapid in oxygen than in air. The addition of 10 mg lipoxidase (from soybeans, approx. 10,000 units/mg) per 100 g flour accelerated slightly the disappearance of both acids in air. From these results we concluded that the decrease in concentration of both polyunsaturated fatty acids is caused by an enzymatic oxidation of *cis,cis*-1,4-pentadiene containing fatty acids. This conclusion is in agreement with results from literature. Smith and Andrews (22) have demonstrated that the uptake of oxygen during mixing is enhanced by the lipids interacting with a water soluble substance (lipoxidase) on the one hand and the gluten proteins on the other. Tsen and Hlynka (23), too, have demonstrated the enzymatic nature of the oxidation.

The oxidation rates of linoleic acid and of linolenic acid as a result of mixing in oxygen are of the order of 8.8×10^{-3} μmole/g sec resp. 1.0×10^{-3} μmole/g sec. The oxidation rate of linoleic acid is almost nine times that of the thiol groups, which latter is approximately 1×10^{-3} μmole/g sec (26).

If we assume that per mole linoleic or linolenic acid 1 mole O_2 is taken up irrespective of the number of double bonds, the total quantity of oxygen used up in the oxidation of the lipids during mixing is approximately 2.0 μmole/g (1.7 μmole/g for linoleic acid, 0.1 μmole/g for linolenic acid and 0.2 μmole/g for α-monoglycerides and carotenoids). Flour contains approx. 1 μmole thiol groups and 10 μmole disulfide bonds per gram, the maximum quantity of oxygen that can be taken up by the thiol groups is therefore 1 μequiv. (26). As we calculated that the oxygen that can be taken up by the lipids is 8.0 μequiv, the total uptake of oxygen by both lipids and proteins is 9.0 μequiv. Smith and Andrews (22) reported actual oxygen uptakes of 26 μequiv/g for a patent and 58 μequiv/g for a first clear flour. Thus much more oxygen is taken up than is actually used by the proteins and lipids. No explanation can be given yet for this fact.

ACKNOWLEDGMENTS

L. L. M. van Deenen (Utrecht) and C. M. van Gent (Leyden) gave suggestions and advice, and Anke Blom gave assistance in experiments and analyses.

REFERENCES

1. Hird, F. J. R., and J. R. Yates, Biochem. J. *80*, 612 (1961).
2. Peifer, J. J., Mikrochim. Acta p. 529 (1962).
3. Freeman, C. P., and D. West, J. Lipid Res. 7, 324 (1966).
4. Lepage, M., J. Chromatog. *13*, 99 (1964).
5. Skipski, V. P., R. F. Peterson and M. Barclay, Biochem. J. *90*, 374 (1964).
6. Skidmore, W. D., and C. Entenmann, J. Lipid Res. *3*, 471 (1962).
7. Wagner, H., L. Hörhammer and P. Wolff, Biochem. Z. *334*, 175 (1961).
8. Mangold, H. K., JAOCS *38*, 708 (1961).
9. Dittmer, J. C., and R. L. Lester, J. Lipid Res. *5*, 126 (1964).
10. Pries, C., A. Aumont and C. J. Böttcher, Biochim. Biophys. Acta *125*, 277 (1966).
11. Privett, O. S., M. L. Blank, D. W. Codding and E. C. Nickell, JAOCS *42*, 381 (1965).
12. Van Wijngaarden, D., Anal. Chem. *39*, 848 (1967).
13. Blank, M. L., J. A. Schmit and O. S. Privett, JAOCS *41*, 371 (1964).
14. Van Gent, C. M., Z. Anal. Chem. 1968, in press.
15. Tsen, C. C., I. Levi and I. Hlynka, Cereal Chem. *39*, 195 (1962).
16. Wootton, M., J. Sci. Food Agr. *17*, 297 (1966).
17. Daniels, N. W. R., J. W. Richmond, P. W. Russell Eggitt and J. B. M. Coppock, Chem. Ind. p. 955 (1967).
18. Daniels, N. W. R., J. W. Richmond, P. W. Russell Eggitt and J. B. M. Coppock, J. Sci. Food Agr. *17*, 20 (1966).
19. Baldwin, R. R., R. G. Johansen, W. J. Keough, S. T. Titcomb and R. H. Cotton, Cereal Sci. Today *8*, 273 (1963).
20. Ponte, J. G., S. T. Titcomb, J. Cerning and R. H. Cotton, Cereal Chem. *41*, 431 (1964).
21. Fisher, N., "Recent Advances in Food Science, Vol. 1, Butterworths, London, 1962.
22. Smith, D. E., and J. S. Andrews, Cereal Chem. *34*, 323 (1957).
23. Tsen, C. C., and I. Hlynka, Ibid. *39*, 209 (1962).
24. Dahle, H. K., and B. Sullivan, Ibid. *40*, 372 (1963).
25. Tsen, C. C., and I. Hlynka, Ibid. *40*, 399 (1963).
26. Bloksma, A. H., J. Sci. Food Agric. *15*, 83 (1964).
27. Coppock, J. B. M., N. W. R. Daniels and P. W. Russell Eggitt, JAOCS *42*, 652 (1965).

[Received April 19, 1968]