HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE SITOSTERYL-β-D-GLUCOSIDE/*sn*-1,2-DILINOLEOYL-3-GALACTOSYLGLYCEROL RATIO AS AN INDICATOR OF STORED WHEAT CONDITION

by

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ABBREVIATIONS

DGDG: digalactosyl diglyceride

DLGG: *sn*-1,2-dilinoleyol-3-galactosylglycerol

FFA: free fatty acid

GC:, gas chromatography

HPLC: high performance liquid chromatography

MGDG: monogalactosyl diglyceride

MS: mass spectroscopy

RI: refractive index

SSG: sitosteryl-β-D-glucoside

TG: triglycerides

TLC: thin layer chromatography

FIGURES

TABLES

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INTRODUCTION

Although lipids are only a small portion of most grain, they are a substantial factor of grain deterioration during storage so much so that, it is desirable to use lipids as an index of deteriorated grain. A great deal of research has been devoted to investigating nutritional values and stability in the storage of lipids of various kinds of wheat and wheat flours. At the same time, the functions of lipids in dough development and baking quality have been proved. When fresh grain and milled products are stored, their lipids are subject to hydrolytic, oxidative, and microbial deteriorations. Several tests have been used to determine the deterioration of stored grain. An increased fat acidity has been proposed as a convenient index of deterioration in stored grain or flour but is now considered to be unreliable (134). Loss of free glycolipids and phospholipids has been suggested as an alternative, but no simple and rapid tests have been available to determine them in stored grain (134).

Recently, the development of high performance liquid chromatography (HPLC) has presented a new method for separation and determination of glycolipids on a nonpolar bonded phase column (167). It gives improved resolution and selectivity. The mono- and digalactosyl diglycerides can be separated from each other. Also, the individual glycolipids with different fatty acid compositions are resolved. Changes in chromatogram of polar wheat lipid extract during storage under refrigerator, suggested that the inventory of lipid deterioration upon storage may serve as

an early warning.

Some problems arose when attempting to find a practical and accurate lipid class as an indicator for the quality of stored grain and to find the changes within lipid classes during storage by HPLC. First, it was necessary to develop a fast and complete extraction technique for lipids that would allow for maximum recoveries of all or most of the components of interest. Second, there was a need to develop a preparative fractionation system which would allow for the purification and quantitation of the components. The third problem was to develop the measurement of the most significant changes during storage in lipid classes which would relate to breadmaking.

For this research, initially the nonpolar lipid fraction was examined for evidence of change by: (A) optimization of the chromatography of nonpolar fractions of lipid extracts, and (B) incorporation of an extraction process which would be practical for a screening use. Effort was then directed to measuring the changes taking place in the glycolipid fraction extracted from whole and ground wheat stored at different temperature by HPLC as a good promising method.

A practical extraction and fractionation of lipids was needed to allow many analyses to be done daily. The free glycolipids from grain were analyzed by HPLC to seek a suitable indicator of condition upon storage. Qualitative and quantitative significance of the results were discussed. This analytical information not only served as an interesting indicator of past deteriorations, but also allowed prediction of the future storability of a given lot of grain. It also provided a means of routinely evaluating

stored wheat for lipids in association with baking quality characteristics.

LITERATURE REVIEW

Wheat and Flour Lipids

Earlier work on lipids in wheat was reviewed by Mecham (101) and Pomeranz (131), on cereal lipids from 1969 to 1976 by Morrison (107), and on the functionality of wheat flour lipids in breadmaking more recently by Morrison (106) and Chung et al. (24, 27). Chung and Pomeranz reviewed the recent finding on wheat and flour lipids primarily between 1976 and 1981 (31, 32).

Morrison et al. (110) reported 2.4% to 3.8% lipids in whole wheat. The free lipids (petroleum-ether extract) averaged 1.63% with a range of 1.2% to 2.2% (127). The extraction of lipids in wheat and wheat products depends, in addition to the type of solvent used, on the moisture content (7), on the type of extractor (25), and on the particle size of the material (20, 87, 178).

Free and Bound Lipids. "Free lipids" are present in a free state and can be extracted with nonpolar solvents such as ether or petroleum ether. The remaining lipids are tightly associated with wheat components and defined as "bound lipids". Polar solvent such as water saturated n-butanol or a mixture of chloroform-methanol-water are required (22, 26, 101, 127, 175).

Starch and Nonstarch Lipids. Lipids can be separated as nonstarch lipids which are outside the starch granules (1, 105, 108, 109). Water saturated n-butanol extracts the nonstarch lipids very rapidly at room temperature, and the starch lipids can then be extracted with water saturated n-butanol at 90 to 100^{o}C.

The lipids can be fractionated according to their elution from a silica gel column (10, 101, 126). The nonpolar lipids

are eluted first with chloroform followed by the polar lipids which are eluted with methanol (86, 147, 148). A further separation can be made in successive steps with chloroform, acetone, and methanol to successively yield nonpolar lipids, glycolipids, and phospholipids (10, 45, 53, 108, 122, 147, 152).

McKillican (93) found 12 classes of lipid (plus 6 unknowns) from wheat. Graveland (60) found 19 classes of lipid (plus 6 unknowns), MacMurray and Morrison (90) identified 23 classes of lipid and 3 unidentified. Lin et al. (87) proved that one of those unidentified was 6-O-acyl-monogalactosyl monoglyceride.

By counter current distribution between n-heptane and 95% methanol, Carter et al. (15-17) first found monogalactosyl diglycerides (MGDG) and digalactosyl diglycerides (DGDG) from wheat flour. The MGDG and DGDG compounds were obtained in roughly 3:7 ratio. Also, sitosteryl-β-D-glucoside (SSG) was isolated from flour lipids. It was mainly comprised of β-sitosterol with a glucose at the third position of the sterol molecule.

Total wheat flour lipids (1.4-2.0%) contain approximately equal amounts of nonpolar (50.9% of the total lipids) and polar (49.1%) components (90, 136). Triglycerides (TG) was a major component of nonpolar lipids. MGDG and DGDG were major components of glycolipids, and lysophosphatidyl cholines and phosphatidyl cholines were major components of phospholipids.

In the hard red winter and hard red spring wheat flour, DGDG content ranged from 10.7% to 17.6% of total lipids, and MGDG ranged from 3.7% to 5.4% (87, 90, 107). Lin et al. (87) found steryl glucoside was about 1.0% to 1.2%. However, free lipids were the major components of concern to the cereal

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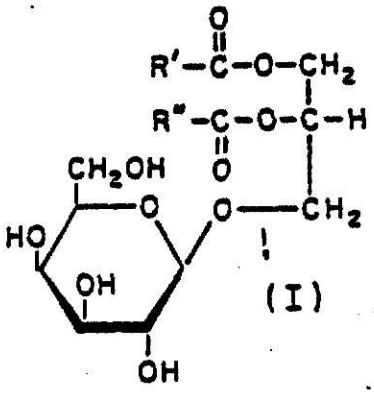
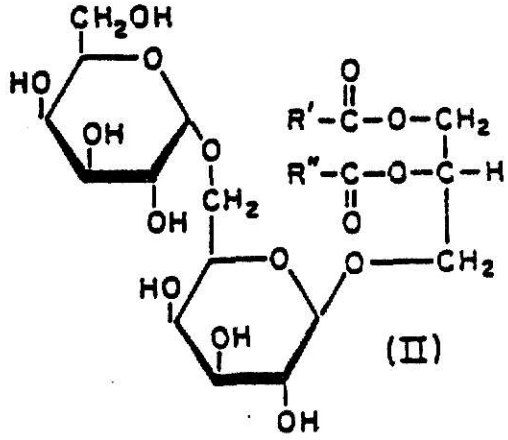
chemist who was exploring the effects of lipid quality and quantity on breadmaking (30, 31, 33, 42, 52, 88, 91). There are approximately 0.8% to 1.0% free lipid in the wheat flour. Almost 70% of the free lipids were nonpolar lipids. TG was a major component. Of the residual 30% of the free lipids, about two-thirds were glycolipids, containing MGDG and DGDG as the major components, and one-third was phospholipids, with phosphatidyl cholines as a major component (136).

Approximately 0.6% to 1.0% bound lipids could be extracted from flour with water saturated n-butanol after petroleum ether extraction. Bound lipids contained about 30% nonpolar lipids and 70% polar lipids (107, 136, 137).



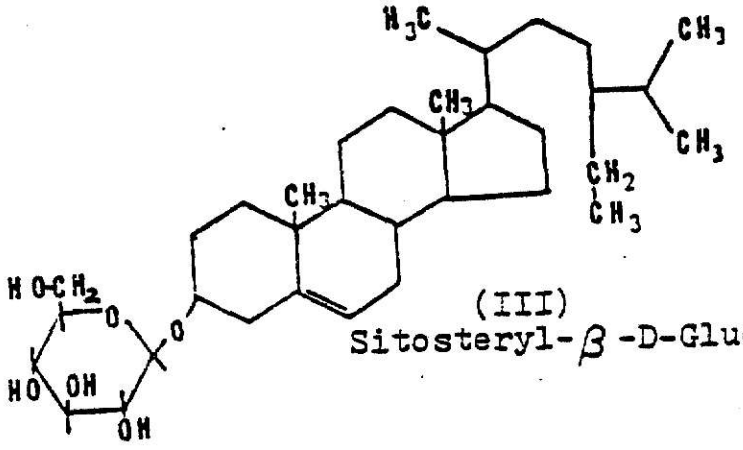
(I)

1,2-Diacyl-3-β-D-galactosyl-sn-glycerol
(Monogalactosyldiglyceride)



(II)

1,2-Diacyl-3-[6-α-D-galactosyl-β-D-galactosyl]-sn-glycerol
(Digalactosyldiglyceride)



(III)
Sitosteryl-β-D-Glucoside

Lipids Changes of Stored Wheat Flour

Several reviews on changes in lipids and breadmaking of stored wheat and flour have been reported (101, 128, 131-134). In stored milled wheat flour, lipid deterioration was faster than in whole kernel wheat. Whole wheat contained a fair amount of natural antioxidatns, and the fats in an unbroken kernel of grain were rather effectively protected against effects of oxygen in the air. The major lipid changes included hydrolysis and oxidation (125, 181). Hydrolysis took place much more rapidly in lipids than in the other wheat flour constituents, such as protein or carbohydrate during storage. Therefore, lipids seemed to be a proper choice as an indicator of deterioration in stored grains (134).

Starch lipids were exceptionally stable and showed no signs of hydrolysis or auto-oxidation under any reasonable storage condition. The exceptional stability of starch lipids was attributed to their being protected inside the starch granules (109).

Cuendet et al. (40) found changes in wheat lipids during storage at 37.8°C. Morrison (104) found the increase in individual free fatty acid (FFA) of several flours of 13% to 14% moisture during storage. Individual fatty acid was liberated at a steady rate, in proportions close to those of the total lipids. Freshly milled flour contained 6% to 12% FFA, and 12% to 16% after 10 weeks of storage at room temperature. About 30% were found in flours under nitrogen for 2-4 years. These values were obtained from gas chromatographic analyses.

Daftary et al. (43) studied changes in lipids of soft wheat and hard wheat stored at elevated moisture levels and high temperature. Mold count increased about two thousand times, accompan-

ied by a 40% decrease in total lipids content. Nonpolar lipids decreased about one-third, and polar lipids decreased about two-thirds. The breakdown of polar lipids was faster and more extensive than formation of FFA or disappearance of TG (41). Deterioration of wheat during storage was also accompanied by the complete breakdown of free lipids and a substantial decrease in bound lipids (41, 43, 123, 129).

The changes in twenty lipid classes of wheat flour at various temperature due to lipolysis on storage have been quantitated by Clayton and Morrison (34). Warwick et al. (173) found changes in individual lipid classes during prolonged storage of wheat flour at room temperature. All results showed the decreases in MGDG and DGDG content. Clayton and Morrison (34) found that steryl glucoside and ceramide monohexoside remained almost constant. Therefore, it can be concluded that the ratios of MGDG to steryl glucoside and ceramide monohexoside on storage were decreased.

Indicator of the Stored Wheat Condition

Several attempts have been made to develop a reliable and convenient index for measuring grain damage during storage. Most have been based on the types of changes occurring in stored grain, including the following: 1) visual observation, 2) increased fungal population, 3) weight loss, 4) decreases in germinability or viability, 5) heating, 6) production of toxins, 7) uric acid content, and 8) various biochemical changes including mustiness, souring, high fat acidity, bitterness, and lipid content (134).

Mold counts and viability tests were good indexes of incipient deterioration. The quantitation of mold infestation was quite

time-consuming and not accurate. A decrease in germinability was one of the first changes detected in grain storage, but the relation between germinability and breadmaking was casual rather than causative (134). Determination of either the heat or the carbon dioxide that were generated required continuous control. The heat did not dissipate rapidly because cereals were poor conductors of heat. Visual observation, mustiness, souring, and bitterness were empirical and failed to detect the early stages of deterioration which preceded the actual appearance of discoloration. A positive germination test might be used as evidence that seeding and malting potentialities had not been impaired by damage in storage (134).

In the past, the determination of uric acid as an index of insect contamination had sensitivity sufficient only to detect gross infestation. Recently, Wehling and Wetzel (176) developed a HPLC method with UV detector absorbed at 280 nm. Ion-pairing with tetrabutylammonium ion in methanol-water was used as mobile phase. The detection limit of 1.5 μg uric acid/g sample in grain and cereal products indicated the contamination of stored product insects.

A decrease in glutamic acid decarboxylase activity during storage accompanied a slight decrease in protein and also a slight increase in free amino acids (89). Rohrlich and Siebert (144) reported that glutamic acid decarboxylase activity would be a more useful index to measure soundness of grain to be germinated than to measure deterioration in stored grain for breadmaking. Bottomley et al. (12) found that the decreases in nonreducing sugar was accompanied by the increase in mold count. However,

accurate information could not be provided on the extent of damage to grain.

Deterioration in stored grain was accompanied by increased acidity, produced by FFA, acid phosphates, and amino acids. At early stages of deterioration, FFA increased at a much greater rate than either of the other two types (180). Consequently, it had been suggested that fat acidity be used as one of the best measures of grain damage. It was a simple and rapid procedure that could be accepted as routine estimations and quality control. However, it was unreliable and no correlation was found between baking quality and fat acidity content or composition (61).

Warwick et al. (173) reported the decrease in total fatty acid was principally due to a decrease in the polyenoic acids (linoleic acid). Tweeten and Wetzel (160) reported an HPLC method for analyzing both FFA and fatty acids derived from TG. They chromatographed p-bromophenyl esters of lipid extracts in a methanol-water mobile phase with a 10 μm, RP-18 column, an UV detector, and an internal standard. The lipids extracted from a number of grains and feedstuffs were converted readily to the derivative without isolation of the intermediate fatty acid salts or necessity of anhydrous conditions. Parallel analysis of the fat extracts by gas chromatography (GC) of the corresponding methyl esters showed that the HPLC analysis described was a comparable alternative.

Daftary and Pomeranz (41) indicated that polar wheat flour lipids decreased, and this decrease was a better index of damage during storage. The decreases in glycolipids and phospholipids measured actual damage to breadmaking postentialities (128).

However, no simple and accurate tests could determine the glycolipids and phospholipids in stored grain (134).

Pomeranz (134) concluded that none of the tests was a tell-all test, as each measured changes in a certain parameter that was of greater significance in some applications than in others.

Recently, Chung and Pomeranz (31) reported in their review paper that changes in individual lipid (especially major) classes during early (18 months) storage of flour with a low moisture at 12°C were more rapid than damage that could be recognized by the baking test. Still, loaf volume was linearly related to the amount of total lipids, nonpolar lipids, polar lipids, glycolipids plus phospholipids in the flour or to the amount of individual lipid classes in flours stored longer than 18 months. To predict baking quality of stored grain or flour, perhaps the ratio of some combined lipid classes such as the nonpolar lipids/polar lipids ratio of lipids extracted with a nonpolar solvent might be a better index than the amount of a single lipid class or the nonpolar lipids/polar lipids ratio of water saturated n-butanol extracts. Hydrolysis of MGDG and DGDG increased the levels of the intermediate products, monogalactosyl monoglyceride, and digalactosyl monoglyceride, which seemed to be a potential index of detecting storage deterioration of flour.

Lipids in Breadmaking

Although at early stages, the baking quality of deteriorated wheat flour could be restored by adding polar lipids or total lipids from undamaged flour (43, 129), at advanced stages this treatment was only partially effective because of damage to gluten protein and water-soluble compounds (44).

Several reviews have been written on the important effect of lipids in breadmaking (24, 27, 101, 131, 136). The presence of shortening (88, 91) and surfactants (23, 24, 28) were important contributors to the overall effect of lipids on breadmaking. The fractions rich in glycolipids increased volume of bread more than phospholipids (24, 27, 42, 88, 101, 177).

The lipids extracted with diethyl ether or petroleum ether had an improving effect on bread quality (52, 69). Recently, the free glycolipids extracted with petroleum ether had been shown to affect breadmaking, and the free DGDG was the best improver (27, 42, 88, 130). Because glycolipids contained both polar (carbohydrates) and nonpolar (long-chain fatty acids) moieties, it could interact with glutenin proteins by hydrophobic bonding and to gliadin proteins by hydrogen bonding. Hoseney et al. (70) indicated that the strength of gliadin-glycolipid-glutenin complex controls the loaf volume during breadmaking.

Chung and Pomeranz (31) correlated the lipid content in stored flour (data from Warwick et al. (173)) to loaf volume of breads baked by the long fermentation process test method from the same stored flour (data from Bell et al. (9)). Irrespective of fat in the formulation, loaf volume was linearly related to the amount of total lipids, of nonpolar lipids, and of polar lipids present in stored flour for different periods at 12°C. The relation of loaf volume to FFA in flour was the reverse of the relation of loaf volume to TG. Loaf volume generally decreased with a decrease in total glycolipids, esterified steryl glucosides, MGDG, and DGDG and with an increase in monogalactosyl monoglycerides in flour. The relationship of loaf volume to esterified MGDG and digalactosyl

monoglycerides were reversed. Loaf volume decreased linearly with a decrease in total phospholipids and individual classes of phospholipids, except for phsophatidyl cholines. Therefore, no single major lipid class (FFA, TG, DGDG, or phosphatidyl cholines) in flour could be used to monitor changes during storage and predict the breadmaking potential of stored flour.

Recently, Chung et al. (29) also found the linear correlation between DGDG of wheat and flour was significantly higher than the correlation between MGDG of wheat and flour. There were significant linear correlations between loaf volume and MGDG, DGDG, and the sum of MGDG plus DGDG for the wheats and flours, respectively. MGDG and DGDG were analyzed by HPLC using a 10 μm Spherisorb ODS column, methanol/water (90:10), and a highly sensitive refractive index (RI) detector with interferometric optics.

Biochemical Behavior of Lipids during Storage

The enzymatic degradation of galactosyl diglyceride was demonstrated first in runner bean leaves. The galactolipases hydrolyzed the ester bonds and galactosidases hydrolyzed the galactosidic bonds of glycolipids (76, 149). Oxidative deterioration of flour lipids was significant only at moisture levels below 8% (40, 63) and microbial deterioration at moisture levels above 15% (8, 43, 63, 132). On storing at the normal moisture level of 11% there was slow hydrolysis of lipids and a corresponding increase in FFA (8, 34, 40, 61, 104, 181).

Clayton and Morrison (34) reported that TG hydrolyzed to diglycerides, monoglycerides, and FFA. Neither diglycerides nor monoglycerides was accumulated: thus, there must have been further hydrolysis of diglycerides and monoglycerides. As an increase

in total FFA exceeded an increase in FFA from the simple glyceride, a portion of FFA must come from the hydrolysis of polar lipids. In the glycolipids there was some hydrolysis of MGDG to monogalactosyl monoglycerides and DGDG to digalactosyl monoglycerides (34).

The measured FFA was lower than calculated FFA because some lipolyzed FFA was resynthesized to partially acylated glyceride in milled flour (5, 34). Some complete hydrolysis to the water soluble products might have occurred to account for the small decrease in total lipids extracted from flour stored at high temperature (34).

Even though the stereo specific distribution of the fatty acids in the glycolipids was not determined, it was known that the 2-position fatty acids were much more unsaturated than those in the 1-position of MGDG and DGDG (5). Since the fatty acids in the monoacyl glycerides of glycolipids, monogalactosyl monoglycerides, and digalactosyl monoglycerides were almost identical to those in the corresponding diacyl glyceride and about half the monogalactosyl monoglycerides and digalactosyl monoglycerides in the stored flours were formed during storage, the galactosyl diglycerides must have been randomly hydrolyzed (34). Galactolipase which hydrolyzed galactosyl diglycerides to galactosyl glycerols were isolated from several plant tissues (64); however, the specificity of the initial hydrolysis to galactosyl monoglycerides has not been studied. Galactosyl diglycerides were specifically hydrolyzed at the 1-position by pancreatic lipases (65). Clayton and Morrison (34) indicated that wheat flour lipids hydrolyzed galactosyl diglycerides either randomly or did not hydrolyze

them at all.

In the control and deteriorated flour, diglycerides were present as *sn*-1,2-diacylglycerol. If TG were attacked by lipase which hydrolyzed 1- and 3- position fatty acids equally, and the diglycerides isomerised (since the 1- and 3-position fatty acids of the TG were very similar in the diglycerides would result in identical fatty acid composition in all positions (5). The fatty acid compositions of diglycerides in the stored flour suggested that the random hydrolysis of TG would give isomerised diglycerides with fatty acid compositions in all positions identical to TG and the resulting diglycerides in the stored flour would also have fatty acid compositions similar to those found. Random hydrolysis was, therefore, equally possible (34).

Separation of Lipid Classes

The extraction, fractionation, and quantitation of lipids has been reported (14, 53, 82). Direct separation of all lipid classes within any single chromatographic system has not yet been achieved (168, 182). For many years, the extracted lipids were fractionated prior to chromatographic analyses, so that the fractionated lipids had more homogeneous physico-chemical properties than the initial complicated mixture (66, 77). Available fractionation techniques were countercurrent distribution (3), dialysis (182), ion-exchange (146, 147), and adsorption chromatography (3, 146, 170).

Separation had most often been done on silica gel (silicic acid) open column, a molecule adsorption chromatography. It was a good technique for the preliminary separation of polar lipids

(146). At present, fractionation of lipids was carried out using the following eluents: hexane-ether or chloroform for neutral lipids, acetone (77) or its mixture with chloroform (170, 174) for glycolipids, methanol (77, 147), methanol-chloroform (146, 170, 174), or methanol-ether (113) for phospholipids. Using a chloroform-acetone mixture which was shown to cause no desorption of phospholipids from silica gel column, the recovery of phospholipids from the column was on the average 95% (139).

A batch adsorption of silica gel with solvent of increasing polarity had been used for fractionation of glycolipids (47). Also, the ascending dry-column chromatography was used to preparatively isolate glycolipids (169).

A directly unidimensional thin layer chromatography (TLC) was adequate for separation of nonpolar lipids, but a complex glycolipid or phospholipid mixture might require preliminary separation into groups by column chromatography or analysis by two-dimensional TLC (35, 46, 90). TLC was much cheaper and simpler than in classical liquid chromatography.

The combination of open column chromatography followed by TLC was used to determine lipids of wheat and flours (126, 127). The lipid composition of different stages of development of wheat was analyzed also (124). The changes in fatty acid and lipid class composition for developing soybean were reported (139).

The two-dimensional TLC was used to determine special glycolipids, MGDG, and DGDG in various source (49, 57). The MGDG extracted with water saturated n-butanol in four varieties of hard red spring and durum wheat ranged from 3.1% to 5.4% and DGDG from 9.0% to 17.3% (87). The technique of TLC had a better separation

and required much less time than in classical liquid chromatography. Also, a large number of samples could be done simultaneously. The individual lipid could be scraped off the TLC plates and quantitated based on fatty acid content or phosphorus content (87, 90, 105, 109).

However, quantitative analysis of TLC required uniform layers of adsorbent and a uniform coating of spraying reagent. Standards of various concentration had to be prepared for comparison. The automation and reproducibility were difficult. The capacity was lower than column chromatography. Under very optimum conditions the relative standard deviation was about 10% (11).

The TLC technique has been improved recently. A programmed multiple development TLC was developed for a better resolution in less time (119). A high performance TLC has been introduced which might displace HPLC from much of its present qualitative work (183).

Because the most time-consuming part in the classic purification procedure of lipid classes was column chromatography at atmospheric pressure, a prerequisite for developing HPLC equipment was the flexibility of the different parts, so that analytical, as well as preparative, columns could be used. A reusable preparative column was run for more than 60 times without loss of capacity and reproducibility has been reported (59).

Analysis of Lipid Composition and Structure

The chromatographic behavior displayed by lipids provided some information about their structure (66, 135). More detailed data could be obtained by examination of other physical and chemical properties of isolated lipids, including UV, IR, mass spec-

troscopy (MS), GC, and HPLC.

Most natural lipids showed no distinctive absorption bands in the UV above 220 nm. A homologous series of saturated fatty acids had a maximum absorption for wavelength in the range of 210 nm (62, 99). Mitchell et al. (103) first published a detailed method of heating lipids in potassium hydroxide to get conjugated isomers. IR had been used more extensively in the analysis of lipids than of any other food components (19, 55, 115). In addition to structure, it was useful in the identification of lipid compositions. IR spectroscopy had been used for identification of *cis* or *trans* compounds and saturated or unsaturated compounds (62, 115). Wehrli and Pomeranz (177) indicated that hydrogen bonds between glycolipids and gelatinized starch and between glycolipids and glutenin. The IR spectra of purified MGDG and DGDG had been studied (168, 169). Various sterol structures had been elucidated by IR spectra (83).

Mass spectroscopy recorded the mass/charge ratio and relative abundance of each ion collected. Several reviews on mass spectral analysis of lipids have been written (112, 145). The present composition of lipids depended upon the energy of the impinging electrons. Usually an energy level of 70 electron volts had been used for lipid analysis. Phospholipids had been analyzed by field desorption MS (112). The chemical compositions of underivatized polysaccharides from biological molecules had been elucidated by pyrolysis followed with field ionization MS (150).

The introduction of GC revolutionized the determination of fatty acid composition of complex lipid fractions. The major requirement was that the sample must be thermal stable and moder-

ately volatile. Lipids could be analyzed directly (81) or by preparation of fatty acid methyl ester (39). Margaric acid was used as an internal standard for analysis of wheat lipid fractions (107, 108). Alkaline hydrolysis followed by borofluoride methylation had been used extensively (6, 21, 102). Acid-hydrolyzed esterification had also been used (75). Most often the flame ionization detector was used for fatty acid determinations because of its extreme sensitivity and broad range. Recently, the MS has been used in series with the GC (111). GC/MS combined a good feature of lipid analysis.

The HPLC technique became gradually important in the quantitative analysis of lipids. The ubiquitous detector problem was largely solved with the advent of spectrophotometer detectors operating down to 190 nm, making possible the sensitive detection of almost any compound type (153). Increased use of fluorescence and electrochemical detectors, plus off-line and on-line derivatization, further pushed detection problems into the background, even for trace analysis of complex samples. Recent developments in microprocessor controlled instrumentation have also produced greatly improved equipment performance. Previously difficult or impossible separations of compounds could be solved by using the small particle, reverse-phase liquid chromatography with gradient elution and special methods such as ion-pair formation. Successful separations that could be obtained within a few tries were now common (153).

An HPLC consists essentially of a solvent delivery system, an injection system, a column, and a detector. Recently, the solvents considerations and properties of HPLC have been reviewed

(13, 140).

Sample injection onto the column might be carried out by two main methods: "on-column" or via a "valve." The former mode led to a greater resolution, but the disadvantages included leakage and needle blockage. Injection valves were more convenient, reproducible, and automatic. However, when sample was finally applied to the inlet bed of the column, some of the sample was still dragging along the wall of the sample loop (140). The valve method gave slightly less efficiency than on-column injection, but did not damage the inlet bed.

The most popular analytical columns were 4.0 to 4.6 mm internal diameter. However, diameters as small as 2.2 mm and as high as 5.0 mm were available (97). Open tubular capillary (30-50 μm. i.d.) columns (71), packed capillary (80-125 μm. i.d.) columns (114), and microbore (0.5-1.0 mm. i.d.) columns (141) have been reported. The smaller internal diameter columns used lower amounts of solvent to achieve the same separation time.

The female fitting was beginning to be favored by many commercial companies. It had the advantage of less damage to threads after numerous makings and breakings when compared to the popular male fitting (97). Cartridge columns without end fittings were developed by Brownlee labs (Santa Clara, CA). Such columns fitted into specially constructed holders which, by the application of only finger-tight pressure on an end external compression nut, could seal up 7000 psi (97).

Although columns packed with 5-10 μm particles had been considered to provide optimal resolution and speed, recently work has shown that further gains in efficiency could be realized by

using 3 μm particles (36). The simultaneous decreases in particle size and column length, which left resolution similar to that of commonly used reverse-phase columns, resulted in a significant decrease in analysis time and solvent.

The principle and theory of liquid chromatography has been reviewed (96, 153). There were four types of chromatography: liquid-solid, liquid-liquid, ion-exchange, and gel permeation.

For liquid-liquid chromatography in most HPLC columns, either polar or nonpolar, the stationary phase was chemically bonded to the solid support (78, 151). Normal partition chromatography was commonly applied to the separation of very polar organic molecules. Reverse phase partition chromatography was most commonly applied to the separation of nonpolar compounds. For a reverse phase partition chromatography, about 70% of available surface hydroxyl groups were used for hydrocarbon attachment. The longer the chain length of hydrocarbon, the longer the retention time, and the better the selectivity.

The reverse phase chromatography is by far the most popular mode of HPLC today for the following reasons: many nonionic, ionic, and ionizable compounds can be separated in the same sample, using a single column and mobile phase; the predominant mobile phase water is plentiful, the most frequently used organic methanol is pure and inexpensive; and the elution order is often predictable as the hydrophobic character of the solute (97).

The method of derivatizing the octadecyl silane and attaching it to the support surface affected the column efficiency. For octadecylsilane packings, the use of di- and trichloro- or ethoxy-octadecylsilanes for the preparation of these phases were being

replaced by monochloro- and monoethoxyoctadecylsilane reactants. In general, the polymeric and highly loaded phases were most useful for the separation of nonpolar species. The monomeric and lower loading (less than 10% carbon) phases were useful for the separation of polar species as well as for ion suppression and ion-pair application. However, for those polar compounds, a better peak symmetry was often observed on shorter chain phase (C 6 or C 8), possibly due to a better wetting of the surface by the mobile phase and resulting in better solute mass transfer (97).

Recently, multidimensional chromatographic techniques (e.g., LC coupled to LC) were developed (98). By selecting a small portion of unresolved components from a primary separation column and automatically directing it to a secondary column with different separation characteristics, most problems were often greatly simplified.

The detectors most used for lipid analysis were the UV detector, RI detector, IR detector, transport detector, and MS (2, 85, 153). Since most lipids had only minor UV absorbence above 210 nm, a variable wavelength detector, ranging down to 190 nm was necessary. Lipids could be determined by this detector without prior derivatization (48, 94, 153). It had a more nearly constant response with molecular weight change of compounds and could be operated at higher temperature (up to about 150^{o}C) with gradient elution (153). The transport detector and MS had good nonselective sample responses and gradient elution capabilities. However, the disadvantages were: sample destructive, sample loss, and a high degree of complexity in establishing optimum operating

parameters.

The RI detector has been called a universal detector, as it responds to all compounds that caused the RI of the column eluent to be different from that of the solvent (153). Commercially available RI detectors were: (1) Fresnel Refractometer--it measured the intensity of the light transmitted or reflected by liquid-glass interface; (2) Deflection Refractometer--it measured the deflection of a light path when a sample passed through the cell; and (3) Interferometric Refractometer--it was based on the shearing-interferometer principle for measurement. The light source beam was divided into two parts by a beam splitter; after passing through the sample and reference cells, the light beams were recombined by another beam splitter. The difference in optical path length was measured by the interferometer in fractions of wavelengths. Advantages of the detectors were linear response and higher sensitivity.

Application of the HPLC on Lipids Analysis

A number of articles have been reported on reviewing the application of HPLC to food analysis (94, 95, 163, 179). As was pointed out earlier, inadequate detection devices had imposed a limit on the use of HPLC in lipid analysis. However, the development of derivatives and new detection devices increased the applications of HPLC in lipids analysis.

Triglycerides had been chromatographed directly on a non-polar bonded phase column and detected with a RI detector (117, 120). The retention time of TG increased with decreasing unsaturation and increasing chain length. Critical pair and isomers of TG were separated with reverse phase chromatography (50).

Nonpolar lipids could be analyzed using an IR detector with an organic solvent which did not interfere (116). Fatty acids were analyzed on HPLC with nonpolar bonded phase columns with an RI detector (4, 67). Analysis of fatty acids by HPLC could be direct (67, 68). or derivatized (118, 160). The derivatization method was more sensitive and a fixed wavelength UV detector could be used. Comparative study of the detect limit of fatty acids with GC and HPLC has been done (160). Also, geometric and positional isomers of long chain monounsaturated fatty acids were analyzed by HPLC and glass capillary GC (156). The *trans* isomer values obtained by the HPLC method were somewhat higher (about 4%) than those obtained by the GC method. HPLC could separate hydroperoxide isomer and hydroxylated isomer of fatty acids (18, 51). It also separated epoxy glycerides of trivernolin (121).

Using HPLC, Tweeten (165) determined TG content and fatty acid compositions of the lipid extract from oilseed. Using the variable wavelength detector at 210 nm and reversed phase column, TG comprised of shorter chain fatty acids as well as fatty acids with higher unsaturation elute earlier than TG with longer chain, saturated fatty acids. Also, a LiChrosorb RP-8, 10 μm, column was used for the separation of total fatty acid composition. Fatty acids were derivatized with p-bromophenacyl bromide. Both TG and fatty acid derivatives can be separated.

A number of studies have been done on HPLC analysis of phospholipids. Twenty-nine components of soybean lipids were resolved on a silica gel column with a gradient elution and transport detector (130, 155). Phospholipids containing primary amino compounds could be converted to biphenyl carbonyl derivatives and

separated on a silica gel column with a 280 nm detector (37, 72). Phosphatidyl cholines and sphingomyelin were analyzed directly at 203 nm (73). A study of unsaturation of phospholipids was done with molar absorptivity (58). Synthetic phosphatidyl cholines and phosphatidyl ethanolamine were resolved on a polar bonded phase column ($-NH_2$) using a polar gradient solvent system with transport detector (80). Separation of oxidized from unoxidized phosphatidyl cholines species, on a reverse phase column with aqueous methanol mobile phase, was reported recently (38).

A lot of HPLC work was done with glycolipids recently. Perbenzyl derivatives of glycosphingolipids and galactosyl ceramides have been analyzed on a silica gel column with UV detector (92). Purification of DGDG from spinach has been done by preparative HPLC (59). Qualitative analyses of DGDG and MGDG has been done with reverse phase ODS column and the variable wavelength UV detector (142). Tweeten et al. (158) using nonpolar bonded phase HPLC partition systems analyzed previously preparative column chromatographic fractionated glycolipids from petroleum ether extracts of wheat flour lipids. Although DGDG peak II and *sn*-1, 2-dilinoleoyl-3-galactosylglycerol (DLGG) (MGDG peak I in their work) were not separated under the HPLC condition, it is a favorable alternative to TLC for analysis of such lipids. They (159) identified three major peaks as DGDG in the separation of polar lipid fractions of wheat flours with nonpolar (C 18) bonded phase column and methanol/water solvent system. TLC gave only a single DGDG spot from the same sample. The fatty acid compositions of the first DGDG subfraction consisted of both palmitic acid and linoleic acid while the third had palmitic acid, oleic acid, and

linoleic acid present on the glycolipids. Using MS, Chromatographic analysis, and wet chemical methods, they (162) elucidated the chemical composition of fractions collected from the chromatographic separation. It required no sample preparation beyond solvent extraction and fractionation. The detection limits of the RI detector with interferometric optics compared well to the variable wavelength UV detector at 200 nm. Approximately 35% of each DGDG subfraction was accounted for as carbohydrate. They (167) found the separation of glycolipids on a reverse phase column had better resolution and selectivity than on silica gel column. Not only MGDG and DGDG could be separated, but also a different fatty acid composition of MGDG or DGDG could be resolved. Using the RI detector or the UV detector at wavelength of 206 nm, a routine quantitative analyses of MGDG and DGDG was developed. The detection limit of the RI detector with interferometric optics was 0.25 μg for both MGDG and DGDG. The first fraction of DGDG or MGDG separated by HPLC contained linoleic acid on both SN-1 and SN-2 positions whereas, in the second fraction, palmitic and oleic acids were preferentially located on the SN-1 position and linoleic acid was on the SN-2 position. A comparison of IR and variable wavelength detectors for the determination of MGDG and DGDG in wheat flour have been developed by Tweeten et al. (161). Using gradient elution, DGDG peak II were further separated to two compounds: SN-1: palmitic, SN-2: linoleic, SN-3: digalactose; and SN-1: oleic, SN-2: linoleic, SN-3; digalactose. They (164) also examined the effects of mobile phase strength, column packing stationary phase, packing particle size, and column temperature on both the efficiency and selectivity of the

glycolipids separation. DGDG peak I and II, DLGG and MGDG peak II were separated from each other with LiChrosorb RP-8 or Spherisorb C 6 column under isocratic or gradient elution. Recently, they (166) developed an automated multidimensional HPLC method to determine the MGDG and DGDG in free lipid extracts from wheat and flour. A short column (Spherisorb C 8, 5 μm, 4.6 mm i.d. x 3 cm), a 6-port switching valve for solvent stream, and an analytical column (Hewlett-Packard RP-8, 5 μm, 4.6 mm i.d. x 20 cm) were used for simultaneous fractionation and analysis of MGDG and DGDG. Both DGDG peak I and II, DLGG, and MGDG peak II in total lipids are completely resolved from each other in the same chromatogram. Also, the absolute and relative amounts of MGDG and DGDG were calibrated.

Recently, application of HPLC to lipid analysis included jojoba liquid wax esters (154), fatty acids (37), lecithin in egg (138), sterols (79), glycerides, nonionic emulsifier (143), malonaldehyde in rancid fat (74), and oxygenated cholesterols and related compounds (157).

MATERIALS AND METHODS

Sample Source

Samples of 8 varieties of sorghum, 2 varieties of oat, 10 varieties of 1977 hard red winter wheat, and 1979, 1980, 1981 hard red winter wheat were taken from the Grain Science Department, Kansas State University, Manhattan, Kansas.

Grain and flour were stored in polyethylene bags. Control samples in polyethylene bags were sealed in tin containers under an atmosphere of nitrogen at -18°C. All samples were stored at ambient temperature and 37°C for various time. Grain was ground on a Wiley experimental mill (1 mm round hold sieve). The moisture content was determined before each lipid analysis by AACC approved methods.

Chemicals and Solvents

Organic solvents and chemicals were reagent grades. Water used for the mobile phase in the liquid chromatography was distilled water redistilled with alkaline potassium permanganate. Anhydrous methanol used for HPLC was distilled over Grignard reagent described by Vogel (171). Acetonitrile was distilled (172). Other solvents for HPLC were HPLC grades (Fisher Scientific Co., Pittsburgh, PA). Ratios of all solvent systems were stated by volume. Reference fatty acid methyl ester and TG for GC and HPLC, respectively were purchased from Applied Science Laboratories, Inc., State College, PA, and were stored at -18°C. Approximate dilutions, when needed, were made at the time of use. Samples were stored in 5 ml reactor vials with polytetrafluroethy-

lene caps to minimize concentration changes. The reference lipids were:

Triglycerides:	Tripalmitin
	Trimargarin
	Tristearin
	Triolein
	Trilinolein
	Trilinolenin
Fatty Acid	
Methyl esters:	Pamitic Acid Methyl Ester
	Margaric Acid Methyl Ester
	Stearic Acid Methyl Ester
	Oleic Acid Methyl Ester
	Linoleic Acid Methyl Ester
	Linolenic Acid Methyl Ester
Glycolipids:	Digalactosyl Diglycerides (plant)
	Monogalactosyl Diglycerides (plant)
	Steryl Glucosides (plant)
Phospholipids:	Phosphatidyl Ethanolamine (plant)
	Lysophosphatidyl Ethanolamine (bovine)
	Phosphatidyl Choline (plant)
	Lysophosphatidyl Choline (plant)
	Phosphatidyl Serine (bovine)

SSG, DLGG, and DGDG Standards

SSG, DLGG, and DGDG standards were fractionated from a wheat flour lipid extract. The method was essentially that described by Tweeten et al. (167) in previous work. Lipids were extracted

from hard red winter wheat flour with Skellysolve F on a large Soxhlet extractor (500 ml thimble) and fractionated by silica gel open column chromatography. After nonpolar lipids were eluted with chloroform, MGDG crude fraction was eluted with chloroform/acetone (1:1), and DGDG crude fraction was eluted with acetone. A spot test was run by TLC to verify that the individual components were eluted completely.

The MGDG and DGDG fractions were subfractionated by semi-preparative HPLC using methanol/water (90:10) on a μ-Bondapak RP-18, 10 μM, column. Two subfractions were collected from the MGDG fraction specifically for SSG and DLGG, and one subfraction was collected for DGDG, from the DGDG fraction, respectively. The ratio of eluting solvent methanol/water changed due to evaporation of methanol at 30°C. A high temperature was avoided to prevent ester bond hydrolysis of the glycolipids as recommended by Tweeten. The glycolipids were extracted from the concentrated subfractions with chloroform, and the aqueous layer was discarded. After chloroform layer was dried over anhydrous sodium sulfate, it was decanted and its solvent was removed on a rotary evaporator. The glycolipids residue was dissolved in a small quantity of chloroform/methanol (1:1) and transferred to a tared vial for solvent removal. Vials were taken to constant weights before and after the lipid was added. Agreement of consecutive weighings was achieved before the final weight was recorded for each lipid and vial. Fractioning standard SSG, DLGG, and DGDG was replicated.

Packing Materials for Preparative Column

The particle size distribution of packing materials was shown

on Table I. Silica gel 100 mesh and Syloid 72 were used disposably. LiChrosorb packing materials were tried reusably.

High Performance Liquid Chromatography

Equipment. The HPLC system consisted of a reservoir, a high pressure pump, an injector, a column, a detector, and a recorder.

Pump. The Waters pump (Model M6000, Waters Associates, Inc., Milford, MA) was a two piston reciprocating with flow control and pulse damper system. It had a satisfactory peak/noise ratio for practical use.

Injector. The lipid concentrations were adjusted to allow 50 to 100 µg in one injection through the valve injection or stop flow injection system. A sample valve of Rheodyne (Model 7010, six ports hand-turned, Rheodyne, Berkeley, CA) was used. A sample volume of minimum 10 µl and maximum pressure 7000 psi could be operated. A model 7011 loop filler port permitted the loading of the valve sample loop by means of conventional microliter syringes so that the sample wastage was minimized. The loop injector was more convenient, accurate, and could be readily automated by pneumatic activation.

The stop flow injector system consisted of a home-made stop flow septumless injector and a low pressure syringe (10 or 25 µl, Hamilton Company, Reno, NV) had been previously described by Tweeten (162). The solvent flow was controlled by two high pressure valves, one for stop flow to the injector and the reference cell; the second to control the flow only to the detector reference cell.

The reproducibility of the sample injection with a syringe

Table I. Particle Size Distribution of Packing Materials of Preparative Column Chromatography

Packing Material	Source	Particle Size Distribution (μm) 90%	50%	10%
Silica gel[a]	Mallinckrodt (Paris, KY)	118	<u>33.7</u>	5.68
Syloid 72[a]	Davison Chemical (Baltimore, MD)	10.6	<u>5.93</u>	2.93
LiChrosorb SI-100 10 μm	EM Laboratories Inc. (Elmsford, NY)	13	<u>10</u>	8
LiChrosorb SI-60 20 μm	EM Laboratories Inc. (Elmsford, NY)	26	<u>21</u>	16
LiChrosorb SI-60 30 μm	EM Laboratories Inc. (Elmsford, NY)	38	<u>30</u>	23

[a]Particle size distributon was determined with Microtrac Histogram Particle Analyzer (Leeds and Northrup, North Wales, PA). The median diameter was underlined.

in the stop flow injector was rarely better than 2%. A stop flow injection could be used at pressure above 1500 psi. The efficiency was not affected since a diffusion in liquids was very slow.

Column. All the analytical columns (4.1 mm i.d. x 15 cm or 25 cm, LiChroma 316 stainless steel, Handy and Harman Tube Company, Norristown, PA) were milled out in the exit to accept a 2 μm stainless steel frit (0.1875 in o.d. x 0.125 in thick, Mott Metallurgical Corp., Farmington, CT). A thin porous film of 5 μm teflon filter (Fluoro-Plastics, Inc., Flexrock Co., G and Venango Sts. PA) was put onto the columns to protect the packing material from contamination of large particles. A 0.5 μm stainless steel removable frit (0.250 in o.d., Alltech Associates, Deerfield, IL) was sandwiched onto the top of the column for back-flush technique. This could prevent the packing material from loss when the mobile phase was reversed. Both ends of the column had reducing unions from 1/4 in to 1/16 in (Swegelock, 316, stainless steel). The reducing unions were milled to give a zero dead volume for the columns.

Column packing materials were obtained from different supplies:

Partisil-10, ODS - Whatman, Inc., Clifton, NJ.

LiChrosorb RP-18, 10 μm - Applied Science Laboratories State College, PA.

LiChrosorb SI-60, 10 μm - EM Laboratories Inc, Elmsford, NY.

LiChrosorb SI-100, 10 μm - EM Laboratories Inc, Elmsford, NY.

Spherisorb ODS, 10 μm - Regis Chemical Co., Morton Grove, IL.

Spherisorb RP-6, 5 μm - Regis Chemical Co., Morton Grove, IL.

μ-Bondapak RP-18, 10 μm - Waters Associates Inc., Milford, MA.

Detector. The differential RI detector, used for comparison of lipid quantitative response, was Multiref 912 with a 10 mm path, 15 μl cell (Optilab AB BO Philip Instrumentation, AB, Vallingby, Sweden; US sales: Lachat Chemicals, Inc., Mequon, WI). The attenuation range was from 2×10^{-7} to 10^{-5} RI units full scale. The Multiref 912 utilized an interferometer for measurement of the RI difference. It provided a high primary optical signal that made extreme electronic amplification unnecessary.

A beam of light (wavelength=546 nm) from the light source was split into two beams of individual intensity by a birefringent beam splitter. After passage through the cells the beams were brought together and a second beam splitter corrected the wave fronts to get the same direction. Thus, it was possible for the two beams to constructively interfere. For many applications it was enough to run the cell at room temperature without thermostating. If extreme stability was needed, the interferometer and the cell could be thermostated.

Recorders. A multirange Omniscribe Strip Chart Recorder (Model 5211, Houston Instrument, Austin, TX) equipped with a preamplifier for a 1 mV full scale response was used for recording detector response. An electronic integrator was used with little success, due to baseline drift, air bubble cause, and inaccurate integration.

Extraction of Lipid from Plant Material

Lipids were extracted by three distinctly different types of processes:

Fosslet extractor. A Fosslet extractor consisted of a hammer with a hole in its center, a cup with a rod welded in its center, and a cap with a rubber o-ring. All were made of stainless steel. The hammer could slide through the rod. When it was operated, the whole extractor moved up and down mechanically. The hammer struck the grain in the cup. It had a 60 Hz vibration frequency, made by A/SN Foss Electric, Denmark. Lipids were extracted from 20 g of ground grain sample with 20 ml of 1,1,1-trichloroethane for 7.5 min, centrifuged and filtered twice, then dried with a rotary evaporator under 40°C. The lipids were dissolved in Skellysolve F to 100 ml, and total lipids was determined by drying two 5 ml aliquots to constant weight. The remaining 90 ml were concentrated under vacuum to dryness, and redissolved in chloroform for further analyses.

Soxhlet extractor. Lipids were extracted exhaustively on a Soxhlet extractor from 100 g (dry basis) of ground grains with 500 ml of Skellysolve F for 16 hrs at a solvent condensation rate of 6 min/cycle. The Skellysolve F extract (free lipids) was filtered and evaporated under reduced pressure. Total lipid content was determined as described in the Fosslet method, and the remaining lipids were used for further analyses.

Stirred extraction. Lipids were extracted from 15 g of ground grains in an Erlenmeyer flask with a magnet stirrer. 100, 50, and 50 ml of 1,1,1-trichloroethane were added, stirred for 20, 10, and 10 min, respectively. The extracts were filtered, combined, and evaporated almost to dryness under vacuum in a glass apparatus under 40°C. Total lipids were determined as above, and the remaining lipids were used for HPLC and other analyses.

Preparative Column Chromatography

Silica gel open column. A 1000 ml Erlenmeyer flask was filled to the 500-ml mark with silica gel (100 mesh, labeled "suitable for chromatographic analysis by the method of Ramsey and Patterson," Mallinckrodt, Paris, KY). A thick slurry was made with deionized water. The slurry was filtered on a large Büchner funnel. Silica gel was dried in an oven at 140°C for 2 hrs, and then cooled to room temperature. For one column, about 20 g of dried water-rinsed silica gel was weighed in a 100 ml beaker. Silica gel was washed with 60 ml of chloroform-methanol (7 to 1) twice and 60 ml of chloroform-methanol (15 to 1) once, and finally washed with 80 ml of chloroform. Each washing was filtered on a Büchner funnel. The silica gel slurry in chloroform was transferred to a column (18 cm in length and 2.2 cm in diameter): up to 400 mg of lipids could be separated.

Preparative high performance lipid chromatography. The eluent delivered by a Milton Roy pump (Beckman Instruments, Inc.) equipped with a sapphire piston operating at a pressure of 600 psi. The piston was lubricated with a drop of glycerine once a week. A 3-4 ml per minute flow rate was used. Alternatively solvent from a nitrogen pressurized (1000 psi) reservoir was used in place of a solvent pump.

A sample (up to 1.2 ml) was injected into a stainless steel column with a stop flow septumless injector. The preparative high pressure column was a stainless steel column (8 mm i.d. x 30 cm) equipped with a 5 μm frit and was dry-packed with silica gel. A spot test was run by TLC to find that the individual components were eluted before the next solvent system was introduced.

Fractionation of Lipid Extracts

Nonpolar and Polar Lipids. 200 mg of lipids were dissolved in chloroform and applied to the preparative column chromatography, and eluted as two fractions: nonpolar lipids with 120-160 ml chloroform, and polar lipids with 100-120 ml methanol. The spot test was run by TLC before the next solvent system was introduced.

Nonpolar, Glyco- and Phospholipids. 200 mg of lipids were fractionated by 120-160 ml chloroform, 100-120 ml acetone, and 100-120 ml methanol, into nonpolar, glyco-, and phospholipids, respectively.

Nonpolar, MGDG, DGDG, and Phospholipids. Lipids were fractionated into nonpolar, MGDG, DGDG, and phospholipids with 120-160 ml chloroform, 100 ml chloroform/acteone (1:1), 100 ml acetone, and 100 ml methanol, respectively.

These fractions were dried with a rotary evaporator at below 40^{o}C, and then dissolved in chloroform and stored in a freezer at -18^{o}C until analyses.

HPLC Column Packing Techniques and Apparatus

Prior to packing, the columns were cleaned with 6N HCl, rinsed with distilled water, dried, and fitted with 1/4 in. Swegelok fittings at the end. The columns were packed by a modified balanced density slurry technique as previously described by Mauro (100). A pneumatic amplifier pump (Model DSTV-122/CP4, Haskel Engineering Products, Div., Burbank, CA) was used. The pump, with precision air controls and manual override valve, was equipped with a two way valve for packing solvent flow direction switching, a ball valve and slurry packing chamber. The amplification ratio was 122:1. This was similar to a simple gas displace-

ment system. However, pressure amplification was obtained by using a large-area gas-driven piston to actuate a small-area liquid piston. Thus, a relatively low inlet gas pressure could be used to create high liquid outlet pressure without directly exposing the solvent to compress gas. The outlet pressure was directly proportional to the ratio of the piston area and to inlet gas pressure.

Slurry Packing Procedure. This procedure was described in part previously by Mauro.

1. Prepare UV-absorption free hexane.

2. Using the apparatus provided, weigh about 1.7 and 2.7 g of packing materials for 4.1 mm x 15 cm and 4.1 mm x 25 cm columns, respectively.

3. Pour the packing material into a clean, dry screw cap bottle (50 ml) with plastic inner cap liners.

4. Packing solvent: mix with 3 parts of tetrachloroethane and 1 part 1,4-dioxane, run through an activated Florisil column (60/100 mesh, Floridin Co., Warren, PA).

5. Dispense packing material in 10 ml of packing solvent. Shake bottle ten times and place in an ultrasonic bath for approximately 60 sec.

6. Remove from ultrasonic bath and wipe traces of water off the bottle.

7. Pour suspension into packing chamber, with stainless steel empty column attached. End frit must be installed.

8. Top off suspension in loading tube to 10 ml of excess packing solvent.

9. Turn on Haskel high pressure pump loaded with degassed

hexane. Extract the slurry from the slurry reservoir into column at a pressure of 8500 psi.

10. Next, collect 100 column volumes of hexane, change the pump pressure to 3000 psi for 5 min, then turn pump off and remove column.

11. Place newly packed column in liquid chromatographic system, condition the column with 20 to 25 column volumes of each solvent of increasing polarity (hexane, chloroform, and methanol).

Column Quality Control and Techniques. The quality of the column was checked with a 20 µl test solution, phenol/2,6 xylanol (1:1). The solution was injected and eluted with methanol/water (60:40) at 1.0 ml/min flow rate. The number of theoretical plates per meter and the capacity factor k' was calculated. Only those columns with ten thousand or more theoretical plates per meter were used. Each used column should be checked every month.

The metal frits and teflon frits of columns required changing when the frits were clogged and the back pressure was too high to work. A periodic cleaning of columns was required for lipid analyses. The columns were washed with 20-25 column volumes each of nitric acid (pH=3), methanol, chloroform, hexane, chloroform, methanol, and finally, the eluting solvent.

When complex lipid classes were run through the HPLC system a backflush technique was used to get quantitative recovery and purge of strongly retained sample components from the column as previously described by Tweeten et al. (167). In some cases, the total quantity of strongly retained materials may be of interest, whereas in other cases it was desired only to clean out the column prior to injecting the next sample.

A six-port hand-turned valve (Model 7010, Rheodyne, Berkeley, CA) was used. The less retained lipid peaks were eluted forward. The remaining more retained compounds were eluted as one large broadened peak as the flow direction was reversed.

Quantitation

Peak areas were determined using the product of the peak height times the width at one-half the peak height. This was a reasonable approach with peaks that closely approximate a Gaussian distribution. Narrow peaks on the recorder trace were measured from the inside edge of one side to the outside edge of the other: it would avoid precision errors due to the recorder pen width. The response factor would be obtained with the total peak area divided by total sample weight.

Fat Acidity

Fat acidity was determined by the procedure of AACC approved methods, revised and reprinted, October, 1969. 02-01. Fat acidity-General Method. Lipids were extracted with Skellysolve F from whole wheat flour.

Gas Chromatography

A gas chromatograph (Model 5750, Hewlett Packard, Avondale, PA) equipped with a hydrogen flame ionization detector was used. Derivatives were prepared by placing a 5-ml aliquot of lipid extract (-500 mg) in a 50 ml round bottom flask and evaporating to dryness under a stream of nitrogen. 4 ml of 0.5 N methanolic NaOH was added. The contents were heated over a steam bath for about 5 min. After 5 ml of BF_3-methanol (14% w/v, Applied

Science) was added, the mixture was heated for another 2 min. 5 ml heptane was added through the condenser and the mixture was heated 1 min longer, cooled, and removed from the condenser. Saturated aqueous NaCl was added to the heptane solution. A mixture was transferred to a 250 ml separatory funnel. The methyl esters were extracted with 2-50 ml portions of redistilled petroleum ether. The petroleum ether fraction was washed with 20 ml portions of distilled water until the wash was acid-free. The product mixture was dried over anhydrous sodium sulfate, filtered and evaporated under a stream of nitrogen gas. The fatty acid methyl esters were dissolved in heptane and analyzed on the gas chromatograph. Duplicate analyses were run on each sample.

The GC column (stainless steel 1/8 in i.d. x 6 feet) was packed with 10% SP-2330 on 100/120 mesh Chromosorb W AW (Supelco, Inc.). The operating temperatures were set: injector at 240^{o}C, column at 200^{o}C, and detector at 240^{o}C. The nitrogen carrier gas flow rate was 25 ml/min. Peak areas were determined by the peak width at half height method. Peaks were tentatively identified by comparing the relative retention times with those from the reference standard mixtures run on the same column under the same conditions.

Mass Spectrometry

Peak 3 of MGDG fraction was analyzed by MS. The MS analysis was done by direct inlet on a Finnigan Model 4021C equipped with a temperature programmed sample probe and data handling system. The instrument was operated at 70 eV for electron impact ionization with an emission current of 1 mA. Chemical ionization spectra

were recorded using CH_4 as the reagent gas.

Interpretation of Chromatographic Data

The chromatographic results were calculated in several different ways. Quantitative data was based on at least duplicated extractions, fractionations, and injections of the sample. A mean, standard deviation, and a relative standard deviation were found. A linear regression was done to define the best linear relationship between related sets of data. A correlation coefficient was calculated to establish the strength of the linear regression.

RESULTS AND DISCUSSIONS

I. Search of an Indicator for the Stored Grain Quality by HPLC

In the course of an earlier wheat lipid project in the analytical laboratory of Kansas State University, unpublished data of Tweeten showed compositional changes in the high performance liquid chromatograms of polar lipid fractions at storage intervals even when refrigerated under nitrogen. In the work Tweeten et al. (167) used an RP-18 column with methanol/water (90:10) for separating previously fractionated wheat lipids. This observation led to the work reported under the heading:

A. Nonpolar Lipid Fractions of Grains

B. Effect of Stored Temperature on the Extracted Lipids

C. HPLC Analyses of Polar Lipids.

Analyses of the polar fractions (C) pertain directly to the title of the thesis, however, the results and discussions include the details of the preliminary investigation with nonpolar fractions and follow the chronology of the overall study. Changes in the nonpolar lipid fractions were looked for because (1) it had not been done before and it was desirable to observe nonpolar lipid changes if any, and (2) nonpolar lipids had been chromatographed before. Also, (3) since not all seeds had much glycolipids etc., the nonpolar lipids fraction methods would be less limited. Sorghum had been chosen first for nonpolar lipids study because of its high content (about 90%).

A. Nonpolar Lipid Fractions of Grains

In order to get the optimum condition for nonpolar lipid fraction analyses by HPLC, various factors were studied: column

length (15 cm vs 25 cm), packing materials (LiChrosorb RP-18, 10 μm, Spherisorb ODS, 10 μm, Partisil-10, ODS, LiChrosorb SI-60, 10 μm, LiChrosorb SI-100, 10 μm, μ-Bondapak RP-18, 10 μm, and Spherisorb C 6, 5 μm), mobile phase (acetonitrile/water system ranging from 50:50 to 100:0), and flow rate (0.5 ml/min to 1.5 ml/min). The best results for the nonpolar lipid fractions were obtained with Spherisorb C 6, 5 μm, column, 4.1 mm i.d. x 15 cm, mobile phase, pure acetonitrile, and flow rate, 0.5 ml/min.

a. Preliminary study of nonpolar lipid

1) Comparison of unfractionated and silica gel open column fractionated nonpolar lipids. Sorghum lipids were extracted with 1,1,1-trichloroethane by a Fosslet extractor: one lipid portion was directly injected into HPLC systems as "unfractionated" and the other portion was fractionated by a silica gel open column before HPLC. Nonpolar lipids were eluted with chloroform as "fractionated". From the two chromatograms (Fig. 1A and B), no difference was found as expected, because nonpolar lipids content in sorghum lipid was more than 90%. In a reverse phase column with acetonitrile as mobile phase, only nonpolar lipids were run through the column. The polar lipids fraction was retained in the column. It could be eluted with backflush technique.

2) Comparison of Folch purification and without Folch purification. The lipids purification by Folch (54) was time consuming in the routine analysis. Nonpolar lipids in whole sorghum lipid extracts with Folch purification and without purification were chromatographed for comparison (Fig. 2A and B). No significant difference had been found, which suggested that the elimination of Folch purification was possible for nonpolar lipids. With

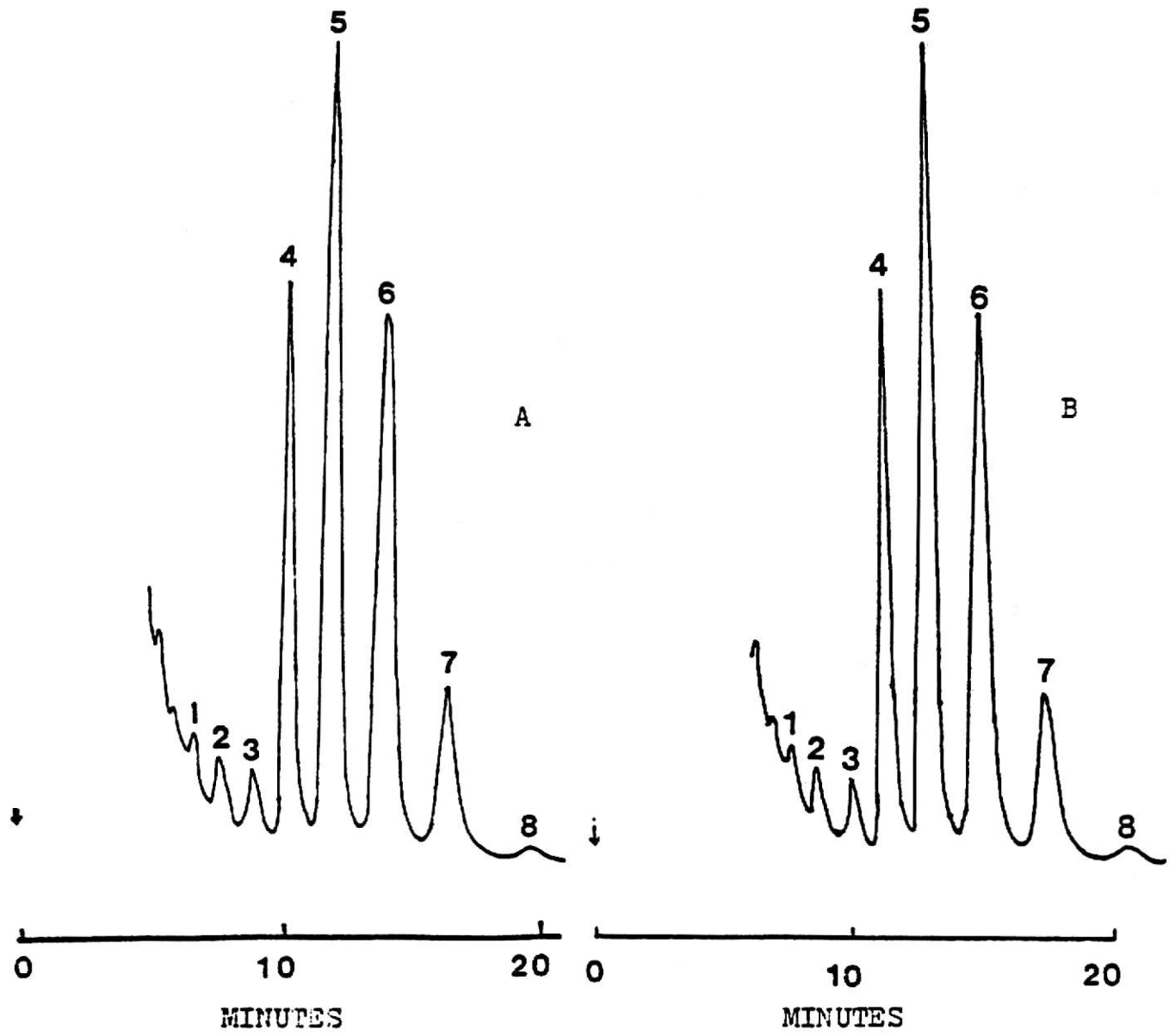


Fig. 1. HPLC separation of sorghum lipid extracted with 1,1,1-trichloroethane by a Fosslet extractor: (A) unfractionation; (B) fractionation with silica gel open column. HPLC conditions: Spherisorb C 6, 5 μm (4.1 mm i.d. x 15 cm), pure acetonitrile, 0.5 ml/min, RI detector.

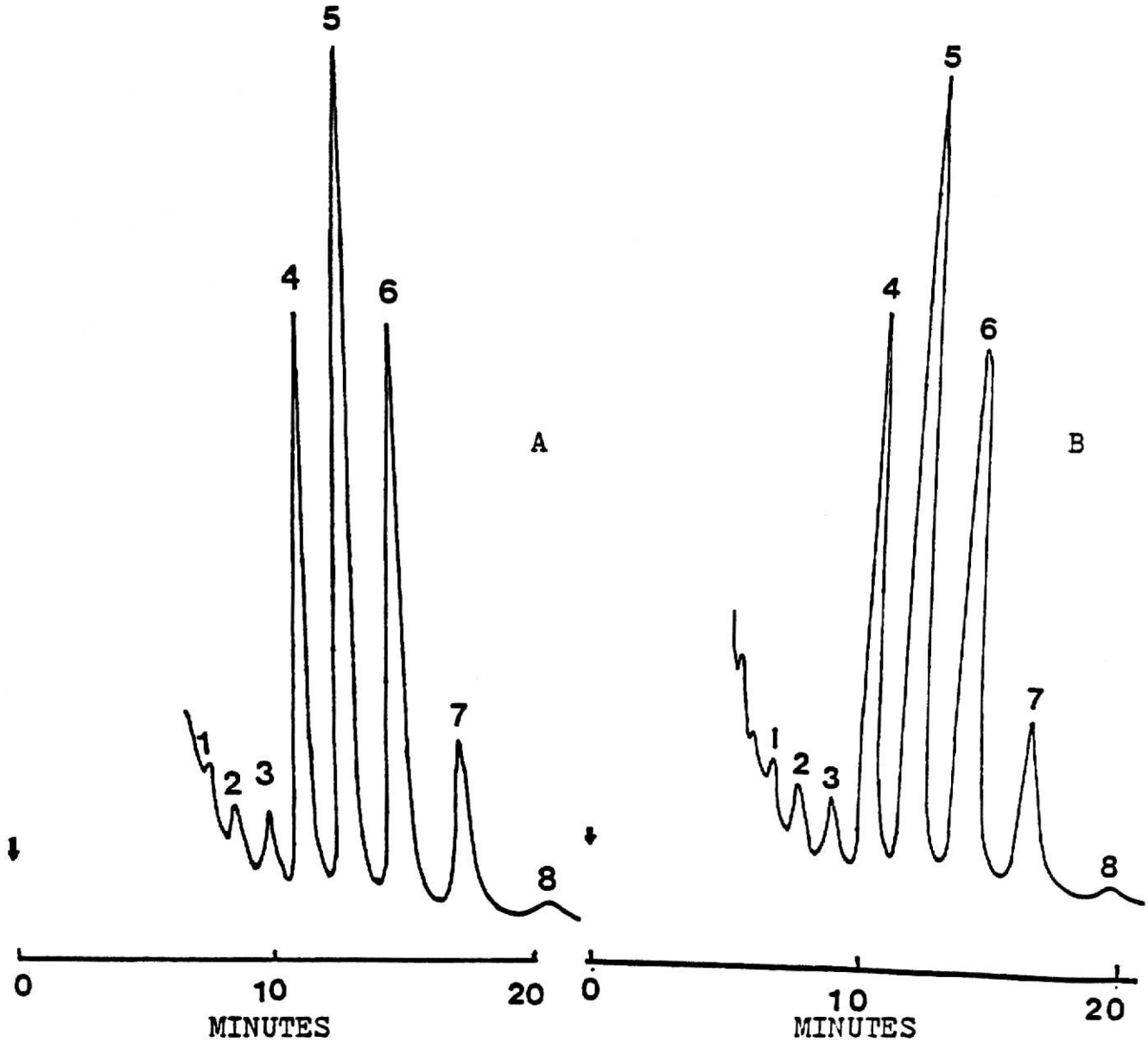


Fig. 2. HPLC separation of sorghum lipid extracted with 1,1,1-trichloroethane with a Fosslet extractor: (A) Folch purification; (B) without Folch purification. HPLC conditions: Spherisorb C 6, 5 µm (4.1 mm i.d. x 15 cm), pure acetonitrile, 0.5 ml/min, RI detector.

the backflush technique, the strongly retaining polar lipids were eluted off, which was supposed to make a difference between these two lipids. In addition to the extremely good resolution, only eight peaks existed in the chromatogram. Forty min were required for analytical separation and backflush. This suggested that HPLC of nonpolar lipids might be a convenient and possible indicator of stored grain.

3) Sample dissolution and concentration. For differential refractometers which measured the refractive index difference of sample and mobile phases, a solvent which could dissolve all of the lipid samples and partially dissolve in the mobile phase was necessary. Pure methanol was not a good solvent for lipids because a lot of white precipitates could be found in the solution. Solvents of different ratios of chloroform to methanol (from 1:1 to 15:1) had been tried: the white precipitates still existed. Pure chloroform could dissolve all lipid sample and the solution was yellow and clear without any precipitates. Comparing the chromatograms of chloroform dissolved and chloroform-methanol dissolved samples showed no apparent difference. This suggested that chloroform solvent could be used.

Sample size could affect the chromatogram by differential RI detector, especially in the Multiref 912 interferometric RI detector. When lipids were dissolved in a solvent that widely differed from the mobile phase,. a large solvent peak could be detected. An injection which contained 50-100 μg of lipids, and 25 μl volume always resulted in a large solvent peak which seriously interferred with the peaks of interest. Also, further dilution produced poor resolution. A more concentrated lipid

solution had been tried, but the resulting viscosity of the sample made it difficult to draw the lipids into the syringe and a significant injection volume measure problem resulted. A 3 µl injection containing 50-100 µg lipids appeared to give the best resolved chromatogram with a satisfactory retention time at the flow rate of 0.5 ml/min.

4) The nonpolar lipids of wheats, oats, millets and corns had also been analyzed by the same HPLC conditions (Fig. 3). Peaks 4, 5, 6, and 7 were always the major peaks in all grains. Peak 5 had the highest peak height in all grains; using it for normalization was convenient for observing changes in relative amounts of individual nonpolar lipids.

b. A short term storage of grain with high moisture content.

1) Sorghum.

i) 8 ml of water were added to 40 g of seven varieties of ground sorghum. The tempered sorghum were stored at 40°C for 8, 16, 32, 64, and 128 hrs. After 8 hrs, a fermentation flavor could be detected. The lipid was extracted with 40 ml of 1,1,1-trichloroethane in a Fosslet extractor. The dried lipids were dissolved in chloroform and injected into HPLC. Only one lipid stored at -18°C and stored at 40°C for 128 hrs is shown in Fig. 4 A and B. Using peak 5 for normalization, Table II showed the peak area ratios of nonpolar lipids extracted from the control and the seven stored sorghum varieties. Only peaks 4, 5, 6, 7, and 8 are shown in the table, because other peaks were small and had baseline drift problems. From Table II, no significant changes could be found.

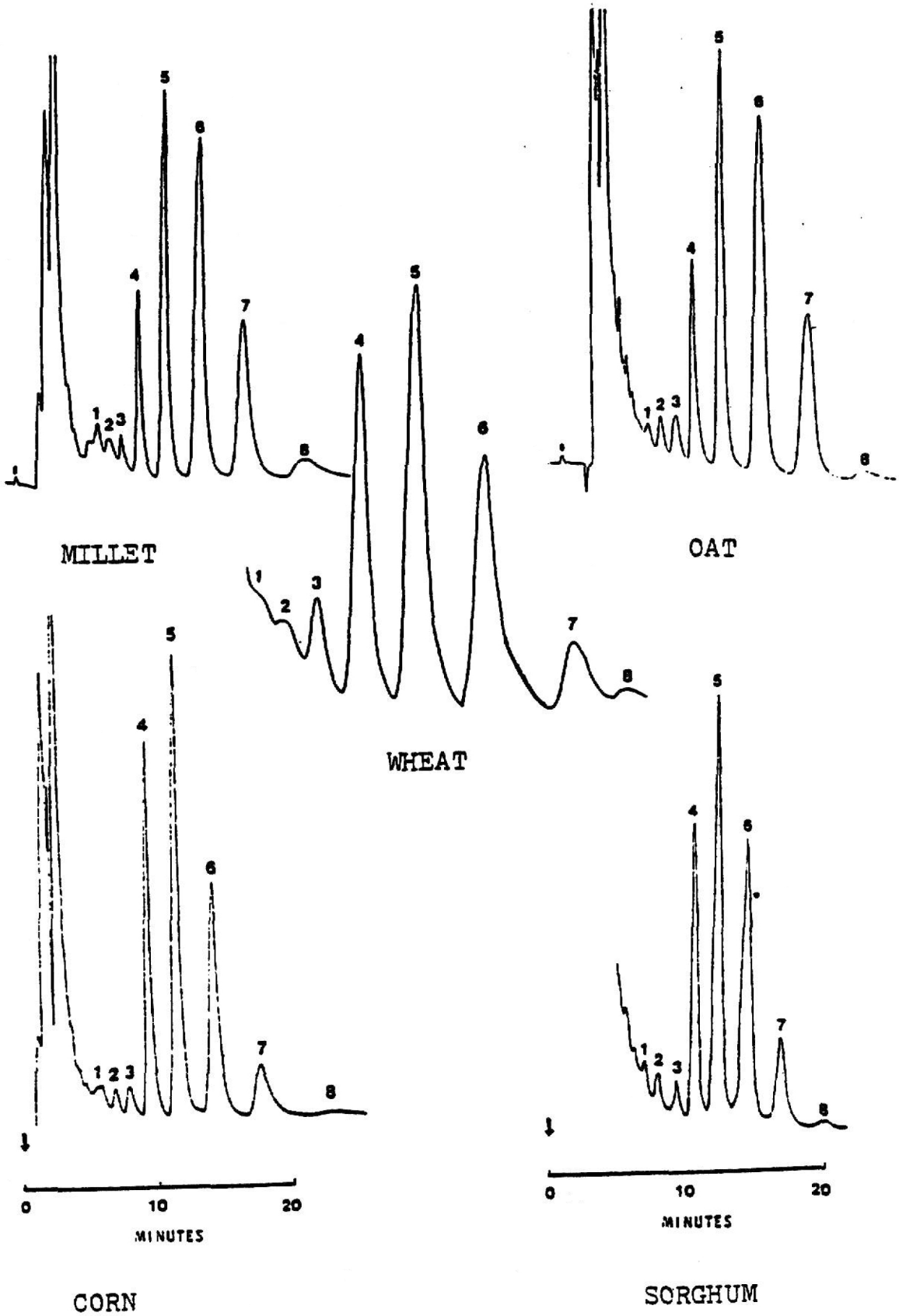


Fig. 3. HPLC separation of nonpolar lipid extracted with Skellysolve F by a Soxhlet extractor and fractionated by an open column, HPLC conditions: Spherisorb C 6, 5 μm (4.1 mm i.d. x 15 cm), pure acetonitrile, 0.5 ml/min. RI detector.

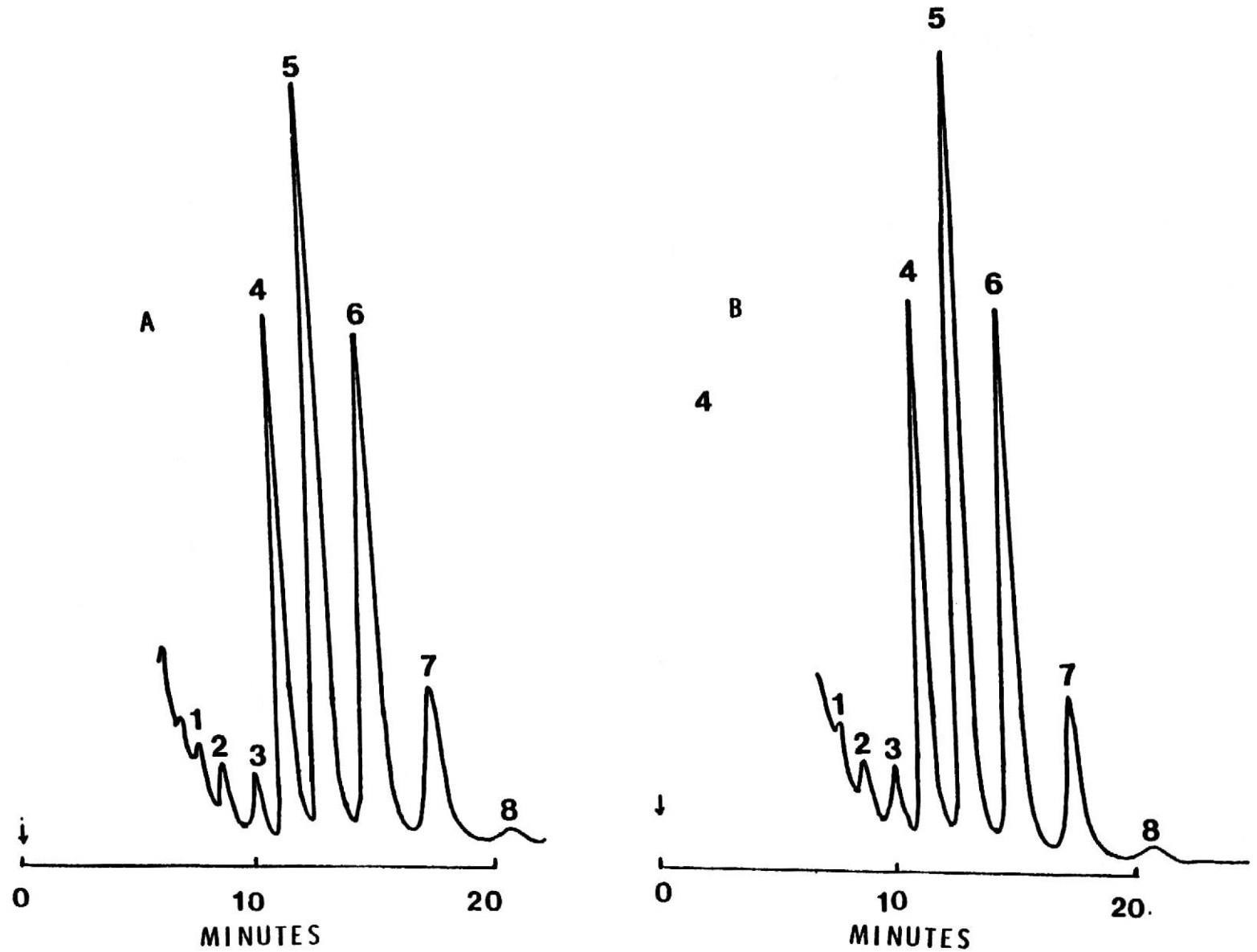


Fig. 4 HPLC separation of sorghum nonpolar lipid: (A) control; (B) stored. HPLC conditions: Spherisorb C 6, 5 μm (4.1 mm i.d. x 15 cm), pure acetonitrile, 0.5 ml/min, RI detector.

Table II. Comparison of Nonpolar Lipid Composition of Control and Ground Whole Sorghum Stored at 40°C for 128 hr[a,b]

Sorghum Variety	Sorghum Lipids	Relative Peak Area to the Area of Peak 5				
		4	5	6	7	8
A	Control	0.55	1.00	0.82	0.27	0.02
	Stored	0.58	1.00	0.85	0.28	0.02
B	Control	0.64	1.00	0.82	0.26	0.02
	Stored	0.62	1.00	0.89	0.31	0.02
C	Control	0.59	1.00	0.88	0.30	0.02
	Stored	0.62	1.00	0.91	0.33	0.02
D	Control	0.67	1.00	1.00	0.33	0.03
	Stored	0.71	1.00	0.93	0.26	0.02
E	Control	0.62	1.00	0.86	0.25	0.03
	Stored	0.62	1.00	0.82	0.28	0.03
F	Control	0.67	1.00	0.91	0.24	0.02
	Stored	0.56	1.00	0.75	0.24	0.02
G	Control	0.65	1.00	0.86	0.32	0.02
	Stored	0.64	1.00	0.96	0.28	0.02

[a]Lipid extracted with Fosslet extractor.

[b]8 ml of water was added to 40 g of ground whole sorghum.

ii) Some standards of commercial TG had been run through the HPLC system. Peaks were tentatively identified by comparing the relative retention volumes with those from the reference standards. Each HPLC subfraction of control sorghum was collected (Fig. 4A). Its fatty acid methyl ester was analyzed by GC. The gas chromatogram of peak 4 subfraction is shown in Fig. 5. Fatty acid composition of subfractions were also shown in Table III.

Tweeten (165) using LiChrosorb RP-8 column, RI detector and variable wavelength detector at 210 nm, to determine the TG content and fatty acid compositions of the sunflower lipid extracts in an oilseed plant breeding program. Individual TG peak fractions have been collected for characterization. TG comprised of shorter chain fatty acid as well as fatty acids with higher unsaturation eluted earlier than TG with longer chain, saturated fatty acids. After comparing the response factor of TG components with RI and UV detectors, different results were obtained. This suggested that a calibration factor must be established between these two detectors.

The separations of nonpolar lipids on HPLC were based on chain length and degree of unsaturation on a Spherisorb C 6 column with acetonitrile as mobile phase. For saturated nonpolar lipids, a linear relationship was observed between the carbon number and the log of the retention volume (120). Each double bond present in the nonpolar lipids decreased the retention volume to approximately that of a saturated nonpolar lipid with two carbon atoms less. Correlations of the fatty acid composition, as determined by GC with HPLC, provided much additional insight about nonpolar

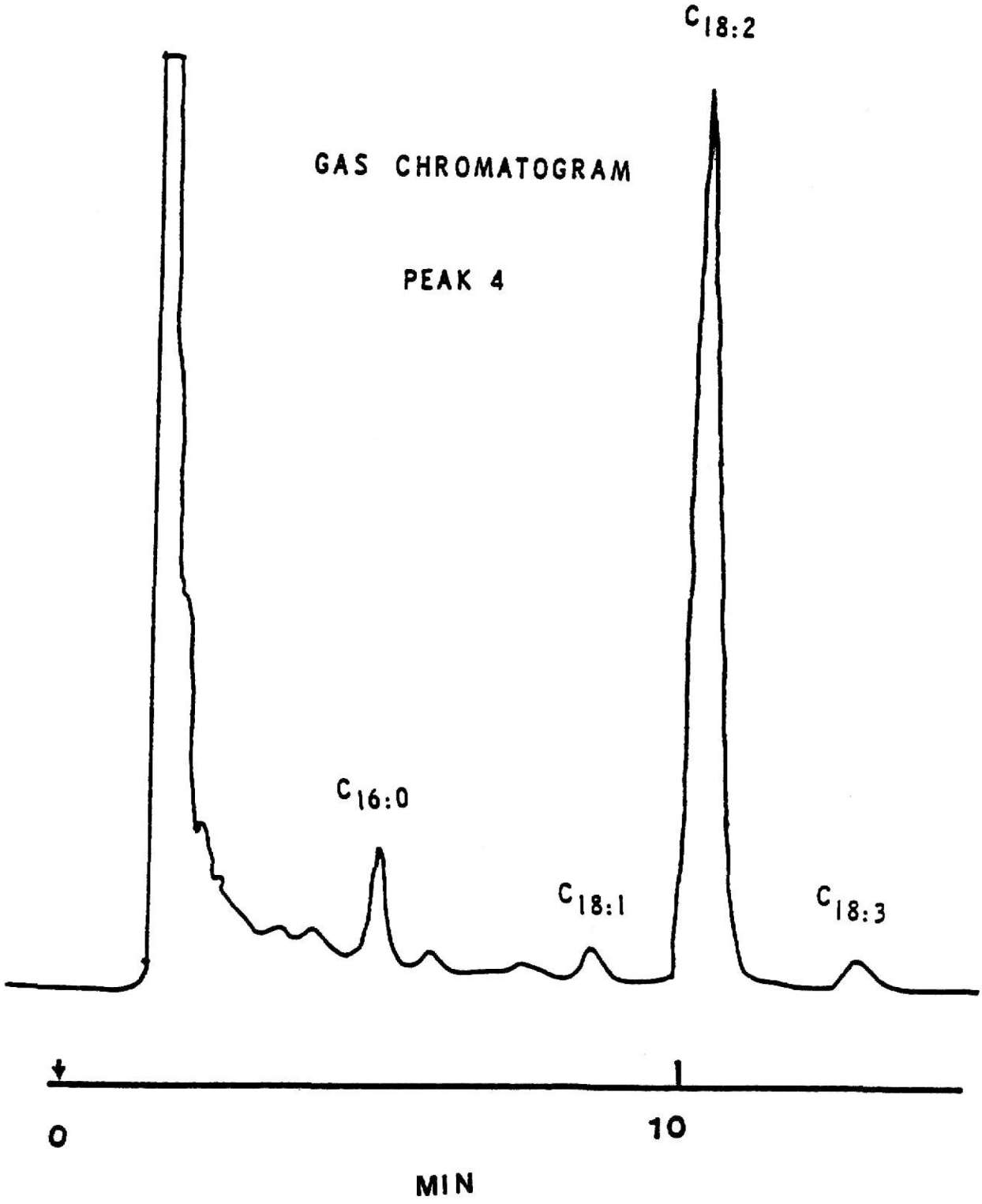


Fig. 5 GC separation of sorghum nonpolar lipid peak 4 (Fig. 4A), GC conditons: 1/8 inch i.d. x 6 feet, 10% SP-2330 on 100/120 mesh Chromosorb W AW. injector: 240°C, column: 200°C, detector: 240°C, nitrogen gas, flow rate: 25 ml/min.

Table III. Fatty Acid Composition of HPLC Subfraction of Control Sorghum Nonpolar Lipids (Mol. %)

Subfraction (Peak No.)	Fatty Acid (% of Total)							
	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
1			22.32	0.47	1.07	26.27	0.49	0.60
2			37.99	4.47	5.40	19.18	32.96	
3			41.58	7.17	13.47	12.30	19.18	6.30
4			5.76	1.07	1.07	2.14	86.98	3.09
5	0.90	1.62	16.03	1.92	1.53	18.02	58.26	1.71
6	0.87	1.74	21.86	1.85	2.13	35.60	35.96	
7	1.51	3.02	27.18	3.01	5.02	48.78	11.49	
8	4.06	4.37	33.20	5.61	9.40	29.77	13.59	

Average of duplicate analyses

lipid composition. The standard trilinolein, tripalmitolein, and trimyristin all had the same retention time as peak 4. Based on the fatty acid composition of subfraction 4 (Table III), the major portion was trilinolein.

iii) The fatty acid composition of control sorghum lipid was: C 16:0 (14.8%), C 16:1 (0.4%), C 18:0 (0.1%), C 18:1 (24.7%), C 18:2 (57.4%), and C 18:3 (2.6%). These values fell within the range reported previously (56). The fatty acid composition of sorghum lipid stored at 40°C for 15 days was: C 16:0 (15.1%), C 16:1 (0.3%), C 18:0 (0.1%), C 18:1 (25.0%), C 18:2 (57.1%), and C 18:3 (2.4%). The relative standard deviation was 4.8%.

Lai and Marston (84) determined the changes in the fatty acid composition of pearl millet free and bound lipids during storage at 42°C and 75% relative humidity. They reported that the fatty acid composition did not change qualitatively during storage. This indicated that the fatty acid methyl ester analyzed composition by gas chromatography was unacceptable as an indicator of stored grain.

2) Oat.

i) Two different varieties of oats (55-8, 55-14) stored at 4°C for 2 years, were used for this study. Fig. 6A and B showed the nonpolar lipids results of two varieties extracted with 1,1,1-trichloroethane in the Fosslet extractor. Different varieties of oats had different chromatograms.

ii) Fig. 7 shows the nonpolar lipids of variety 55-8 oats extracted with different extractors (Fosslet and Goldfisch). The Goldfisch extraction used Skellysolve F as an extractant and

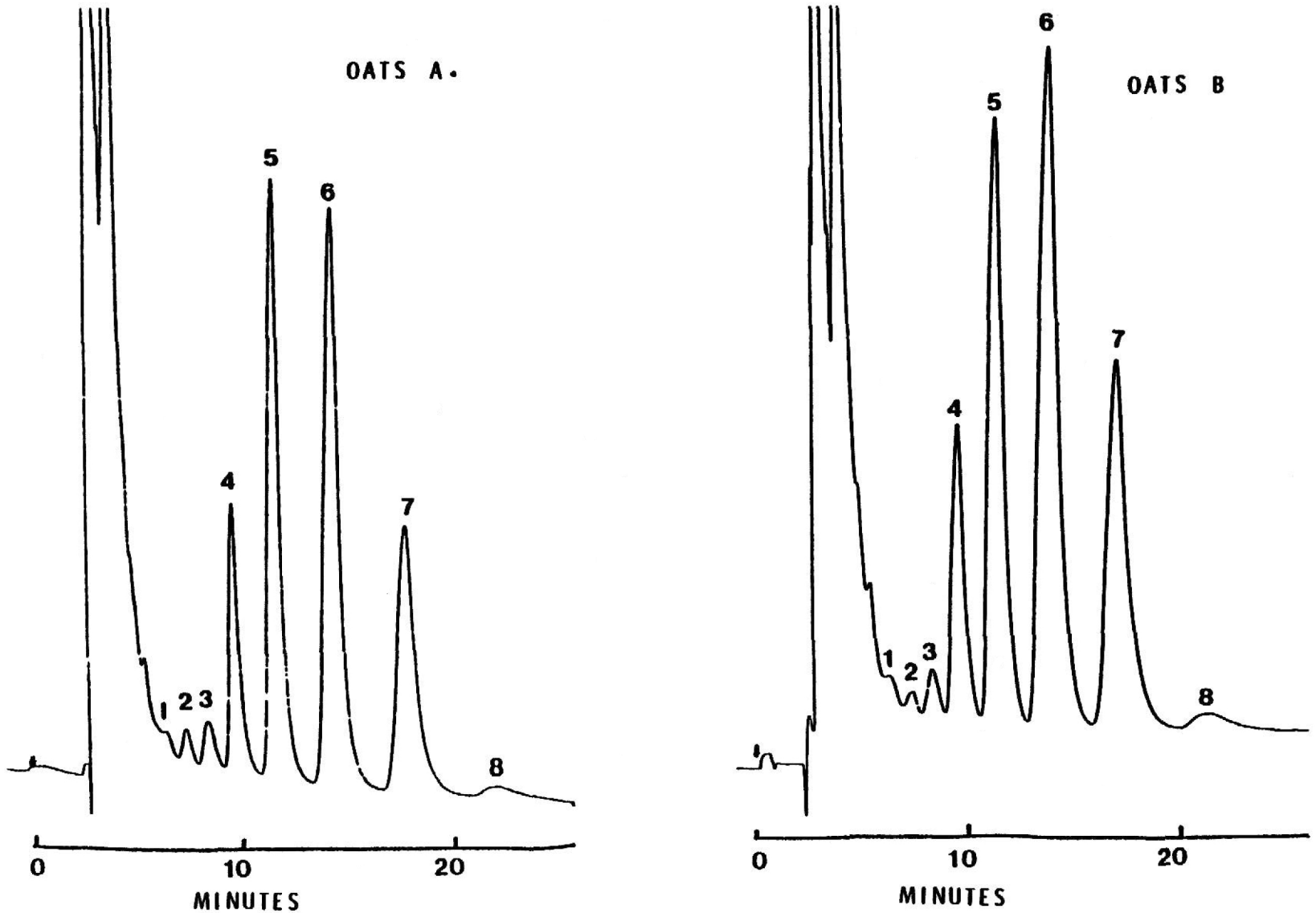


Fig. 6 HPLC separation of oats nonpolar lipids extracted with Fosslet extractor: (A) 55-8; (B) 55-14. HPLC conditions: Spherisorb C 6, 5 µm (4.1 mm i.d. x 15 cm), pure acetonitrile, 0.5 ml/min, RI detector.

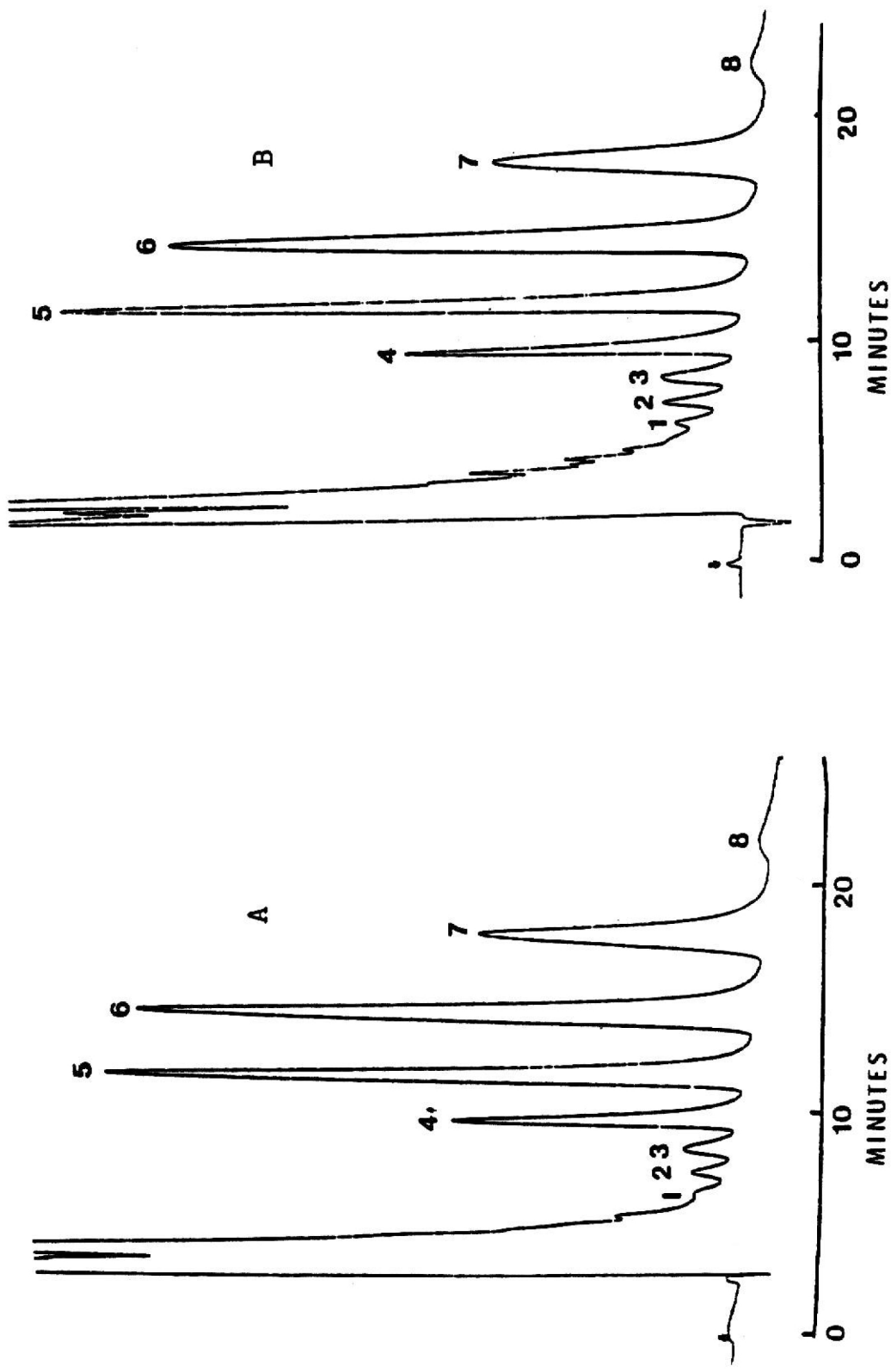


Fig. 7 HPLC separation of oat (55-8) nonpolar lipid extracted with (A) Fosslet and (B) Goldfisch extractors. HPLC conditions: Spherisorb C 6, 5 μm (4.1 mm i.d. x 15 cm), pure acetonitrile, 0.5 ml/min, RI detector.

and the Fosslet extraction used 1,1,1-trichloroethane as an extractant. These two solvents seemed to have a similar extraction effect for lipids, because two extractions gave a similar nonpolar lipid composition.

iii) 8 ml of water were added to 40 g of two oat varieties; oat samples were stored at 40°C for 128 hrs. A fermented odor could be detected after storage of more than 8 hrs. The control and stored lipids were extracted with Fosslet extractor and Goldfisch extractor. The HPLC chromatograms of control and stored nonpolar lipids of oat (55-8 variety) are shown in Fig. 8: the lipid was extracted by the Fosslet extractor. Using peak 5 for normalization Table IV shows the peak area ratios of nonpolar lipids extracted from the two control and stored oat varieties by both Fosslet and Goldfisch extractors. No significant changes were found for the relative peak ratios of nonpolar lipids extracted from the control and stored oats.

From the nonpolar lipids chromatograms of oats and sorghums, there were no significant changes between the control sample and deteriorated sample stored at 40°C in a short term. Even when 8 ml of water was added to the 40 g ground grain sample and a substantial fermentation flavor was detected, the chromatograms still had similar peak ratios. It indicated that no measurable deterioration happened in such a short storage. The other possibility might be that the deterioration rate for each nonpolar lipid peak had the similar reaction rate. It was suggested that nonpolar lipids would not be a sufficiently sensitive indicator of stored grain quality.

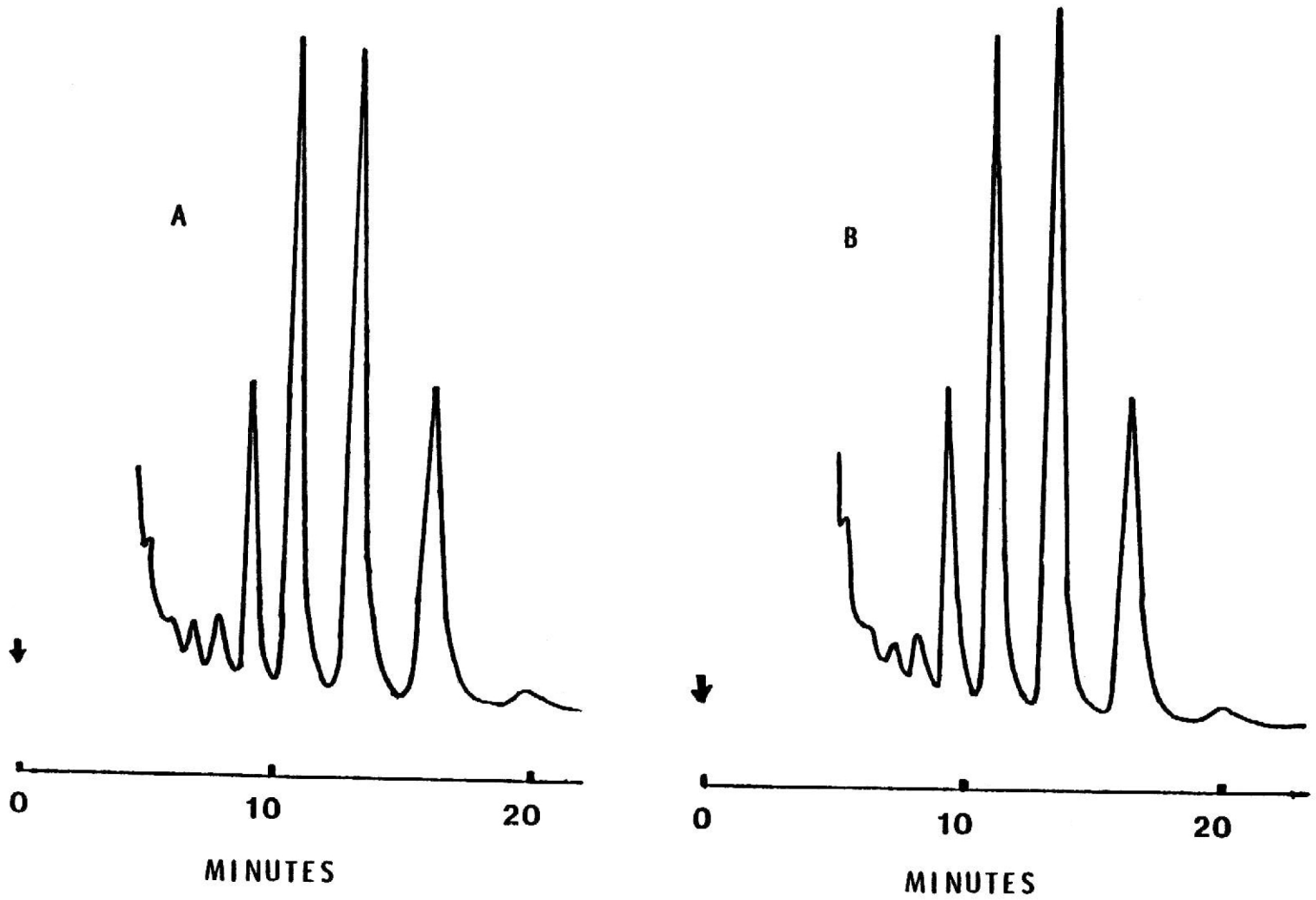


Fig. 8 HPLC separation of oat nonpolar lipid (55-8 variety): (A) control; (B) stored. HPLC conditions: Spherisorb C 6, 5 μm (4.1 mm i.d. x 15 cm), pure acetonitrile, 0.5 ml/min, RI detector.

Table IV. Relative Peak Areas to the Area of Peak 5 of Nonpolar Lipids Extracted from Control Whole Oats Flour and Oat Flour Stored at 40°C for 128 hrs.[a/b]

Oats Variety	Lipid Source	4	5	6	7
I. Fosslet Extraction					
55-8	Control	0.41	1.00	1.14	0.74
	Stored	0.38	1.00	1.34	0.66
55-14	Control	0.38	1.00	1.30	0.78
	Stored	0.41	1.00	1.38	0.79
II. Goldfisch Extraction					
55-8	Control	0.39	1.00	1.28	0.81
	Stored	0.39	1.00	1.32	0.79
55-14	Control	0.44	1.00	1.53	0.83
	Stored	0.59	1.00	1.49	0.96

[a] 8 ml of water was added to 40 g of ground oats

[b] Average of duplicate analyses

c. A long term storage of wheat with normal moisture content.

Up to this point in the studies described, no long term storage of grains had been tested. A 1979 hard red winter whole wheat flour stored at 37°C for 8 months was used as a long term storage sample. Lipids were extracted with 1,1,1-trichloroethane by a Fosslet extractor from control and stored whole wheat flour. The HPLC results of the control and stored nonpolar lipids are shown in Table V. Fig. 9 shows the chromatograms of nonpolar lipids extracted from the control wheat and stored (37°C for 8 months) wheat. It also shows the chromatogram of nonpolar lipids extracted from a mixed wheat (10 varieties) stored at the ambient temperature for 5 years. Although no significant changes in the peak area ratios could be found due to storing wheat at 37°C for 8 months (Table V), a small shoulder on peak 6 appeared with the nonpolar lipids extracted from the stored wheats (Fig. 9, indicated by the arrows). The shoulder on peak 6 was even more pronounced with lipids extracted from the mixed wheat which had been stored for 5 years.

B. Effect of Stored Temperature on the Extracted Lipids.

Because no significant changes were found in the peak area ratios of nonpolar lipids extracted from control and stored grain flours, the concentrated lipids stored at different temperature and different pH were tested.

a. Nonpolar lipid fractions.

1) Lipids stored at different temperatures. Sorghum lipid extracts evaporated to dryness had been stored at -18°C, 0°C, 23°C, and 35°C, for 15 days and 30 days. The HPLC results of nonpolar lipids in which peak 5 was used for normalization are

Table V. Relative Peak Areas to the Area of Peak 5 of Nonpolar Lipids Extracted from Control Whole Wheat Flour and Whole Wheat Flour Stored at 37°C for 8 months

Nonpolar Lipid Source	4	5	6	7
Control	0.67	1.00	0.70	0.20
Stored	0.71	1.00	0.67	0.22

Average of duplicate analyses

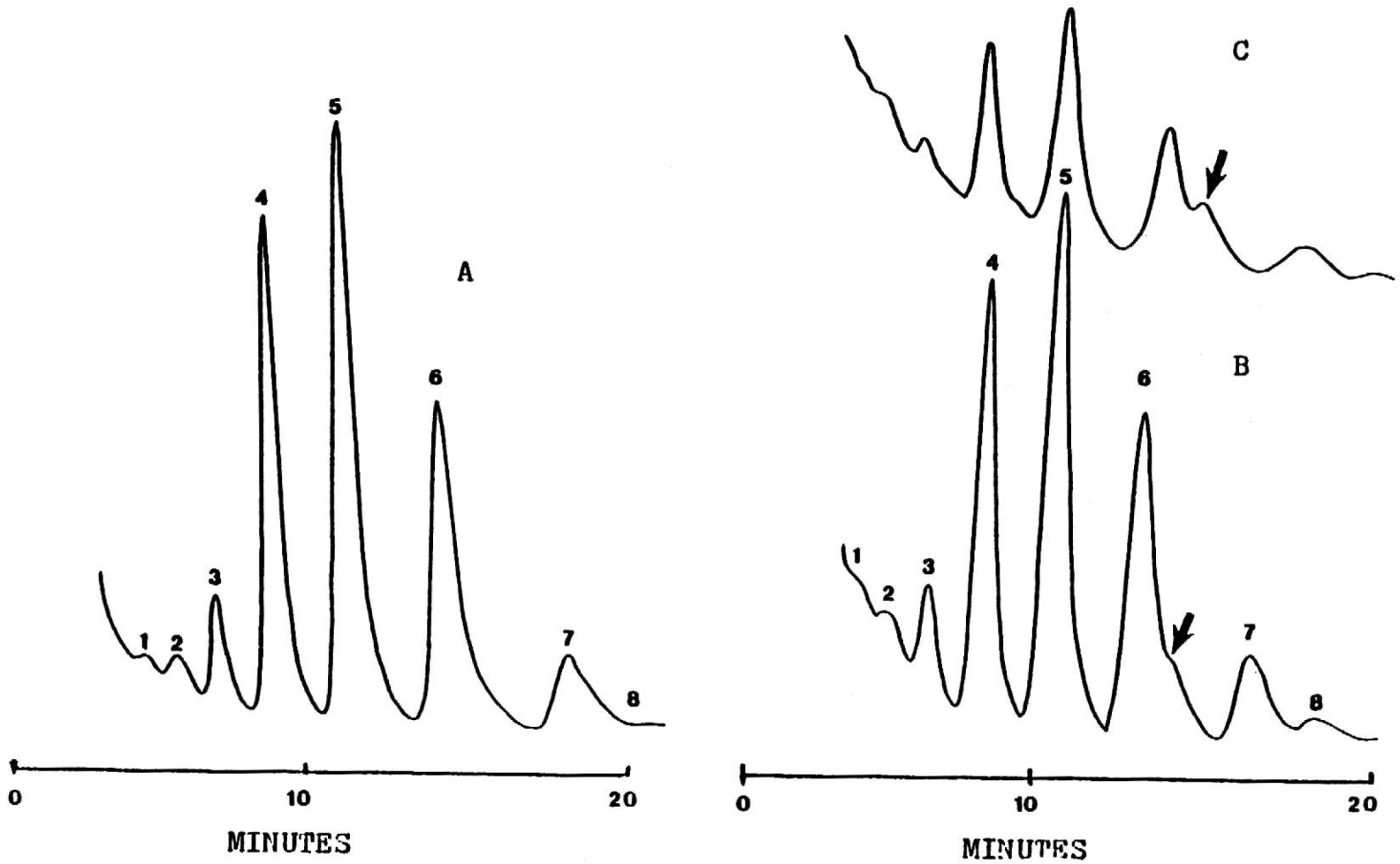


Fig. 9 HPLC separation of nonpolar lipid extracted from wheats: (A) control; (B) stored at 40°C, 8 months; (C) stored at ambient temperature, 5 years. HPLC conditions: Spherisorb C 6, 5 μm (4.1 mm i.d. x 15 cm), pure acetonitrile, 0.5 ml/min, RI detector.

shown in Table VI. From Table VI, the ratios of the area of peak 6 to peak 5 and peak 7 to peak 5 of the lipids, stored at 23°C and 35°C for 30 days, were larger than those of the control. However, the difference was not very significant, and the ratios at 23°C were larger than at 35°C and were not reasonable.

2) Lipids stored at different pH. 200 mg of sorghum lipids were dissolved in 1 ml chloroform/methanol (1:1) then mixed with 1 ml of distilled water of different pH values (from 1 to 13). The mixtures were stirred hard with a magnetic stirrer in reactor vials. They were hydrolyzed at room temperature for 24 hrs. Removed the mixtures, extracted the lipids with 2-20 ml chloroform, decanted the water layer, and dried the chloroform layer. Using peak 5 for normalization, the HPLC results of non-polar lipids are shown in Table VII. No significant changes of peak ratio were found.

b. Polar lipid fractions.

In order to get the optimum conditions for polar lipids analyses by HPLC, various factors were studied: column length (15 cm vs 25 cm), packing materials (LiChrosorb RP-18, 10 µm, Spherisorb ODS, 10 µm, Partisil-10, ODS, Lichrosorb SI-60, 10 µm, LiChrosorb SI-100, 10 µm, Spherisrob RP-6, 5 µm, and µ-Bondapak RP-18, 10 µm), mobile phase (methanol/water system ranging from 50:50 to 100:0), and flow rate (from 0.5 ml/min. to 1.5 ml/min). The best results were obtained with µ-Bondapak RP-18, 10 µm, column, 4.1 mm x 25 cm, mobile phase, methanol/water (90:10), and flow rate, 1.0 ml/min.

Using the backflush technique, the control and stored lipids of wheat were fractionated by HPLC (Fig. 10A and B). Lipids were

Table VI. Changes in the Relative Peak Areas to the Area of Peak 5 of Sorghum Nonpolar Lipids at -18°C, 0°C, 23°C, and 35°C[a]

Storage Temp (°C)	Storage Time (days)	4	5	6	7
Control		0.56	1.00	0.76	0.18
-18	15	0.61	1.00	0.66	0.21
	30	0.61	1.00	0.77	0.19
0	15	0.63	1.00	0.76	0.15
	30	0.59	1.00	0.73	0.17
23	15	0.56	1.00	0.89	0.22
	30	0.48	1.00	0.90	0.32
35	15	0.49	1.00	0.80	0.19
	30	0.53	1.00	0.88	0.27

[a]Average of duplicate analyses

Table VII. Changes in the Relative Peak Areas to the Area of Peak 5 of Sorghum Nonpolar Lipids Compositions under different pH[a/b]

ph	4	5	6	7
Control	0.56	1.00	0.76	0.18
1	0.56	1.00	0.81	0.23
3	0.56	1.00	0.78	0.22
5	0.59	1.00	0.78	0.20
7	0.59	1.00	0.66	0.20
9	0.60	1.00	0.77	0.19
11	0.56	1.00	0.75	0.18
13	0.61	1.00	0.76	0.17

[a]Lipids are incubated at 23°C for 24 hrs

[b]Average of duplicate analyses

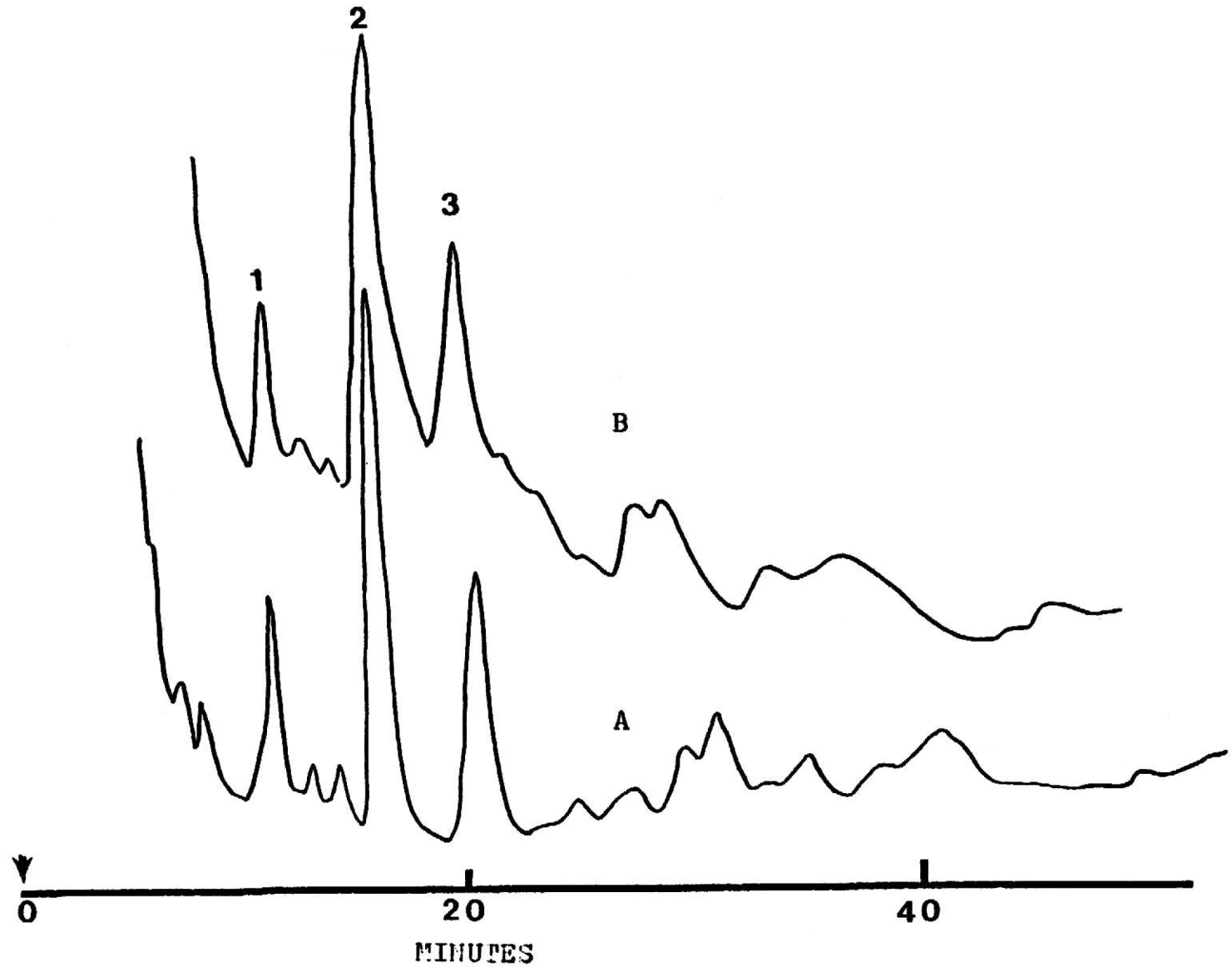


Fig. 10 HPLC separation of wheat lipid extracted with 1,1,1-trichloroethane by a Fosslet extractor: (A) control; (B) stored. HPLC conditions: μ-Bondapak RP-18, 10 μm (4.1 mm x 25 cm), methanol/water (90:10), 1.0 ml/min, RI detector.

extracted with 1,1,1-trichloroethane by a Fosslet extractor: one lipids portion, stored at -18°C, was termed "control" and the other portion, stored at 37°C for 15 days, was termed as "stored" lipids. Chromatograms showed only polar lipids fractions because the packing material was reverse phase and the mobile phase was methanol/water. Using the backflush technique, the strongly retaining nonpolar lipids fraction was eluted off.

The 1,1,1-trichloroethane was used as extractant, because it was low flammable and similar to chloroform. The extracted lipids might be related to breadmaking, because chlorofrom extracted lipids (free lipids) could improve the baking quality.

From the chromatograms, there were 3 major peaks and 15 minor peaks; the retention time was about 50 min. After the backflush was finished, the injection was made after 100 min. Comparison of the control lipids and stored lipids was difficult, because peaks were broadened and tailing. However, the ratios of peak area of 1 to 3 were changed.

General conclusions on the HPLC analyses of nonpolar lipids of various grains (sorghums, oats, and wheats) were as follows.

1. There were no significant changes in the peak area ratios of nonpolar lipids extracted from the control grain flours and their stored grain flours. Also, there were no significant changes in the peak area ratios of control nonpolar lipids and the stored nonpolar lipids (lipids stored at different pH and temperature). However, small shoulders on peak 6 were found on the chromatograms of nonpolar lipids of the stored wheats (Fig. 9). Because the resolution of the shoulder from peak 6 was poor and the shoulder was very small for the wheat stored at 37°C for 8 months, quanti-

tating the shoulder was difficult and also it seemed impractical to measure the early stage of deterioration in the stored grain quality. Therefore, nonpolar lipids were not very promising to be used as an indicator to measure the stored grain quality.

2. For the nonpolar lipids studies, the Fosslet extractor (1,1,1-trichloroethane, b.p. 75°C, as an extractant) and Goldfisch extractor (Skellysolve F as an extractant) were used. The extraction time was 7.5 min per sample (20 g) with the Fosslet extractor and 4 hr per 6 samples (5-6 g each) with the Goldfisch extractor. Lipids extracted with either extractor showed similar chromatograms, indicating that both extractors extracted lipids in similar compositions.

Although the extraction time was shorter with the Fosslet than the Goldfisch extractor, the Fosslet extractor had several disadvantages. They were: it made high noise during operation; the extraction temperature was not constant because the stainless cup was heated as high as 90°C by the fast-moving hammer; the high temperature of extraction resulted in possible decomposition of lipids as well as changes in the extracted lipid concentration due to a partial volatilization of the extractant; and as the result of inconsistent temperature of extraction, the lipid recovery might be also inconsistent.

For the Goldfisch extraction, a high temperature was required and the sample was partially immersed in the boiling solvent. Thus, grain quality might be changed during the extraction procedure. Although the Soxhlet extraction might take 16 hrs with relatively slow condensation rate (2-3 drops/sec), the temperature of the extractor (thimble containing grain samples) could maintain

36°C. The constant and low extraction temperature by a Soxhlet would overcome the disadvantage of a longer extraction time required by a Soxhlet. Therefore, a Soxhlet extractor was decided to be used for the next phases of the works.

3. Results obtained with stored lipids (at -18°C vs 37°C), verified that more substantial changes during the high temperature-storage might show with the polar lipid fraction rather than with the nonpolar lipid fraction.

C. HPLC Analyses of Polar Lipids.

a. Free polar lipids.

When free lipids were extracted from ground wheat with Skellysolve F by a Soxhlet extractor, the extracted lipid content was 1.68 ± 0.08 g/100 g sample (on dry weight basis). About 50-100 µg of polar lipids fractionated by an open column (silica gel), was injected into HPLC system. The chromatogram is shown in Fig. 11. Compared with Fig. 10, the resolution and retention time were not improved. Under the present condition, most phospholipids were retained in the analytical column and deteriorated column efficiency.

b. Free glycolipids.

Because polar lipids showed too many peaks on the chromatogram and large amounts of phospholipids retained in the HPLC-analytical column, glycolipids were fractionated from an open column of silica gel by eluting with acetone after eluting nonpolar lipids with chloroform. It was reported that about 95% of phospholipids retained in the silica gel open column and glycolipids fraction was free of nonpolar lipids and phospholipids (139). About 50-100 µg of glycolipids was injected into HPLC

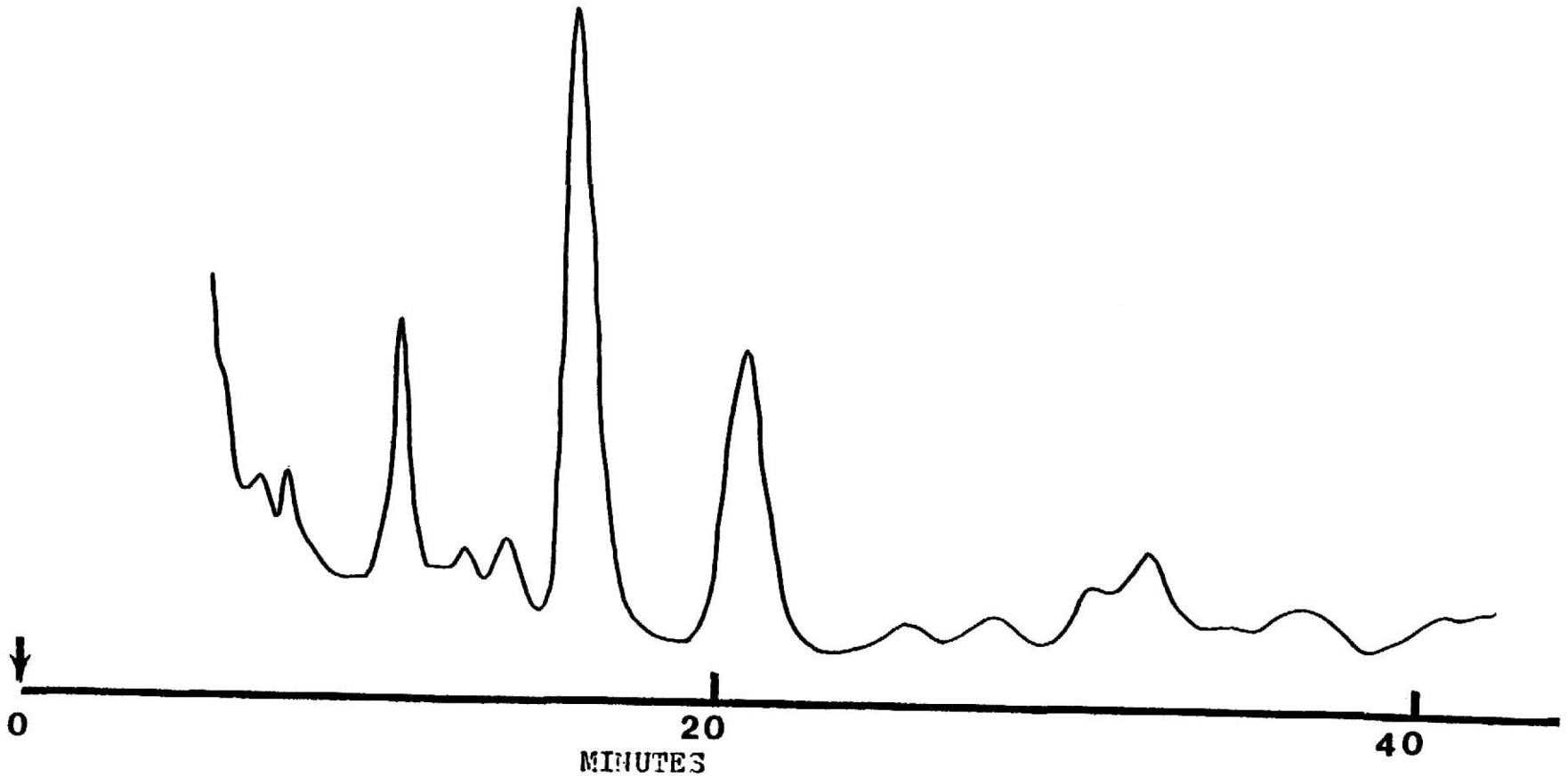


Fig. 11 HPLC separation of wheat polar lipids extracted with Skellysolve F by a Soxhlet. HPLC conditions: μ-Bondapak RP-18, 10 μm (4.1 mm i.d. x 25 cm), methanol/water (90:10), 1 ml/min, RI detector.

system. Fig. 12A gives the chromatogram of purified glycolipids of control whole wheat flour. Fig. 12B shows the chromatogram of wheat lipids stored at 37°C for 15 days. However, the quantitation was difficult for the stored lipids.

c. Free DGDG fraction.

Because the free DGDG was the most important bread-making improver (27), it seemed to be proper to check if there were any significant changes in DGDG fraction during storage. Lipids extracted from whole wheat flour with Skellysolve F by a Soxhlet extractor were fractionated through a silica gel open column. After nonpolar lipids was eluted with chloroform, the MGDG fraction was eluted with chloroform/acetone (1:1), the DGDG fraction was eluted with acetone, and the phospholipids fraction was eluted with methanol.

The individual polar lipid fractions (obtained from the open column chromatography) were alalyzed by HPLC. The phospholipids fraction did not show any major peak at the same HPLC conditions.

Different ratios of mobile phase had been tried to separate peaks 1 and 2, and peaks 4 and 5. At the methanol/water ratio of 90/10, the better the separation of peaks 1 and 2, and the worse the separation of peaks 4 and 5. At the methanol/water ratio 95/5, the better the separation of peaks 4 and 5, and the worse the separation of peaks 1 and 2. The retention time was about 20 min.

The free DGDG fraction of control whole wheat flour stored at 37°C for 4 months was checked by HPLC every month. The mixture of 10 varieties of whole wheat flour (1977), each stored at ambient

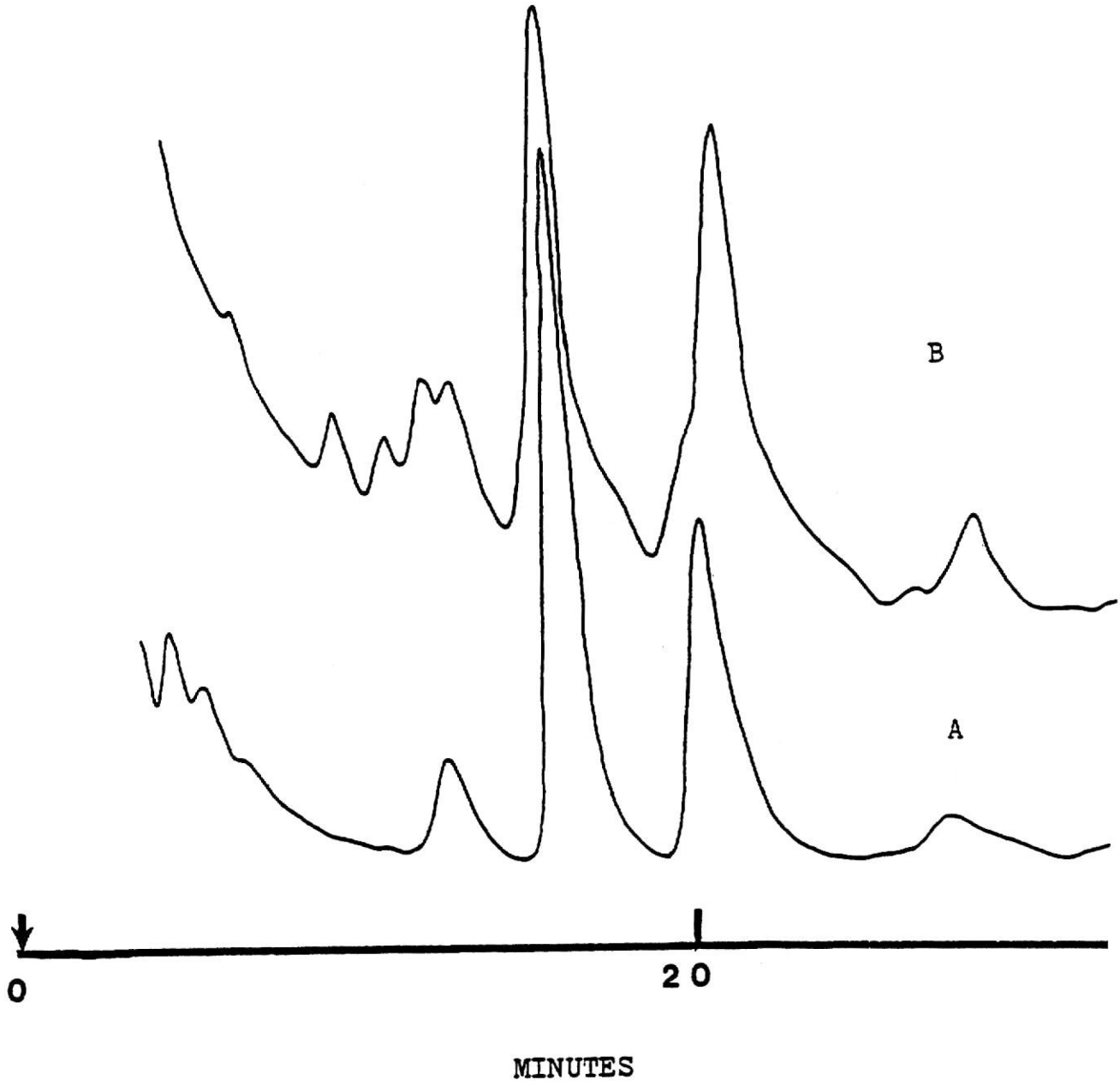


Fig. 12 HPLC separation of glycolipid: (A) control; (B) stored at 37°C for 15 days. HPLC conditions: μ-Bondapak RP-18 10 μm, (4.1 mm i.d. x 25 cm), methanol/water (90:10), 1.0 ml/min, RI detector.

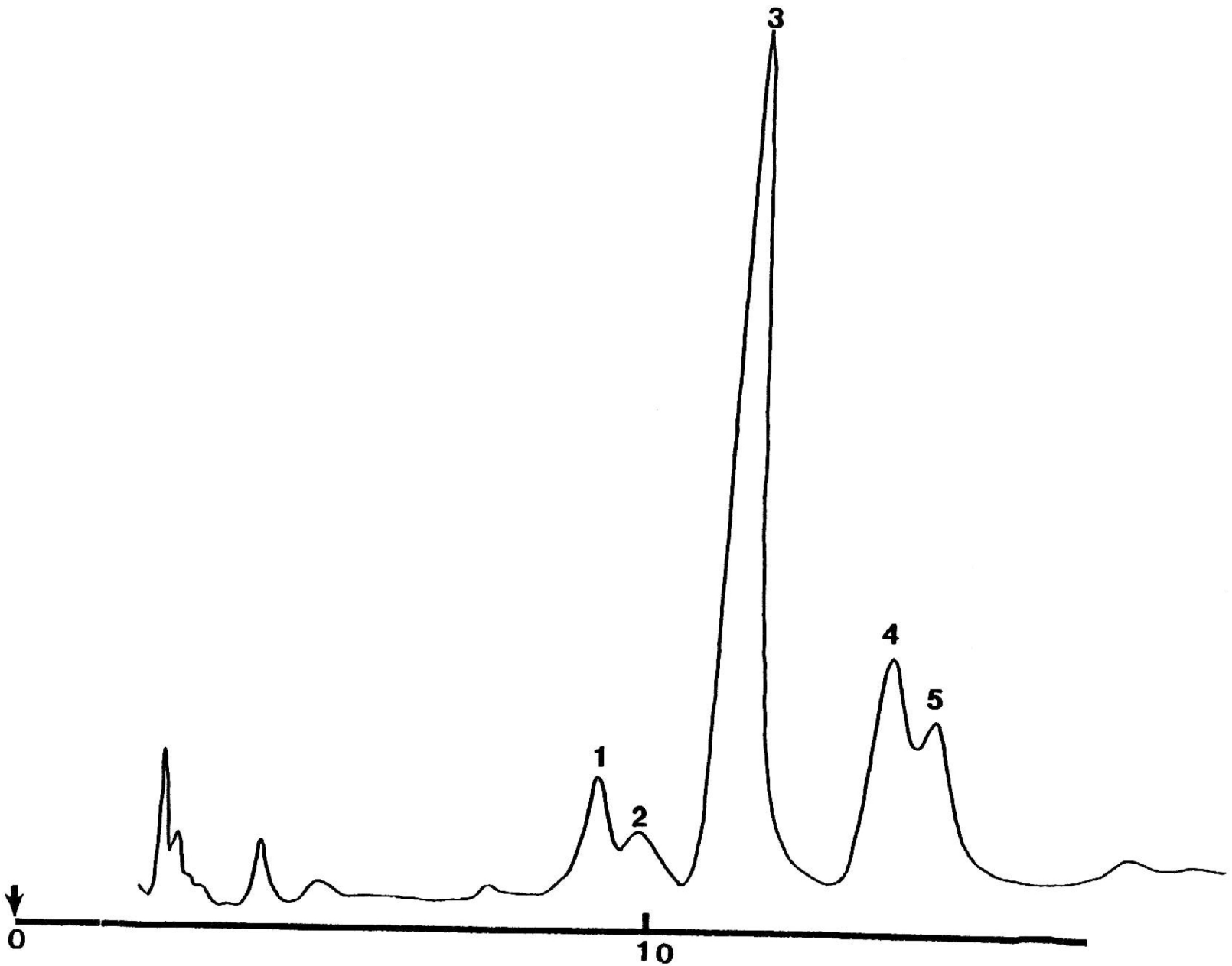


Fig. 13 HPLC separation of DGDG fraction. HPLC conditions: μ-Bondapak RP-18, 10 μm (4.1 mm i.d. x 25 cm), methanol/water (93:7), 1.0 ml/min, RI detector.

temperature (most 24°C) for 5 years, was also used for reference. Table VIII shows the relative peak of DGDG fraction by using peak 3 as standard and normalized. Although peaks 4 and 5 were larger than peaks 1 and 2, the poor resolution made the quantitation difficult. Also, peak 2 was not completely resolved from peak 1 or 3; its area was affected by baseline drift. Chromatograms are compared in Fig. 14 for the DGDG fractions of control wheat (A), wheat flour stored at 37°C for 4 months (B), and 1977 HRW wheat flour stored at ambient temperature for 5 years (C).

Although separating DGDG fractions had advantages of short retention time, few peaks, and no need for backflush procedure, there were some disadvantages to be used as an indicator of stored wheat quality because: 1) as the resolution of peaks 1 and 2 and peaks 4 and 5 were poor, it was difficult to quantitate them: 2) no significant changes of relative peak area ratio in DGDG fractions of control and stored wheats; 3) one more step was required in fractionation of silica gel open column to obtain DGDG than to obtain MGDG fraction. Thus, it was time consuming and extra solvent was needed; and 4) the absolute amount of peak 3 (DGDG peak I in Tweeten's work) could be measured only by using several concentrations of standard DGDG which had to be from the wheat source.

d. Free MGDG fractions

About 50-100 µg of the MGDG fraction in 3 µl was injected into HPLC system. Total retention time was about 30 min, and peaks 3 and 6 were completely resolved from the other peaks (Fig. 15).

Using TLC, GC, and MS, Tweeten et al. (167) had identified peak 6 was DLGG, SN-1; linoleic, SN-2; linoleic, SN-3; monogalac-

Table VIII. Relative Peak Areas of DGDG Fraction of Control and Stored Whole Wheat Flour at 37°C

Storage Time (Months)	Lipid Source	Relative Peak Areas to the Area of Peak 3		
		1	2	3
1	Control	0.11	0.06	1.00
	Stored	0.13	---	1.00
2	Control	0.11	---	1.00
	Stored	0.11	---	1.00
3	Control	0.13	---	1.00
	Stored	0.15	---	1.00
4	Control	0.11	---	1.00
	Stored	0.11	0.02	1.00
60	1977 HRW Wheat*	0.13	0.03	1.00

*A mixture of 10 varieties of 1977 whole wheat flour stored at ambient temperature for 5 years

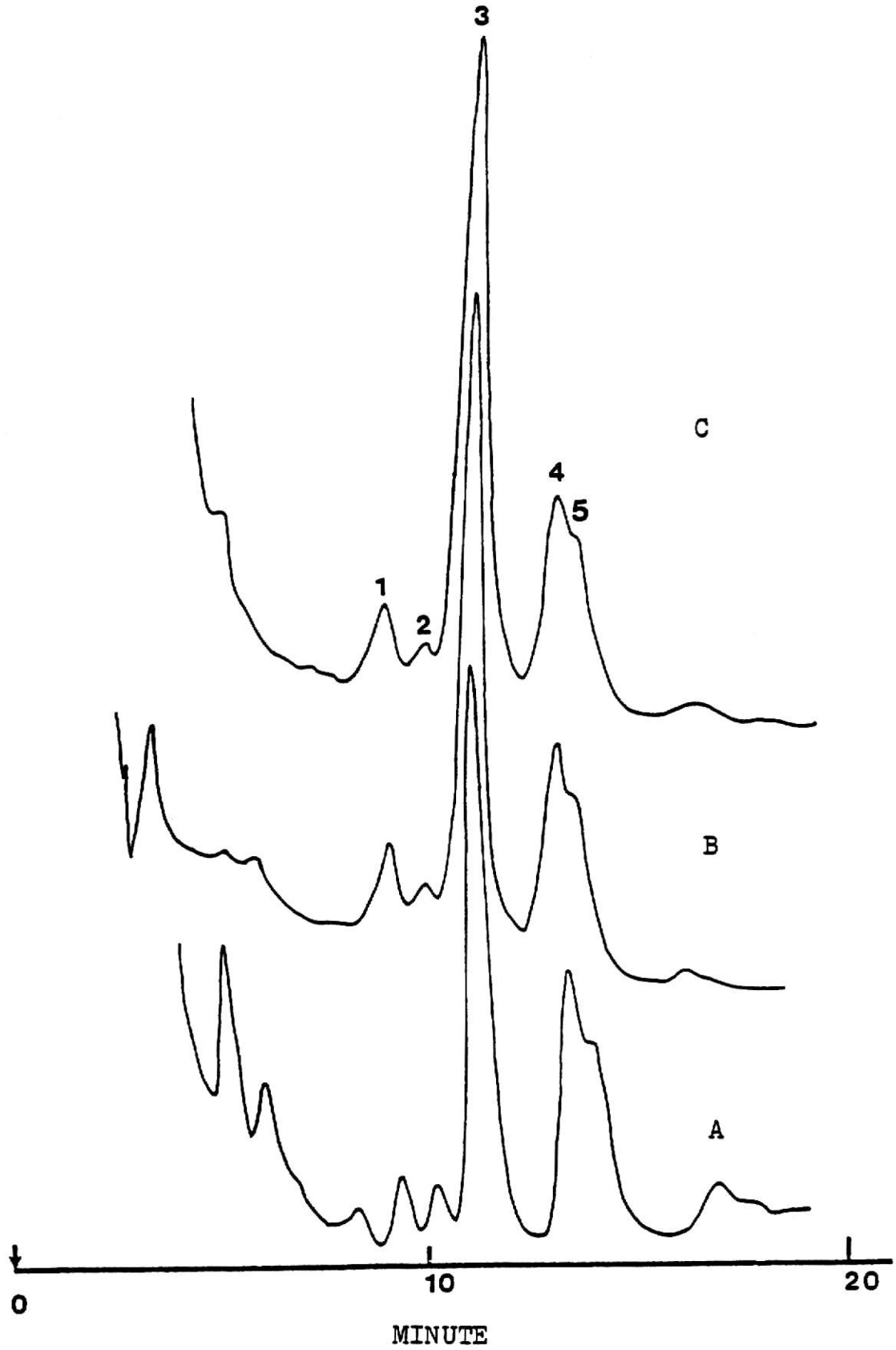


Fig. 14 HPLC separation of DGDG fractions obtained from whole wheat flour: (A) control; (B) stored at 37°C, 4 months; (C) stored at ambient temperature, 5 years. HPLC conditions: μ-Bondapak RP-18, 10 μm (4.1 mm i.d. x 25 cm), methanol/water (93:7), 1.0 ml/min. RI detector.

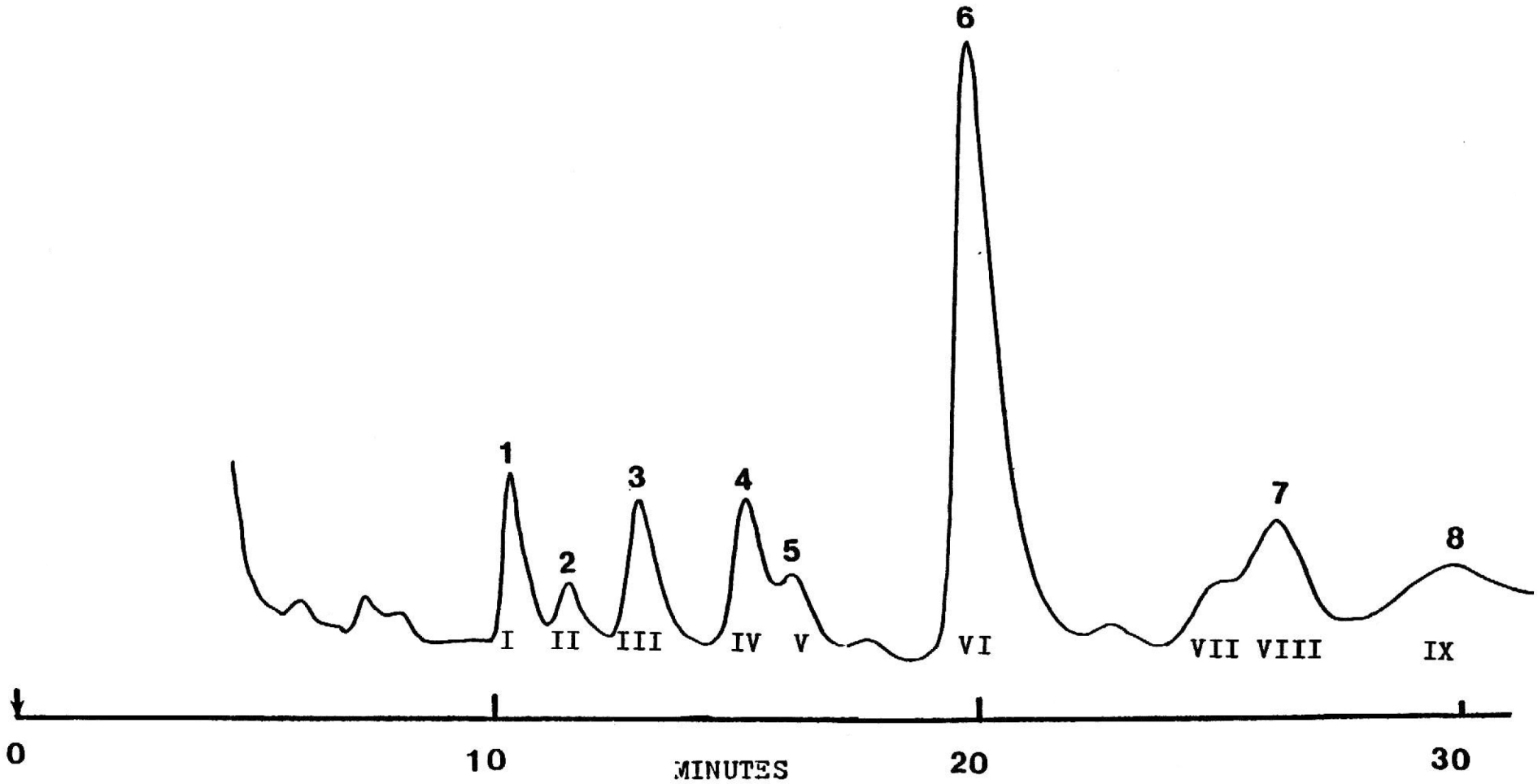


Fig. 15 HPLC separation of MGDG fraction of control whole wheat flour stored at -18°C for 2 weeks. HPLC conditions: μ-Bondapak RP-18, 10 μm (4.1 mm i.d. x 25 cm), methanol/water (90:10), 1.0 ml/min, RI detector.

tose in their work (MGDG peak I), and peak 7 was a combination of SN-1; palmitic, SN-2; linoleic, SN-3; monogalactose and SN-1; oleic, SN-2; linoleic, SN-3; monogalactose (MGDG peak II).

Tweeten et al. also identified peak 3 as SSG with GC and TLC. The commercial standard steryl glucoside had been run through the column under the same HPLC conditions. The retention time of standard steryl glucoside was the same as peak 3. Mass spectra were obtained for peak 3 in this research. The subfraction was collected and analyzed directly without prior derivatization using a direct inlet at 70 eV for electron impact ionization. A number of ion fragments observed in these mass spectra were useful in interpreting the structure of SSG (Fig. 16). Some of the characteristic ion fragments formed from the underivatized carbohydrate portion of the SSG are also summarized (Fig. 17). In general, the parent molecular ion was found in the mass spectra (577), glucose (179), glucose-16 (163), and β-sitosterol (397) were the major fragments of primary importance in interpreting the data. Other fragments shown on Fig. 17 were also important factors (fragment: 85, 141, 256).

e. <u>The change of the free MGDG fractions stored at higher temperature</u>

In an attempt to know the change of free MGDG fractions itself instantly, a dried control free MGDG fraction was stored in a 5 ml reactor vial at 37°C for 15 days. Its HPLC result is shown in Fig. 18B. Compared with the chromatogram of the control free MGDG fraction (Fig. 18A), it could be found that the ratio SSG to DLGG increased in the stored MGDG fraction. The tailing and unseparated peaks made the resolution bad. Checking the

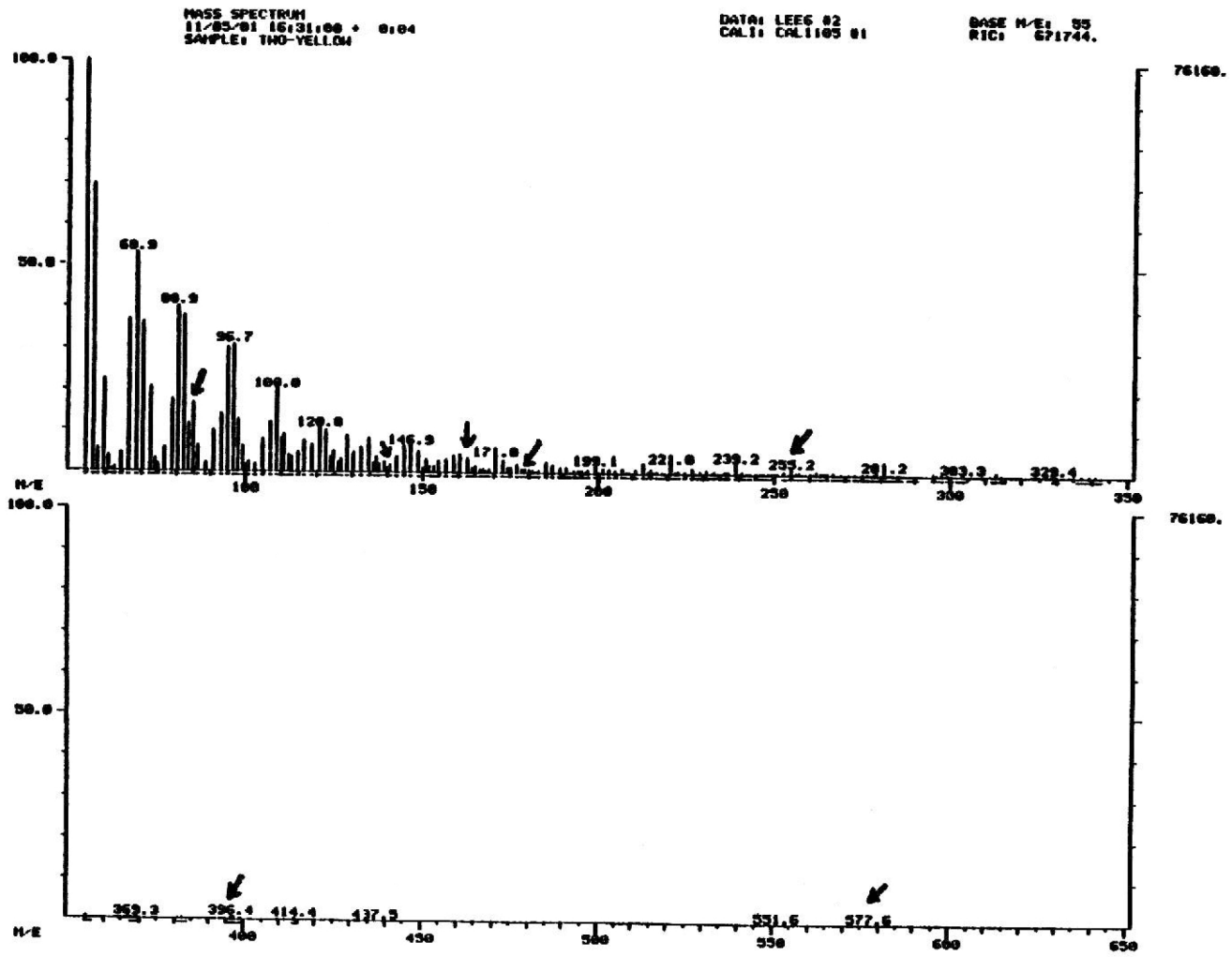


Fig. 16 Mass spectrum of sitosteryl-β-D-glucoside (peak 3 in MGDG fraction). Sampling by direct inlet at 70 eV for electron impact ionization.

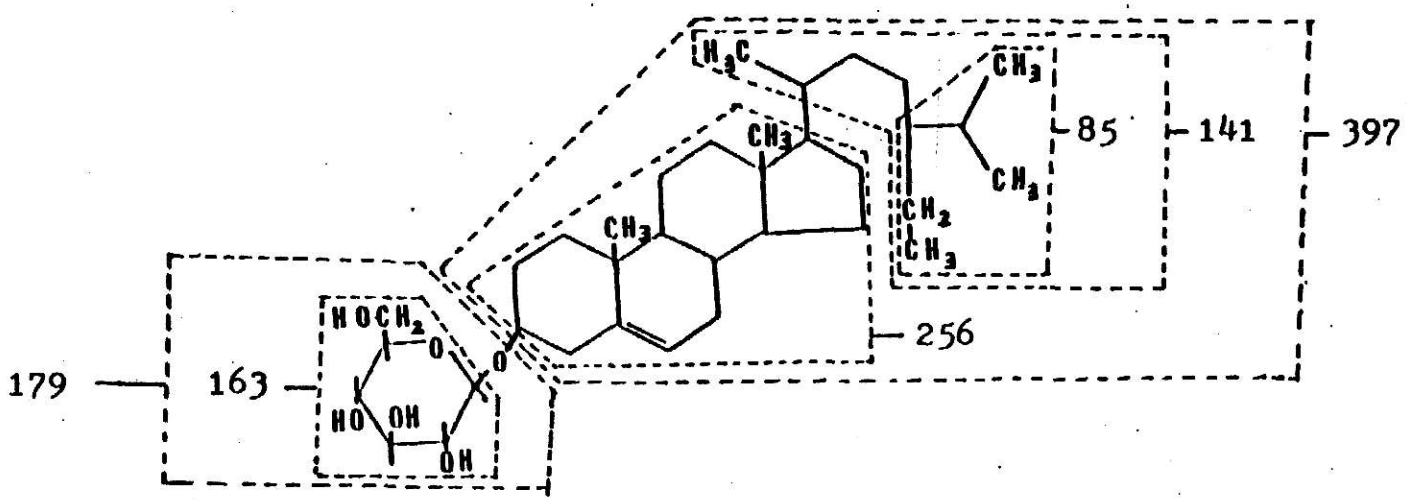


Fig. 17 Molecular ion fragments used to interpret the mass spectrum of sitosteryl-β-D-glucoside.

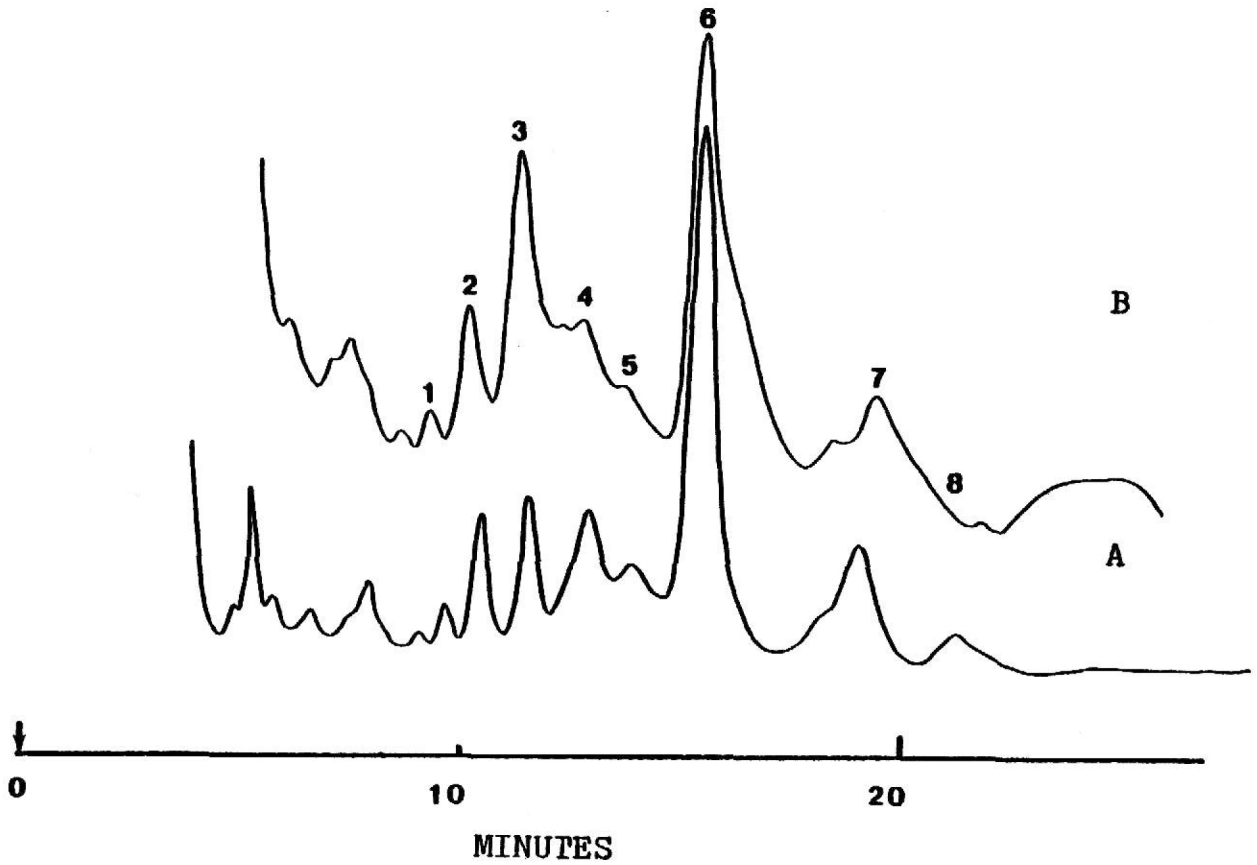


Fig. 18 HPLC separation of MGDG fraction of wheat lipid: (A) control; (B) MGDG fraction stored at 37°C, 15 days. HPLC conditions: μ-Bondapak RP-18, 10 μm (4.1 mm i.d. x 25 cm), methanol/water (93:7), 1.0 ml/min, RI detector.

lipids stored at higher temperature (Fig. 10B and Fig. 12B), it could be found that the bad resolution was easily obtained under unnatural conditions.

f. The change of free MGDG fraction in stored wheat

The hard red winter whole wheat flours were stored at 37°C for 8 months. Their free MGDG fractions were checked by HPLC every 4 weeks (Fig. 19). Using peak 6 as standard, other peaks were normalized at different stages as shown on Table IX. Whole kernel wheat was stored at 37°C for 48 weeks; its free MGDG fraction is also shown on Table IX.

The whole wheat flour stored at 37°C for 104 weeks was also checked after 2 years (Table IX). A mixture of 10 varieties of whole wheat flour, each stored at ambient temperature (most 24°C) for 5 years is shown in Table IX and Fig. 20C. These two longer stored wheat flours were used as guideposts of the storage change of free MGDG fractions.

All HPLC conditions were the same as before except the mobile phase which was methanol/water (93:7). From Fig. 19B and C, the peak 1/peak 6 and peak 4/peak 6 ratios in the chromatograms increased significantly, but peak 3/peak 6 ratios increased only a little. From the chromatograms of 12 weeks storage (Fig. 19D), peak 1/peak 6 and peak 4/peak 6 ratios decreased unexpectedly, but peak 3/peak 6 ratios still increased slowly. These results indicated that at an early stage of deterioration (37°C, 10 weeks), peak 1/peak 6 or peak 4/peak 6 ratios were a more sensitive indicator than peak 3/peak 6 ratios. The decreases of the ratios after 12 weeks suggested it was not a proper indicator to estimate the deterioration of long term storage. After checking the storage

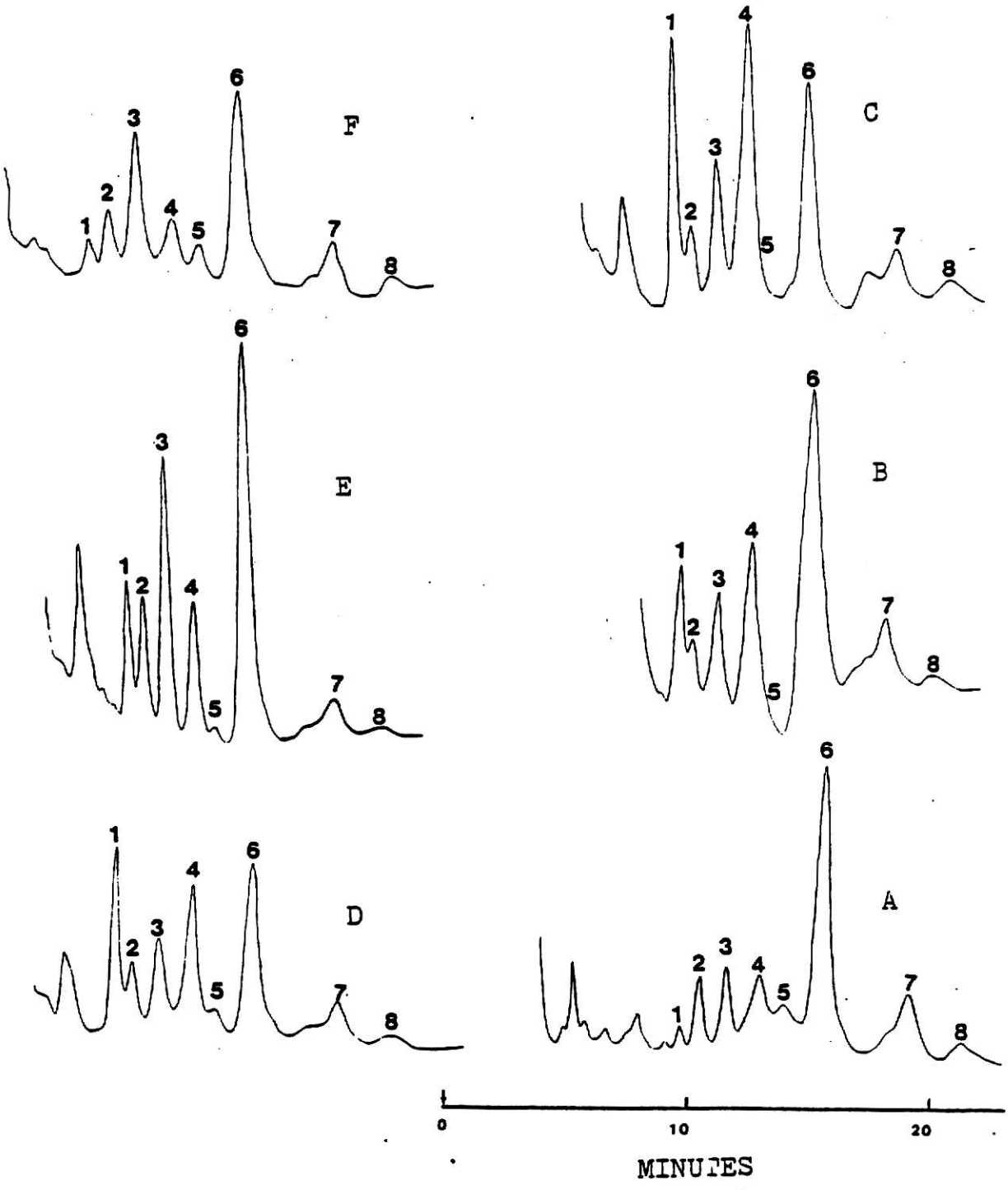


Fig. 19 HPLC separation of MGDG fractions obtained from whole wheat flours stored at 37°C: (A) control; (B) 4 weeks; (C) 8 weeks; (D) 12 weeks; (E) 6 months; (F) 8 months. HPLC conditions: μ-Bondapak RP-18, 10 μm (4.1 mm i.d. x 25 cm), methanol/water (93:7), 1.0 ml/min, RI detector.

Table IX. Relative Peak Areas to The Area of Peak 6 of MGDG Fraction of Control and Stored Whole Wheat Flour at 37°C

Storage Time	1	2	3	4	5	6	7	8
Starting Wheat	0.139	---	0.103	0.164	---	1.000	---	---
First Week								
Control[a]	0.184	---	0.112	0.194	---	1.000	0.174	0.033
Deterioration	0.343	---	0.133	0.269	---	1.000	0.171	0.042
4th Week								
Control	0.132	---	0.128	0.171	---	1.000	0.227	0.080
Deterioration	0.926	---	0.148	1.251	---	1.000	0.276	0.123
8th Week								
Deterioration	1.069	---	0.160	1.062	---	1.000	0.362	---
10th Week								
Deterioration	0.317	---	0.215	0.493	---	1.000	0.286	0.227
12th Week								
Deterioration	0.511	---	0.238	0.943	---	1.000	0.175	---
20th Week								
Control	0.146	---	0.153	0.156	---	1.000	0.181	0.038
Deterioration	0.655	0.137	0.375	0.632	0.118	1.000	0.316	0.034
32nd Week								
Deterioration	0.089	0.169	0.519	0.167	0.095	1.000	0.305	0.160
34th Week								
Control	0.586	---	0.207	0.746	---	1.000	0.204	---
Deterioration	0.094	0.204	0.532	0.171	0.065	1.000	0.333	0.240
48th Week								
Whole Wheat[b]	0.294	---	0.329	0.506	---	1.000	0.160	0.420
104th Week								
Deterioration	---	---	(1.23)	---	---	(1.000)	---	---
1977 HRW Wheat[c]	0.094	0.245	0.786	0.181	0.228	1.000	0.225	0.487

[a]Stored at -18°C.

[b]Only whole kernel wheat in this table.

[c]A mixture of 10 varieties of 1977 whole wheat flour stored at ambient temperature five years.

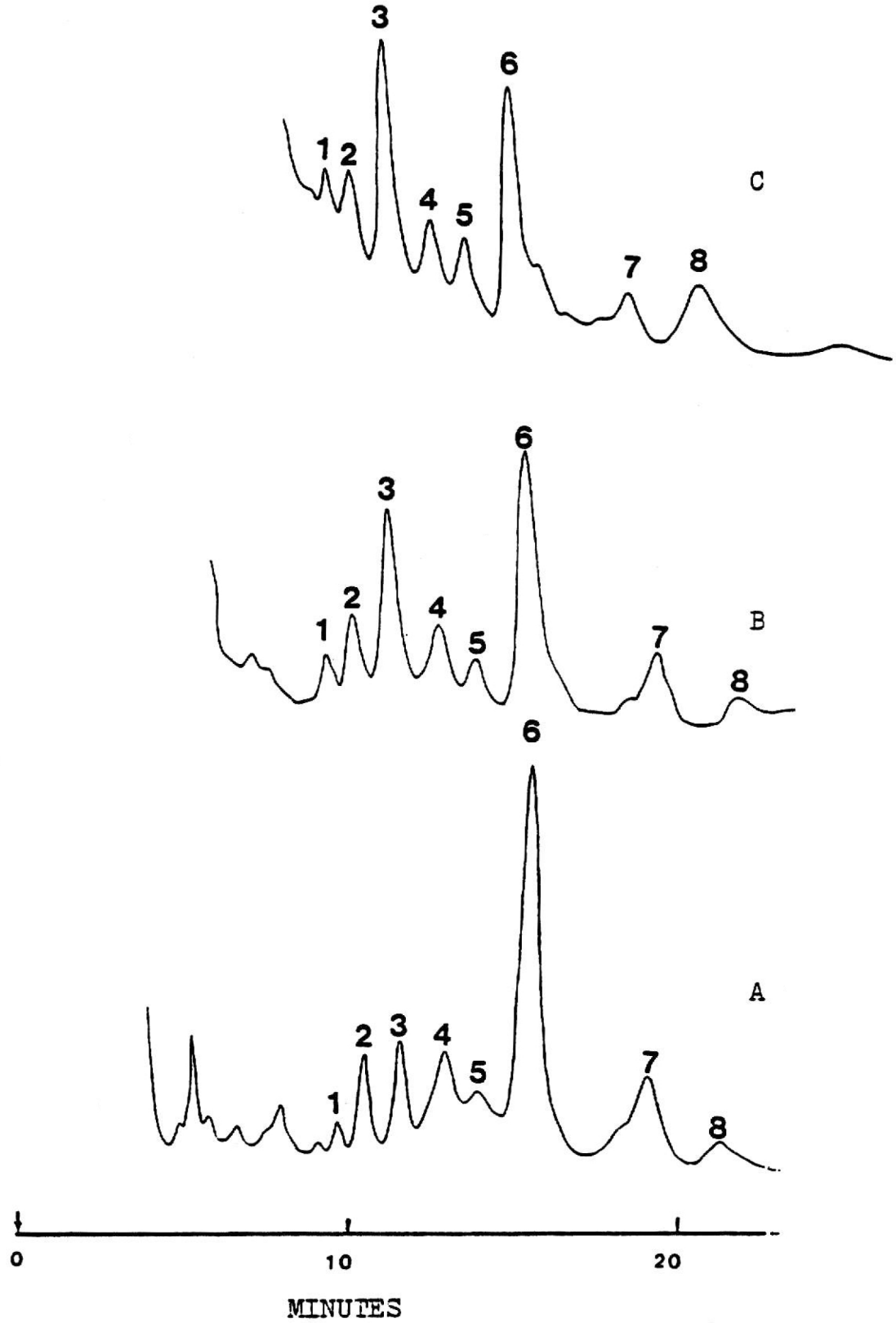


Fig. 20 HPLC separation of MGDG fractions obtained from whole wheat flours: (A) control; (B) stored at 37°C, 8 months; (C) stored at ambient temperature, 5 years. HPLC conditions: μ-Bondapak RP-18, 10 μm (4.1 mm i.d. x 25 cm), methanol/water (93:7), 1.0 ml/min, RI detector.

of wheat at 6 months (Fig. 19E), 8 months (Fig. 19F), and 5 years (Fig. 20C), a significant increasing trend of peak 3/peak 6 ratios was found. This indicated that peak 3/peak 6 ratios might be a good indicator of deterioration for long term storage.

A linear relationship between peak 3/peak 6 ratio (SSG/DLGG) and storage time (weeks) has been shown on Fig. 21. A correlation coefficeint of 0.95 was obtained. It could be concluded that SSG/DLGG ratios were a favorable indicator. From Table IX, the whole kernel wheat stored at 37^{o}C, 48 weeks had a value of 0.329; it was equal to the whole wheat flour stored for 16 weeks. These two samples were stored at the same conditions (except one was whole kernel and the other was whole wheat flour). It indicated that the deterioration rate in whole kernel was one third of whole wheat flour. Although one third may not be suitable for all varieties of wheat, the slower deterioration of whole kernel versus whole wheat flour was reasonable.

Because the wheat varieties, previous history of wheat, extraction, purification, and analytical method were different, the quantitative comparison of the lipid changes during storage with other authors was difficult.

Warwick et al. (173) found that when "medium grist flour" was stored at 12 $\pm$ 2^{o}C for 54 months, the MGDG extracted with water saturated n-butanol decreased (from 64 mg to 42 mg/100 g of flour). Clayton and Morrison (34) found the lipid classes change of four different flours (high-grade winter, high-grade spring, low-grade winter, and low-grade spring) stored at 37^{o}C for 3 months or 25^{o}C for 4 months. Using water saturated n-butanol extractant, the lipid was run through a silica gel open column

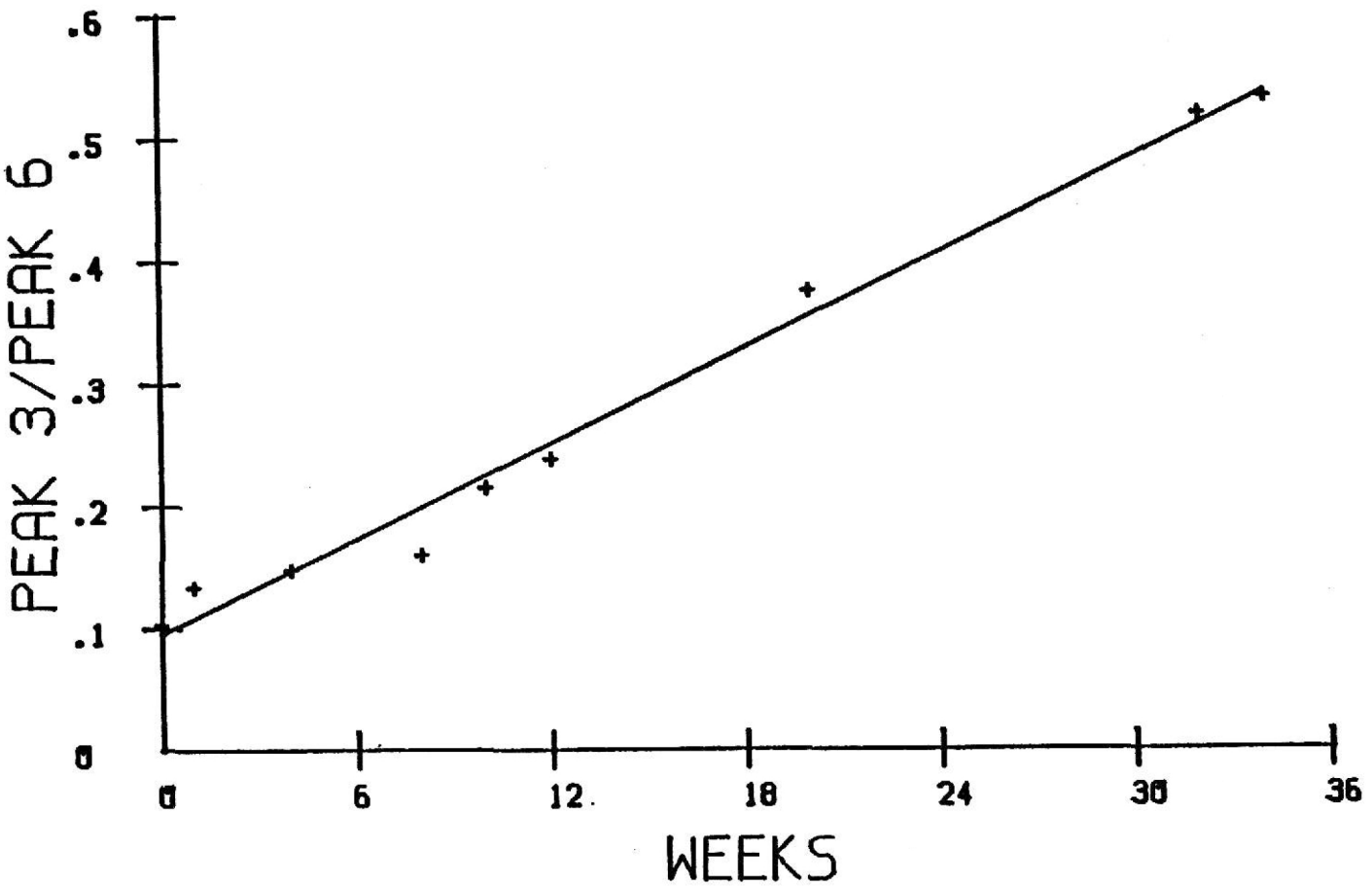


Fig. 21 The relationship between the ratio SSG to DLGG (peak 3/peak 6) and storage time.

into neutral, glyco, and phospholipid fractions. Glycolipids was further separated by TLC. They found that MGDG decreased about one-third in four varieties of wheat flour. Steryl glucoside and ceramide monohexoside were not separated in their results. The total amount of steryl glucoside and ceramide monohexoside in control and deteriorated flour showed almost no significant change. The ratios steryl glucoside plus ceramide monohexoside/MGDG in control flour were from 0.31 to 0.38; in deteriorated flour were from 0.45 to 0.68. The increasing trend of the ratio in deteriorated wheat was the same as our report. Lin et al. (87) separated steryl glucoside from ceramide monohexoside in 3 varieties of hard red spring wheat flour. They reported 5.4% MGDG and 1.0% steryl glucoside in Red River 68. The ratio of steryl glucoside/MGDG was 0.1850; it was close to our results. They also reported the composition of steryl glucoside by GC. In the steryl glucoside of Red River 68, 26.2% was cholesterol, 15.8% was campesterol, and 58.0% was β-sitosterol. In my earlier MS work, peak 3 contained only glucoside of β-sitosterol. Therefore, when only 58.0% β-sitosterol was considered, the ratio steryl glucoside/MGDG of their results was very close to ours. It could be concluded at this point that the combination of open column and HPLC had the best potential in the lipid quantitative work.

g. GC of MGDG subfractions

The MGDG fraction of wheat was run through HPLC (Fig. 15). The individual subfractions were collected for fatty acid determination (Table X). These GC results verified that peak 6 (subfraction VI) of the MGDG fraction contained mostly linoleic acid.

Comment: Using SSG/DLGG ratios as an indicator of deteriora-

Table X. Fatty Acid Composition of MGDG Subfractions of Wheat[a]

Subfractions	Fatty Acid (%)					
	C 14:0	C 16:0	C 18:0	C 18:1	C 18:2	C 18:3
I.	(Trace)	---	---	---	---	---
II.	---	25.9	---	18.3	51.9	3.9
III.	---	39.1	4.1	15.4	41.4	---
IV.	---	26.0	2.0	15.3	51.6	5.1
V.	---	33.0	1.9	10.0	46.2	8.9
VI.	---	9.8	---	3.1	87.1	---
VII.	5.5	20.8	3.5	10.0	57.5	2.8
VIII.	---	19.6	4.9	19.2	54.5	1.8
IX.	---	28.2	---	16.7	55.2	---

[a]HPLC separation of μ-Bondapak RP-18, 10 μm; values reported are the average of two determinations.

ted wheat had many advantages: 1) The reasonable retention time made the routine analyses of wheat possible; 2) The completely resolved SSG and DLGG from other peaks made the quantitative work easy and accurate; 3) The isocratic solvent system was convenient and cheap; 4) No external standard was required. Using only the original two-compound concentration (SSG and DLGG) in the fraction produced less error from the preparation of external standard concentrations. 5) It gave a better reproducibility than other methods; 6) The SSG/DLGG ratio might represent an actual level of baking quality. Two different varieties of new harvested wheat might have different values and baking quality. The lower the ratio, the better the baking quality; and 7) The incomplete extraction of total lipids could make an error in the absolute or external standard method. However, the error was very low in this SSG/DLGG method.

Although peak tailings, unresolved shoulders, and low DLGG concentration in the longer stored wheat made the quantitation difficult, this method was linear and good for wheat stored at 37°C for 8 months. Beyond this point, the wheat might be too bad to be recovered; any measurement was meaningless.

h. Check of control wheat and new crop wheat

The ratio of new crop hard red winter whole wheat (1981) was 0.110. The ratio of control whole wheat (1980) was 0.104. The ratio of control whole wheat (1980) stored at -18°C under nitrogen for 34 weeks was 0.106 (Table XI). Also, the ratio of the MGDG chromatogram in Tweeten's thesis was 0.100.

From these data, it could be concluded that the new harvest hard red winter wheat had a ratio very close to 0.100. It showed

Table XI. Relative Peak Areas to The Area of Peak 6 of MGDG Fraction of the Starting and the Control Wheat

	1	2	3	4	5	6	7	8
Control Wheat (1980)	0.140	---	0.103	0.165	---	1.000	---	---
Whole Kernel Control Wheat Stored at -18°C for 34 weeks under Nitrogen	0.116	---	0.106	0.161	---	1.000	---	---
New Crop Wheat (1981)	0.122	---	0.110	0.179	---	1.000	---	---

Average of duplicate analyses

that the SSG/DLGG method could indicate the stored time since harvest. Also, it showed a good reproducibility.

D. Other Analytical Methods

a. Fat acidity titration

The fat acidity of three whole wheat flours had been titrated by the AACC approved method 1969, 02-01 fat acidity; general method. Whole wheat flour stored at -18°C for 34 weeks had a fat acidity of 21.1 (mg KOH/100 g flour). This value was higher than fresh harvest wheat 6-10 (mg KOH/100 g flour), because the control sample was taken out of the freezer every 2 weeks for 34 weeks. Hydrolysis might have occurred during this period. The whole wheat flour stored at 37°C for 34 weeks was 130.9 and the mixture of 10 varieties of wheat (each stored at ambient temperature for 5 years) was 190.4. The results are shown on Table XII.

All of the data were based on the duplicate extraction with Skellysolve F and triplicate titration. The emulsion formed during the titration masked the end point. The end point matching color with standard reagent color was dependent on personal technique. Because the color change was not very sensitive, a relative standard deviation of 15% was possible. In order to eliminate the error in the fat acidity titration method, it might suggest that the SSG/DLGG method replace it.

b. Gravimetric determination of control and deteriorated whole wheat flours

The moisture contents, Skellysolve F extracts, nonpolar lipids, MGDG fractions, DGDG fractions, and phospholipid fractions of the control and stored flours (37°C, 8 months) were investigated (Table XIII). Both Skellysolve F extracted lipids had

Table XII. Fat Acidity in Skellysolve F Extracts of Stored Whole Wheat Flour*

	Moisture Content (%)	Fat Acidity (mg. KOH)
Control (Flour stored -18°C, 34 weeks)	10.78	21.1
Deterioration (Stored at 37°C, 34 weeks)	8.80	130.9
Deterioration (Stored at amibient temperature, 5 years)	9.70	190.4

*Approved methods of the American Association of Cereal Chemists. Revised and reprinted October, 1969. 02-01. Fat Acidity - General Method

Table XIII. Moisture Content, Total Skellysolve F Extract, Nonpolar Lipids, MGDG, DGDG, and Phospholipids in Hard Red Winter Wheat Flour Stored at 37°C for 8 months[a/b/c]

	Moisture Content (%)	Skellysolve F Extract (%)	Nonpolar Lipid Fraction(%) (Dry Basis)	MGDG Fraction(%) (Dry Basis)	DGDG Fraction(%) (Dry Basis)	Phospholipid Fraction(%) (Dry Basis)
Control	10.78	1.66	1.48	0.06	0.02	0.08
Deterioration	8.82	1.63	1.46	0.05	0.02	0.08
1977 HRW Wheat[d]	9.70	1.44	0.98	0.26	0.06	0.14

[a]Original sample was 11.40% moisture content.

[b]Lipid was duplicate extraction and triplicate fractionation with silica gel open column.

[c]Average of duplicate analyses.

[d]A mixture of 10 varieties of 1977 HRW whole wheat flour stored at ambient temperature for 5 years.

the same clear yellow color, but the relative gravimetric determination of each fraction did not show any significant difference. However, when the MGDG fractions chromatographed from the lipids (extracted from the same amount of control and stored wheat) were dissolved in the same volume of solvent, the stored wheat had a lower response of DLGG than control wheat. This indicated that the absolute amount of DLGG was decreased as the storage time increased.

General conclusions on the HPLC analyses of polar lipid fraction of wheat were as follows:

1. Polar lipids and glycolipids fractions both showed substantial changes during the high temperature storage. However, complicated and unsymmetrical peaks made the quantitation very difficult. The phospholipid fractions did not show any major peak in the same HPLC condition. The DGDG fractions had a simple chromatogram, but no significant change had been found during the storage.

2. MGDG chromatograms showed that peaks 3 and 6 were completely resolved from other peaks. Peak 3 was sitosteryl-β-D-glucoside and peak 6 was *sn*-1,2-dilinoleyol-3-galactosylglycerol. The higher the ratio, the longer the storage. It suggested that the ratio could be a good indicator of time-temperature storage. However, the extraction and fractionation were time consuming, a fast procedure needed to be developed.

3. Different varieties of wheat have different ratios of SSG to DLGG when harvested and the hydrolysis rate of SSG and DLGG are probably different. For the specific compound stored at constant conditions, a constant reaction rate is possible.

Therefore, it is reasonable that the ratio SSG/DLGG increase at a constant rate. From Fig. 21, it can be concluded that different varieties of wheat will probably have same slope but different intercepts. Projection of the future condition of stored wheat should be possible upon calibration to certain temperature and humidity storage conditions.

4. Recently, using HPLC Tweeten and Wetzel (184) determined individual FFA present in the crude oat lipid extracts by selective derivatization under controlled pH conditions. With p-bromophenacyl bromide derivatives, both individual FFA and total FFA were determined. Using HPLC determined FFA as an indicator of stored grain condition requires carefully 1) pH adjustment, 2) complete recovery of free lipids, and 3) accurate internal standard (margaric acid) measurement. With this approach the relationship of the increase of FFA with storage time is curvelinear and the FFA content may in fact decrease at some stage due to oxidation to hydroxy-fatty acids.

5. This project was a series of the research of Tweeten et al. (158). They used nonpolar bonded phase HPLC partition systems to analyze previously preparative column chromatographic fractionated glycolipids from petroleum ether extracts of wheat flour lipids in 1977. They (159) identified three major peaks as DGDG in the separation of polar lipid fractions of wheat flours. TLC gave only a single DGDG spot from the same sample. Using MS, chromatographic analysis, and wet chemical methods, they (162) elucidated the chemical composition of fractions collected from the chromatographic separation. Approximately 35% of each DGDG subfraction was accounted for as carbohydrate. The separation of glycolipids on a reverse phase column had better resolution

and selectivity than on a silica gel column (167). Different fatty acid composition of MGDG or DGDG could be resolved. A routine quantitative analyses of MGDG and DGDG was developed. The first fraction of DGDG or MGDG separated by HPLC contained linoleic acid on both SN-1 and SN-2 positions whereas, in the second fraction, palmitic and oleic acids were preferentially located on the SN-1 position and linoleic acid was on the SN-2 position. All of the results make the resolution of SSG and DLGG good and make this research possible.

A comparison of IR and variable wavelength detectors for the determination of MGDG and DGDG in wheat flour have been developed (161). Using gradient elution, DGDG peak II were further separated to two compounds: SN-1: palmitic, SN-2: linoleic, SN-3: digalactose: and SN-1: oleic, SN-2: linoleic, SN-3: digalactose. They (164) also examined the effects of mobile phase strength, column packing stationary phase, packing particle size, and column temperature on both the efficiency and selectivity of the glycolipids separation. DGDG peak I and II, DLGG, and MGDG peak II were separated from each other with LiChrosorb RP-8 or Spherisorb C 6 column under isocratic or gradient elution. Although all of the efforts improved the resolution of glycolipids, the resolution of SSG in polar lipids or total lipids fraction was still poor.

6. Recently, Tweeten et al. (166) developed an automated multidimensional HPLC for simultaneous clean-up and determination of wheat glycolipids. Both DGDG peak I and II, DLGG, and MGDG peak II were resolved from each other. Although quantitation of DLGG was not difficult, quantitation of SSG was not convenient,

because SSG was not completely resolved from other peaks. Also, peak 1 and 4 in MGDG fraction were increased when whole wheat flour was stored at 37°C for 12 weeks. These all increase the difficulty of the quantitation of SSG. It is doubtful that the multidimensional HPLC can be applicable to the SSG/DLGG method now. However, a further fractionation of pure MGDG fraction is desired to solve the resolution problem.

II. Improvement of Sample Preparation and Fractionation Efficiency

A. Attempted Aspirator Chromatographic Fractionation

Because the normal open column fractionation was time consuming (2 days) and lipid decomposition was possible in fractionation procedure, a fast silica gel open column fractionation connected with aspirator was used. The MGDG fractionation could be obtained in 30 minutes. The HPLC results of aspirator fractionation was the same as normal open column fractionation. Although this fractionation procedure was very fast, fractions from it caused a clog problem in the HPLC system. The filter in the HPLC column needed to be changed every 5-6 injections.

In order to solve the clog problem, subfraction of the MGDG fraction was attempted. It was desired to elute the pure SSG (peak 3) and DLGG (peak 6) in one subfraction and eliminate other lipids. After checking the composition of the different MGDG subfractions, it was found that the best result was obtained with the following fractionation procedure: eluting the nonpolar lipid with 350 ml chloroform, using 100 ml chloroform/acetone (8:2) to elute other lipids in the MGDG fraction, then eluting most of the SSG and DLGG with 100 ml chloroform/acetone (5:5) in the last sub-

fraction. After normalizing the attenuation and concentration of each component, Table XIV and Fig. 22 shows the results of the components in each subfraction. Most SSG and DLGG existed in the 100 ml chloroform/acetone (5:5) subfraction; they were completely absent in the 100 ml chloroform/acetone (8:2) subfraction.

Table XIV. Lipid Composition in the MGDG Subfraction

Peak	Other	0	1	2	3	4	5	6	7	8
Chloroform:Acetone										
8 : 2	large	4.0	7.3	1.3	0	7.6	0	0	1.6	0
5 : 5	1.4	0.4	0.4	1.3	5.7	0.5	1.4	12.3	1.5	4.2

B. Test of the Minimum Requirements of Silica Gel Packing Material and Solvent for Fractionation

5 g, 10 g, and 16 g portions of silica gel had been tested for the fractionation of extracts containing 400 mg wheat lipids. The lipid fractions were eluted with 350 ml chloroform, 100 ml chloroform/acetone (8:2), and 100 ml chloroform/acetone (5:5). Fig. 23 and 24 show the HPLC results of (8:2) and (5:5) subfractions of different amounts of silica gel, respectively.

Fig. 23C and Fig. 24C show the HPLC results of the (8:2) and (5:5) subfractions eluted from 5 g silica gel. Fig. 24C is similar to a chromatogram of the DGDG fraction (Fig. 13). It indicated that 5 g silica gel did not have enough capacity to retain the DGDG fraction. Fig. 23B and Fig. 24B are the results of HPLC chromatogram of 10 g silica gel. SSGtcould be completely retained in the column with (8:2) solvent. However, DLGG was only partially

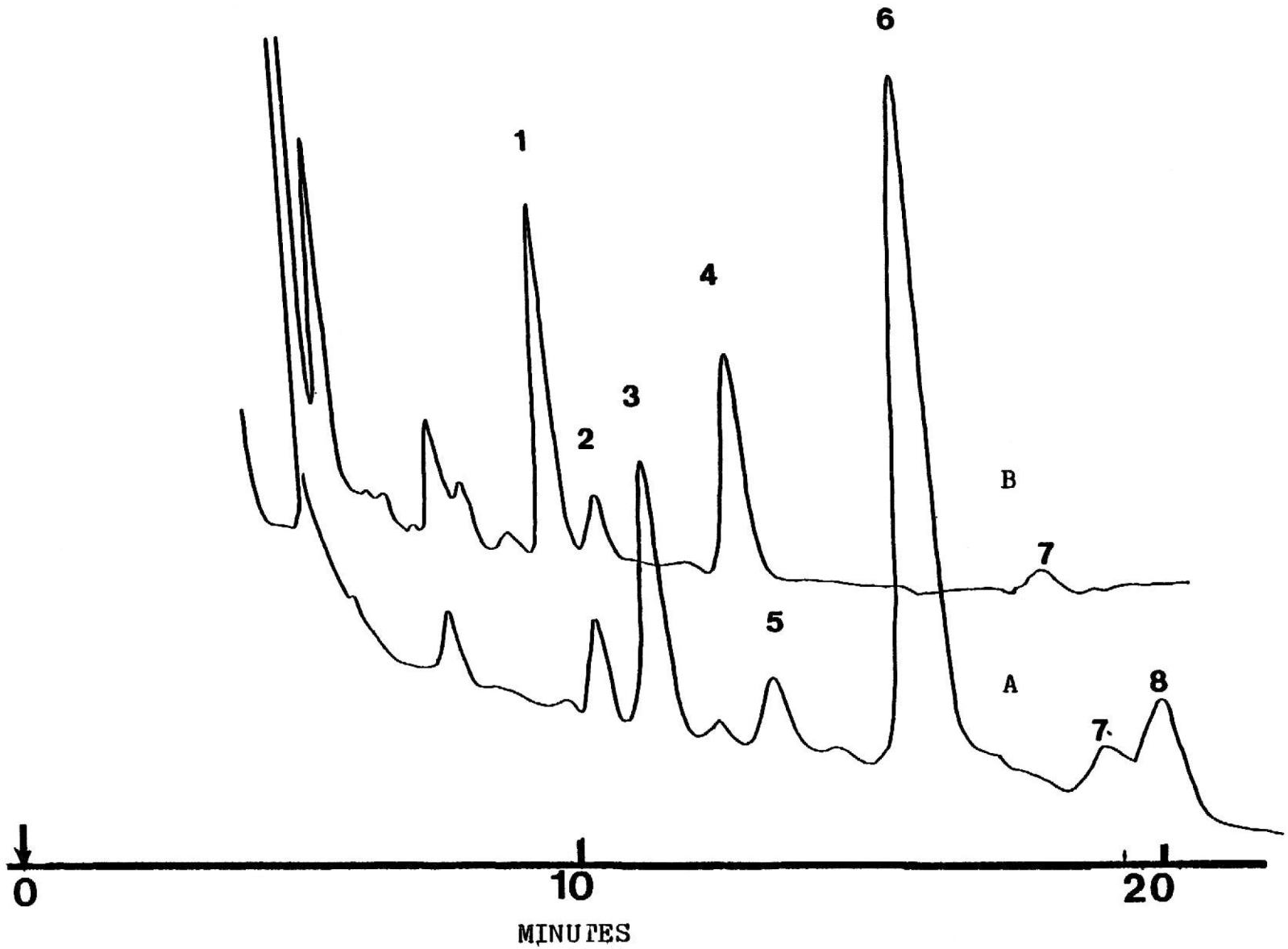


Fig. 22 HPLC separation of wheat MGDG (chloroform/acetone) subfraction: (A) 5:5 MDG subfraction; (B) 8:2 MGDG subfraction. HPLC conditions: μ-Bondapak RP-18, 10 μm (4.1 mm i.d. x 25 cm), methanol/water (93:7), 1.0 ml/min, RI detector.

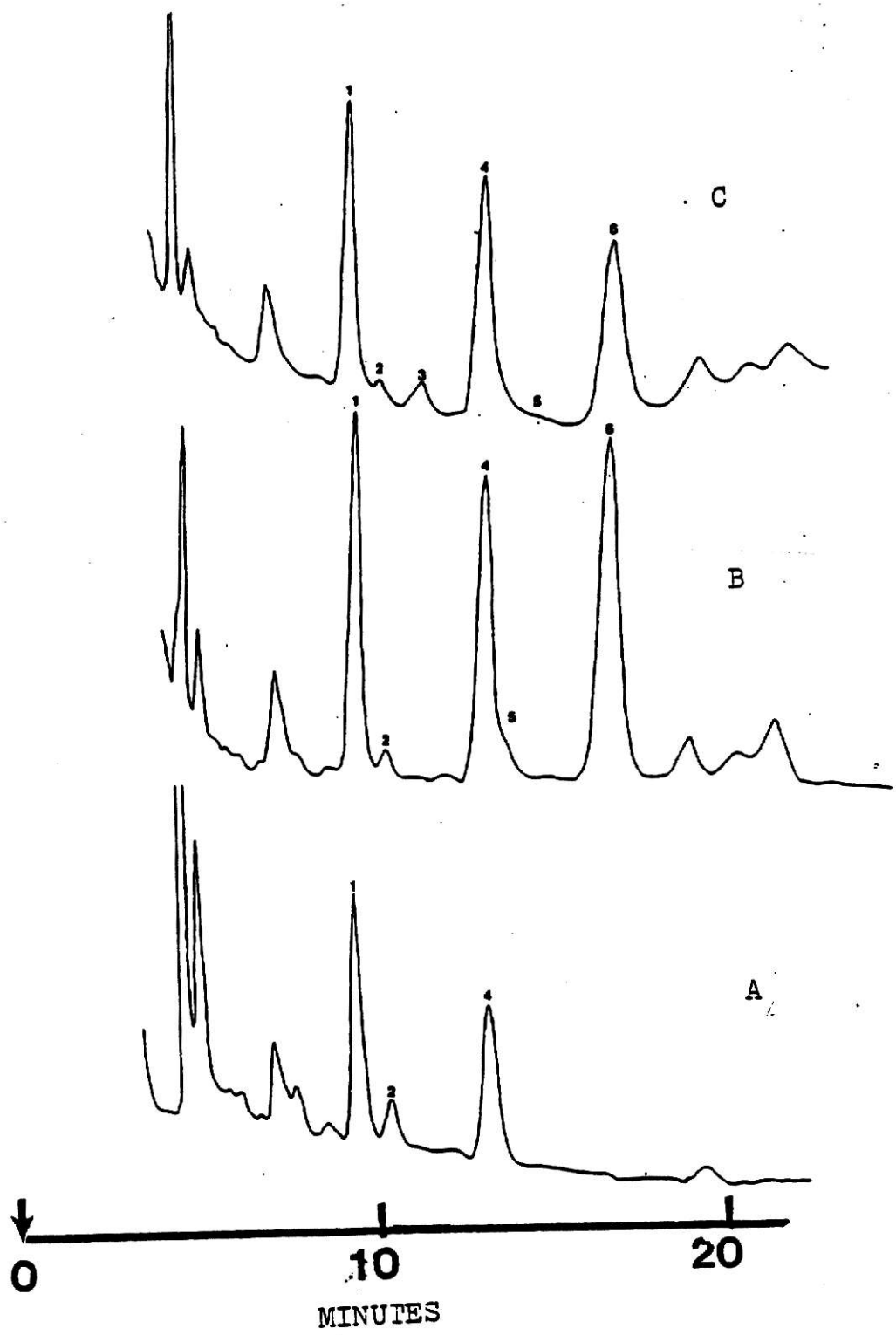


Fig. 23 HPLC separation of chloroform/acetone (8:2) MGDG subfraction of wheat lipid: (A) 16g; (B) 10 g; (C) 5g silica gel. HPLC conditions: μ-Bondapak RP-18, 10 μm (4.1 mm i.d. x 25 cm), methanol/water (93:7), 1.0 ml/min, RI detector.

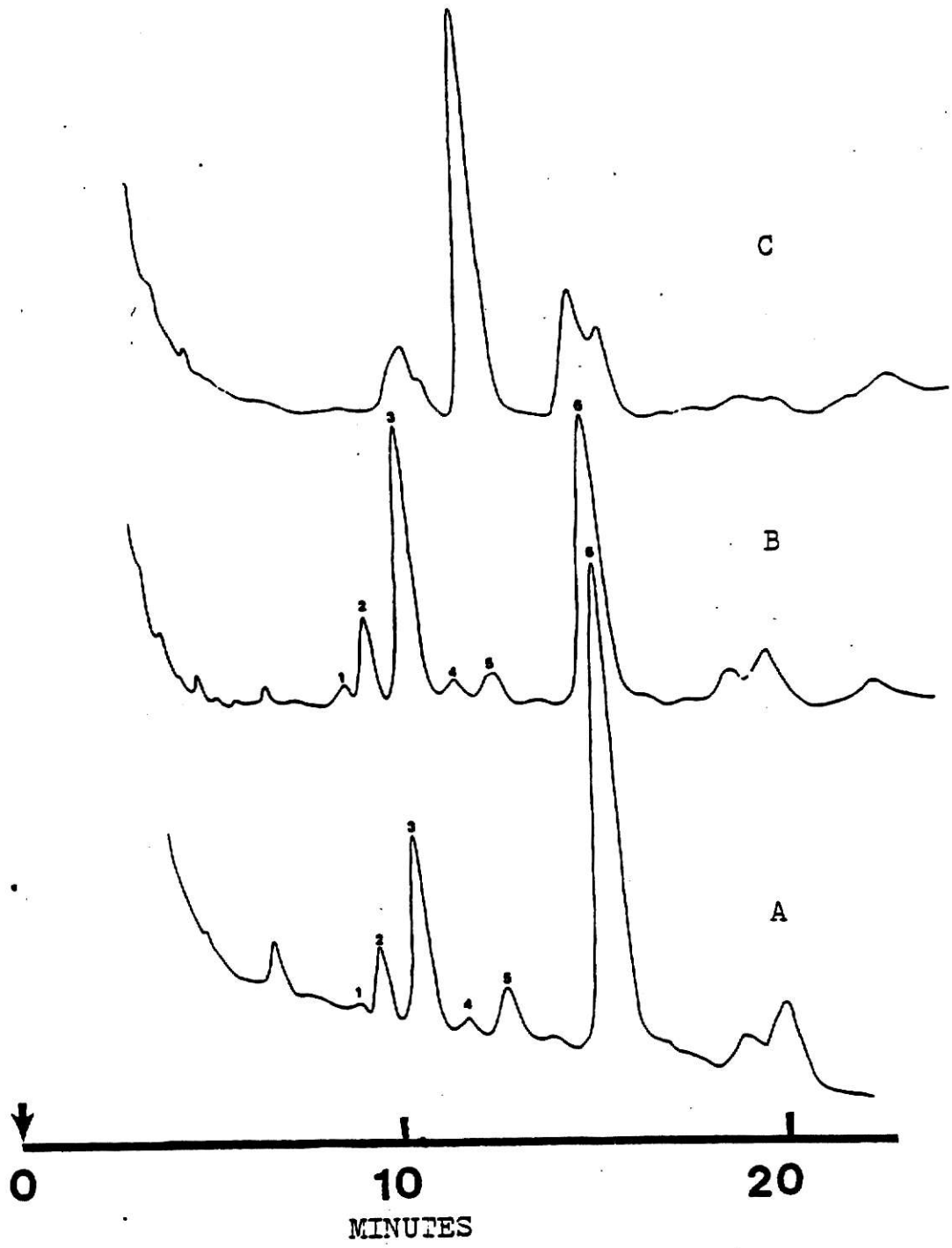


Fig. 24 HPLC separation of chloroform/acetone (5:5) MGDG subfraction of wheat lipid: (A) 16 g; (B) 10 g; (C) 5 g silica gel. HPLC conditions: μ-Bondapak RP-18, 10 μm (4.1 mm i.d. x 25 cm), methanol/water (93:7), 1.0 ml/min, RI detector.

retained. Part of peak 6 was eluted with the (8:2) subfraction. This indicated that the capacity of 10 g silica gel was still not enough. The 16 g silica gel could completely retain SSG and DLGG in the column when the (8:2) subfraction was eluted (Fig. 23A and Fig. 24A). Complete retention was very important for the quantitative work with SSG/DLGG method. It could be concluded that 16 g of silica gel was enough for fractionation of extracts containing 400 mg of wheat lipids.

Different volumes of solvent had been tested to fractionate the wheat lipids in a 16 g silica gel column (Chloroform: 140 ml-350 ml for nonpolar lipid fraction; chlorofrom/acetone (8:2): 40 ml-100 ml for (8:2) subfraction; chloroform/acetone (5:5): 40 ml-100 ml for (5:5) subfraction).

The minimum requirement of chloroform was 160 ml, chloroform/acteone (8:2) was 60 ml, and chloroform/acetone (5:5) was 60 ml. Under this eluting system, it had the same chromatogram as 350 ml, 100 ml, and 100 ml eluting system. With 160 ml, 40 ml, and 40 ml eluting systems, some of the (8:2) subfraction components were eluted with the (5:5) subfraction. With 140 ml, 60 ml, and 60 ml eluting systems, the nonpolar lipids were not completely eluted with 140 ml chloroform (a yellow ring or spot remained at the bottom of the column).

Comment: The MGDG fraction was separated into two subfractions successively. However, SSG and DLGG existed in the second subfraction (5:5). The additional fractionation step resulted in time, labor, and solvent waste. Also, although it partially improved the clog problem, clogging still happened. This suggests that the clog problem may come from other sources (small silica

gel, impure materials in lipids or silica gel).

In order to solve the clog problem in the fractionation step, a sinter glass filter was set at the bottom of the column. The flow rate was very slow, even connected to the aspirator (0.8-1.0 ml/min). A lot of water was consumed in the aspirator making the procedure impractical.

C. Attempted On-Line HPLC Cleanup and Analysis of Lipids

The on-line HPLC method was tried to simplify and accelerate the fractionation procedure. Wheat lipids were chromatographed using two columns in series, a preparative column and an analytical column. The preparative column was LiChrosorb SI-60, 30 μm, 4.1 mm i.d. x 15 cm, flow rate, 3 ml/min. The nonpolar lipid fraction was eluted with 50 ml chloroform into waste. The MGDG fraction was eluted into analytical column with 30 ml chloroform/acetone (5:5).

The analytical column was μ-Bondapak RP-18, 10 μm, 4.1 mm i.d. x 25 cm, mobile phase, methanol/water (93:7), flow rate, 1 ml/min. No peaks were detected in the chromatogram because the MGDG fraction was diluted in the HPLC column and buried in the baseline. Different portions of the MGDG fraction were run into the analytical column separately (0-3 ml, 3-6 ml, ----, 27-30 ml). Most of the portions did not show any peak. Some portions had peaks but they were too broad or unsymmetrical to be quantitated. Also, large amounts of wheat lipids were injected (5 mg-100 mg), but no chromatograms of analytical quality were obtained. Different particle diameter (10-30 μm) preparative column packing materials were tested, but none of the chromatograms were useful.

Comment: The major problems were: 1) a gradient solvent system was formed in the analytical column due to the change of solvent system from chloroform/acetone to methanol/water, it did not work with the RI detector, and 2) when MGDG fraction was run into the analytical column, it was diluted furthermore in the HPLC system; only broad and unsymmetrical peaks could be found.

D. Attempted Batch Adsorption Fractionation

In order to decrease the time consumed in the fractionation, a batch adsorption method was tried. 400 mg of wheat lipids were mixed with 16 g of silica gel in 300 ml chloroform, stirred 30 min, filtered then washed with 50 ml chloroform. The residue was mixed with 200 ml chloroform/acetone (5:5), stirred 30 min, and filtered then washed with 30 ml chloroform/acetone (5:5) solvent.

The (5:5) fraction was concentrated and injected into the HPLC with a 10μl valve injector. Fig. 25A shows the results of the batch adsorption method. Fig. 25B shows the results of the silica gel open column method. The completely different chromatograms indicated that the batch adsorption method was not useful for the replacement of the silica gel open column chromatographic method.

E. Preparative High Pressure Liquid Chromatography

Several preparative grade packing materials had been tested in the preparative high pressure liquid chromatography. Their results were shown as follows:

a. Syloid 72 (Davison Chemical)

Its mean particle size was 6 μm (90% < 10.6 μm, 10% < 2.93 μm). The advantages were: the chromatogram of MGDG frac-

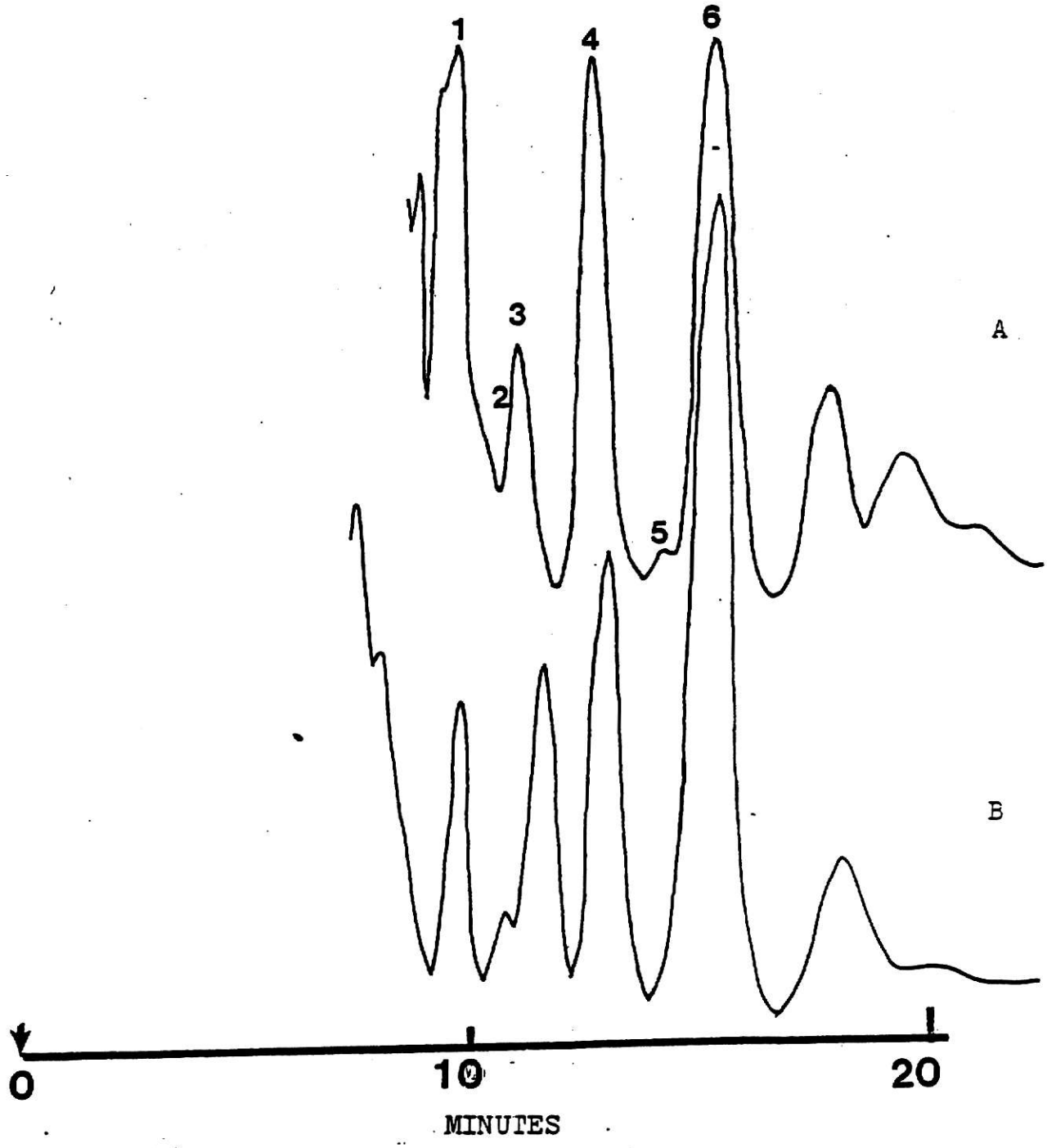


Fig. 25 HPLC separation of wheat MGDG fractionated with (A) batch adsorption and (B) silica gel open column. HPLC conditions; μ-Bondapak RP-18, 10 μm (4.1 mm i.d. x 25 cm), methanol/water (93:7), 1.0 ml/min, RI detector.

tion was the same as silica gel open column chromatography, the price was cheap (US $5.00/lb), and no pretreatment of packing materials was required. The disadvantages were: the flow rate was very slow and back pressure was too high (0.3 ml/min at 1000 psi). It was not useful for preparative purposes. A large mean particle size (15 μm) might solve the problem.

b. LiChrosorb SI-100, 10 μm, LiChrosorb SI-60, 20 μm, and LiChrosorb SI-100, 30 μm for HPLC (EM Laboratories, Inc.)

All of these three dry packing materials got the same chromatograms as the silica gel open column method. However, these packing materials were expensive; the reusability, flow rate, back pressure, and column efficiency were checked. The 10 μm packing material flow rate was not satisfied (1.0 ml/min at 1000 psi). The 30 μm packing material had a good flow rate (3.5 ml/min at 600 psi) but unsatisfied reusability (once). The SI-60, 20 μm had the best reusability (6 times) (Fig. 26) and flow rate (3.5 ml/min at 800 psi). Although the preparative high pressure column could be used many times, it still had some disadvantages. The column must be cleaned and conditioned each time before the next run. Cleaning the column with methanol to elute the polar fraction and conditioning with chloroform to original condition require time, labor, and solvent.

c. Silica gel 100 mesh (Mallinckrodt)

7.5 g silica gel was dry packed in the column (8 mm i.d. x 30 cm). As the test of the capacity of silica gel open column chromatography revealed, 16 g silica gel was enough for 400 mg wheat lipids. Therefore, 7.5 g silica gel was enough for

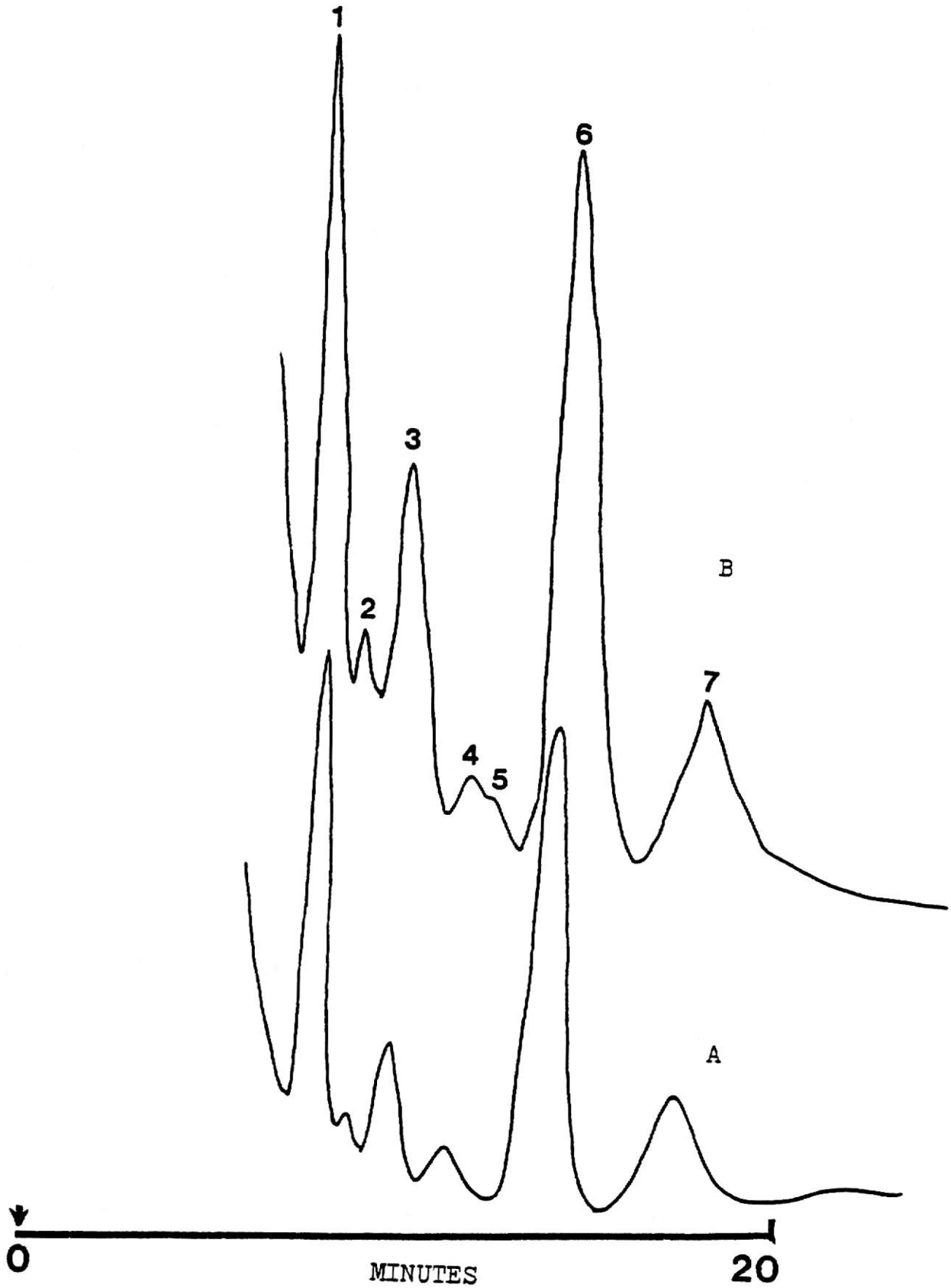


Fig. 26 HPLC separation of wheat MGDG fractionated with LiChrosorb SI-60, 20 µm: (A) first time; (B) sixth time. HPLC conditions: µ-Bondapak RP-18, 10 µm (4.1 mm i.d. x 25 cm), methanol/water (93:7), 1.0 ml/min, RI detector.

fractionation of 180 mg of wheat lipids. The result was the same as the silica gel open column. The clog problem was solved also. The disadvantage was that the silica gel packing material required time consuming in-lab sizing purification and activation pretreatment.

Comment: So far, the most economical and convenient fractionation procedure was preparative high pressure liquid chromatography with silica gel 100 mesh. Bulk ready to use and previously sized Syloid silica gel could possibly be substituted. The inexpensive and disposable Syloid silica gel may be the best cost and labor efficient packing material for routine analyses.

F. Stirred Extraction

15 g of fresh whole wheat flour or 25 g of whole wheat flour stored at 37°C for 8 months were tested, each mixed with 100 ml 1,1,1-trichloroethane in a 250 ml Erlenmeyer flask. The mixture was stirred with a magnetic stirrer for 20 min. Using a suction filter for the total lipids, the residue was mixed with 50 ml 1,1,1-trichloroethane, stirred for 10 min, then filtered out. Another 50 ml 1,1,1-trichloroethane was added, stirred for another 10 min, then filtered out. These three filtrates were combined, and then dried with a rotary evaporator. Six extractions of the result were 1.62 ± 0.08 g total lipids in 100 g dry basis fresh whole wheat flour (1981 hard red winter wheat).

The extracted lipid was fractionated with preparative high pressure liquid chromatography. A comparison of the MGDG fraction with the Skellysolve F extracted MGDG fraction is shown in Fig. 27A and B. No significant difference was found. It suggested that the stirred extraction method could be used for the replace-

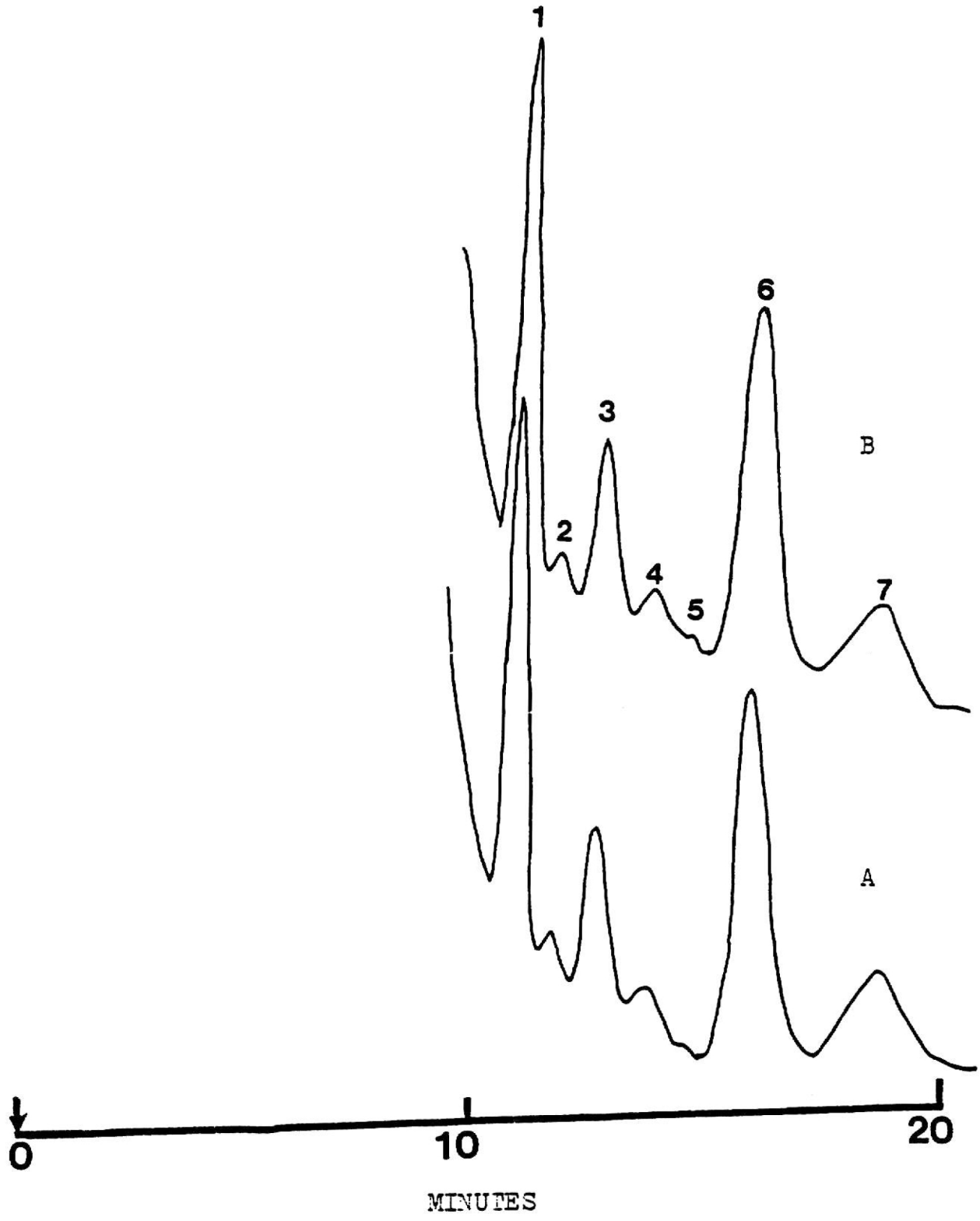


Fig. 27 HPLC separation of wheat MGDG fractions: (A) Stirred extraction; (B) Soxhlet extraction. HPLC conditions: μ-Bondapak RP-18, 10 μm (4.1 mm i.d. x 25 cm), methanol/water (93:7), 1.0 ml/min, RI detector.

ment of the Skellysolve F Soxhlet extraction.

A bulk ready to use and previously sized Syloid 620 (Davison Chemical, Baltimore, MD) had also been tested. The mean particle diameter is 15 μm, the pores is 150 Å, and the surface area is 320 sq in per gram. The flow rate is 3.5 ml per min at 950 psi. The relative standard deviation for SSG/DLGG method is less than 3% in six replicates. This result suggested that the Syloid 620 might be a good preparative column packing material. It is not necessary to presize and prewash the fine packing material (silica gel 100 mesh) as in the procedure given on page 36.

Comment: Using the stirred extraction and preparative high pressure liquid chromatography, the analysis time decreased to about 2 hours for one sample instead of 2 days. Also, the SSG/DLGG indicator of wheat deterioration made the prediction of wheat quality possible.

RECOMMENDED PROCEDURE

1. Grind wheat with Wiley experimental mill (1 mm sieve).
2. Weigh 15-25 g of ground wheat into 250 ml Erlenmeyer flask.
3. Add 100, 50, and 50 ml of 1,1,1-trichloroethane: stir for 20, 10, and 10 min, respectively: decanting between each successive extraction.
4. Combine the extracts, filter with suction into a 500 ml boiling flask, and evaporate the extract to dryness with rotary evaporator under 40°C.
5. Dissolve in a premeasured portion of chloroform (0.5-0.8 ml), inject into a preparative high pressure liquid chromatography column: 8 mm i.d. x 30 cm equip with a 5 µm frit dry packed with 7.5 g silica gel pretreated as previously described.
6. Elute the nonpolar lipids with 100 ml chloroform; then elute with 60 ml chloroform/acetone mixture (5:5) and collect, flow rate: 3.5 ml/min.
7. Evaporate the chloroform/acetone eluate to dryness and dissolve in chloroform (0.1-0.5 ml).
8. Inject the purified sample into a high performance liquid chromatography: µ-Bondapak RP-18, 10 µm, (4.1 mm i.d. x 25 cm) column, mobile phase: methanol/water (93:7), flow rate: 1 ml/min, quantitate by peak width at half height method with Multiref 912 interferometric refractive index detector, attenuation: x 50, or other detector with capability to detect SSG or DLGG, 0.25 $\pm$ 0.01 µg.

CONCLUSIONS

The high performance liquid chromatograms of wheat MGDG fractions show a significant change between the control and stored wheat flours. The DLGG (peak 6) decreases with storage time, whereas the SSG (peak 3) remains approximately constant. A linear relationship of the ratio SSG/DLGG with the time stored was found. The longer the storage, the higher the ratio. A quality factor that deteriorations with storage showed in theory, be correlatable with the ratio at SSG/DLGG.

The development of a fast and convenient stirred extraction with 1,1,1-trichloroethane and practical fractionation with preparative high pressure liquid chromatography of lipids improved the analysis time. With the automatic injection and record system, the analysis time for each sample is about 100 min for duplicate analysis. This makes the routine analysis possible. The maximum replicate analysis is 18 samples in 24 hours. The major replicate procedure is the preparative high pressure liquid chromatography.

In the SSG/DLGG method, the detectable quantity for SSG or DLGG is 0.25 μg. The method has a relative standard deviation of less than 5%. Also, the SSG/DLGG method requires no external standard. The end point of fat acidity method is not clear and always depends on the operator technique. In general, fat acidity titrates not only the free fatty acids, but also the phosphates and amino acids in the grains. The further oxidation and subsequent hydrolysis and cleavage of a large molecular fatty acid to several small molecular fatty acids makes the titration value

large. It is difficult to predict the wheat quality by the unspecific, insensitive, and imprecise fat acidity method. However, the SSG/DLGG method is specific, it measures directly two pure compounds (SSG and DLGG) in grains. Because the glycolipids is a good improver of baking quality, measurement of DLGG and SSG is meaningful. Also, the linear relationship between SSG/DLGG ratio and storage time suggests that this method may be used to replace the fat acidity method.

In order to get the optimum conditions for polar lipids analyses by HPLC, various factors were studied: column length (15 cm vs 25 cm), packing materials (LiChrosorb RP-18, 10 μm, Spherisorb ODS, 10 μm, Partisil-10, ODS, Lichrosorb SI-60, 10 μm, LiChrosorb SI-100, 10 μm, Spherisorb C 6, 5 μm, and μ-Bondapak RP-18, 10 μm), mobile phase (methanol/water system ranging from 50:50 to 100:0), and flow rate (from 0.5 ml/min to 1.5 ml/min). The best results for the wheat polar lipid fraction were obtained with μ-Bondapak RP-18, 10 μm, column 4.1 mm i.d. x 25 cm, mobile phase, methanol/water (93:7), and flow rate, 1.0 ml/min. However, in this work, the DLGG and DGDG peak II in the polar lipids or glycolipids are not separated. Also, SSG peak is not resolved from other peaks. A subfractionation of the polar lipids and glycolipids before the HPLC was developed. The subfraction for analysis was eluted with chloroform/acetone (5:5) in preparative high pressure chromatography after removal of nonpolar lipid with chloroform. With preliminary subfractionation SSG (peak 3) and DLGG (peak 6) are completely resolved from other peaks. More than 200 samples have been injected into analytical column before the column is regenerated. The column can be regenerated by wash-

ing with solvent of decreasing polarity, then increasing polarity. After regenerated, the column can be used more than 50 times. The refractive index detector with interferometric optics provides the sufficient sensitivity with nonselective and nondestructive detection to describe SSG/DLGG ratio.

Mass spectroscopy and gas chromatographic analysis were used to elucidate the chemical composition of fractions collected from the semipreparative HPLC separation. Peak 6 of MGDG fraction is comprised of linoleic acid in both SN-1 and SN-2 positions; and a monogalactose in SN-3 position. Peak 3 is steryl glucoside mainly comprised of β-sitosterol with a glucose connects to the sterol molecule. The different retention times of individual glycolipid components are related to fatty acid composition and isomeric position, as well as to the polar functional group. The elution order of glycolipids on the μ-Bondapak RP-18, column is: SSG, DGDG (peak I), DLGG, DGDG (peak II), and MGDG (peak II). The standards of SSG, DLGG, and DGDG are fractionated from a wheat flour lipid extract.

The dried control free MGDG fraction was stored in a reactor vial at 37°C for 15 days. The ratio SSG/DLGG is increased, however, the bad resolution makes the quantitation difficult. The whole wheat flour was stored at 37°C for 8 months, its SSG/DLGG ratio increased from 0.103 to 0.532. Whole kernel wheat stored at 37°C for 48 weeks, its SSG/DLGG ratio is 0.329. It indicated that the deterioration rate in whole kernel wheat is slower than whole wheat flour stored at the same condition. Although peak tailing, unresolved peaks, and low DLGG concentrations occurring in the longer stored wheat may make quantitation difficult, this

method is found to be linear and good for wheat stored at 37° for 8 months. Beyond this point, the wheat may be too bad to be recovered and any measurement is meaningless. The SSG/DLGG method was chosen after studying other lipid classes, because the chromatograms of other lipid classes may show a difference, but quantitation is difficult.

In the process SSG/DLGG condition monitoring technique by HPLC, other lipid classes and their effect in storage have been studied by HPLC. There were no significant changes in the peak ratios of nonpolar lipids extracted from the control grain flours and their stored grain flours. Also, there were no significant changes in the peak ratios of control nonpolar lipids and the stored nonpolar lipids (lipids stored at different pH and temperature). However, small shoulders on peak 6 were found on the chromatograms of nonpolar lipids of the stored wheats. Because the resolution of the shoulder from peak 6 was poor and the shoulder is very small for the wheat stored at 37°C for 8 months, quantitating is difficult and impractical for the measurement of deterioration in the stored grain quality.

Polar lipids and glycolipids both showed substantial changes during the high temperature. However, tailing and unsymmetrical peaks make the quantitation very difficult. The bad chromatograms are from: DLGG is unresolved from DGDG peak II, SSG (peak 3) is unresolved from other peaks, and many minor peaks exist. The phospholipid fraction did not show any major peak in the same HPLC condition. The DGDG, while a minor component of wheat flour, is of great importance in the breadmaking quality of wheat. Although the DGDG fraction has a simple chromatogram (1 major peak,

4 minor peaks), the distribution of components in the chromatogram showed no significant change in the stored wheat flour (37°C, 4 months).

Although lipids are only a small portion of most grain, they are a substantial factor of grain deterioration during storage. The fat acidity has been proposed as a convenient index of deterioration in stored grain but is now considered to be unreliable. Loss of free glycolipids and phospholipids has been suggested as an alternative, but no simple and rapid tests have been available to determine them in stored grain. The distribution of the components in the chromatograms of stored wheat MGDG fraction showed a significant change. The SSG/DLGG method not only served as a good indicator of past deteriorations, but also allowed prediction of the future storability of a given grain. It also provided a means of routinely evaluating stored wheat for lipids in association with baking quality. Although the SSG/DLGG method is applicable to stored wheat condition, it may be applicable to any other grain rich in SSG and DLGG compounds.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE SITOSTERYL-β-D-GLUCOSIDE/*sn*-1,2-DILINOLEOYL-3-GALACTOSYLGLYCEROL RATIO AS AN INDICATOR OF STORED WHEAT CONDITION

by

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AN ABSTRACT OF A MASTER'S THESIS

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The high performance liquid chromatograms of wheat monogalactosyl diglyceride (MGDG) fractions have a significant change between the control and stored wheat flours, because the *sn*-1,2-dilinoleyol-3-galactosylglycerol (DLGG) (peak 6) is more reactive than the sitosteryl-β-D-glucoside (SSG) (peak 3) during the storage. A linear relationship of SSG/DLGG ratio with time stored was found. The longer the storage time, the higher the ratio, and the worse the quality. Therefore, the quality of wheat can be predicted from the ratio. Besides the MGDG fraction, the other lipid extract from different grains have been checked. However, no significant change in the stored grains has been found.

The development of fast and convenient stirred extraction with 1,1,1-trichloroethane and practical fractionation with preparative high pressure liquid chromatography of lipids improved the analysis time. With the automatic injection and record system, the analysis time for each sample was about 100 min for duplicate analysis. This makes the routine analysis possible.

In the SSG/DLGG method, the relative standard deviation is less than 5%. The detectable quantity for SSG or DLGG is 0.25 μg. Also, the SSG/DLGG method required no external standard. Compared with the fat acidity titration method, the fat acidity method is not sensitive in predicting the wheat quality, because its titration end point is not clear. It must depend on the operator's technique. Also, fat acidity does not have any significant relationship with baking quality. Therefore, it is possible that the SSG/DLGG method can be used to replace the fat acidity titration method.

The best chromatographic condition of determining the ratio of SSG/DLGG in wheat flour lipids using HPLC was developed. A μ-Bondapak, RP-18, 10 μm, column elute with methanol/water (93:7) was the chromatographic system choice. The column could be regenerated by washing with solvent of decreasing polarity, then increasing polarity. The refractive index detector with interferometric optics provided the greatest sensitivity with nonselective and nondestructive detection.

Mass spectroscopy and gas chromatographic analysis were used to elucidate the chemical composition of fractions collected from the chromatographic separation. The different retention times of individual glycolipid components were related to the fatty acid composition and isomeric position, as well as to the polar functional group. The elution order of glycolipids on the μ-Bondapak RP-18, column is: SSG, digalactosyl diglyceride (peak I), DLGG, digalactosyl diglyceride (peak II), and MGDG (peak II). The standards of SSG, MGDG, and digalactosyl diglyceride are fractionated from a wheat flour lipid extract.