Determining Thioctic Acid (Lipoic Acid) in Wheat Flours and Germs by Thin-Layer Chromatography[1]

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ABSTRACT

Thioctic acid in wheat flours and germs was extracted by a rapid-extracting method with a ternary mixture of chloroform, methanol, and water. The extractability of thioctic acid by the ternary mixture indicates that thioctic acid is more likely bound to protein in wheat flour and germ through hydrogen bonding than through covalent bonds. The thioctic acid was separated from the extract by ascending thin-layer chromatography (TLC) (silica gel) with a solvent system of ethyl ether:petroleum ether:acetic acid (60:40:2,v./v./v.); charred with a saturated solution of potassium dichromate in 55% sulfuric acid; and determined by densitometry. Extracting added thioctic acid in flour by acid hydrolysis showed a lower recovery as compared with the rapid method. Identification of thioctic acid spots was further confirmed by two-dimensional TLC. The thioctic acid content, determined by the present TLC method, ranged from 1.38 to 2.85 μmoles per g. for wheat flour and 36.59 to 41.04 μmoles per g. for wheat germ. However, no reduced thioctic acid was detected.

Thioctic acid in wheat flour was first reported by Dahle and Sullivan (1,2), who isolated a few mg. from 8.9 kg. of flour (2), confirming the acid's identification by its R_f value of ascending strip chromatography, ultraviolet maximum absorbance at 334 nm., melting point of S-benzyl thiouranium derivative, and sulfur content. They found that flour contained 1 to 10 p.p.m. of thioctic acid, and wheat germ contained about 200 to 300 p.p.m. Morrison and Coussin (3) confirmed the presence of thioctic acid in wheat flour, bran,and germ by gas chromatography.

Dahle and Pinke (4) attempted to detect free thioctic acid in wheat flour by spotting their extract on a Gelman ITLC Type SC glass microfiber sheet, developing the chromatograph in petroleum ether:ethyl ether (90:10), and then using 5,5′ dithiobis-(2-nitrobenzoic acid) for coloration; but they found no free thioctic acid.

In view of the probable role of thioctic acid in oxidizing wheat flour and in determining the rheological properties of dough (1,2,4), we developed a sensitive method to determine the thioctic acid contents in wheat flours and germs.

MATERIALS AND METHODS

Chemicals

Reagent-grade chemicals were used. DL-6,8-thioctic acid (reduced or oxidized) was purchased from Sigma Chemical Company; 2,2′-dithiobis-(5-nitropyridine), from Nutritional Biochemical Corporation.

Samples

Two varieties each of hard red winter, hard red spring, and soft white wheats—all milled on a Miag Multomat—were used. Proximate analysis of untreated,

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straight-grade flours and germs (Table I) was determined as described in AACC Methods (5), except for the fat which was determined by the AOCS Method (Aa 4-38) using petroleum ether as extracting solvent (6).

Total Lipid Extraction of Wheat Flours or Germs

Using the acid hydrolysis method of Morrison and Coussin (3), 200 g. of flour was digested with 100 ml. of 1N hydrochloric acid under reflux for 3 hr. The suspension was cooled and centrifuged at 1,000 X g for 20 min. The supernatant was extracted with three portions of chloroform, using one part chloroform to five parts supernatant liquid. The combined extracts were evaporated to dryness under vacuum.

Using a rapid-extracting method of Tsen et al. (7), total lipids (including thioctic acid) were extracted from flours and germs. The solvent system was a mixture of methanol:chloroform:water (2:1:0.8). Throughout this study, this rapid-extracting method was used to extract total lipids and thioctic acid from wheat flours and germs except when otherwise stated.

Thin-Layer Chromatography (TLC)

Glass plates (20 X 20 cm.) were coated with a 0.5-mm. layer of silica gel G, then activated at 130°C. for 2 hr. Quadruplicate spots of 4 μl. (0.133 μmole) of standard oxidized thioctic acid and 2 to 8 μl. of chloroform-diluted lipid extracts (containing 0.033 to 0.167 μmole of thioctic acid) were spotted on the TLC plate; the plates were developed ascendingly in a saturated chamber containing ethyl ether:petroleum ether (b.p. 35° to 60°C.):acetic acid (60:40:2, v./v./v.). After a 15-cm. solvent migration on the TLC plate, the plates were removed, air-dried, and sprayed with a saturated solution of potassium dichromate in 55% of aqueous sulfuric acid; they were then charred at 180°C. for 20 min.

Quantitative TLC

The densities of the thioctic acid spots were measured by a Photovolt Densitometer (Model 530) with scanning stage and varicord recorder. The peak

TABLE I. PROXIMATE ANALYSIS OF WHEAT FLOURS AND GERMS

Sample	Protein %	Fat %	Moisture %	Ash %
Flour				
HRW				
Eagle	10.8	0.8	13.7	0.49
Bison	11.5	0.6	14.6	0.41
HRS				
Waldron	14.6	0.7	12.8	0.43
Chris	13.3	0.7	12.6	0.53
SW				
Arthur	10.4	0.8	12.7	0.37
Logan	10.7	0.8	12.6	0.37
Germ				
HRW				
Eagle	25.4	7.9	8.6	4.4
Bison	29.2	6.8	9.7	5.6
SW				
Arthur	20.3	7.9	9.3	4.4
Logan	24.4	9.0	9.1	4.6

areas were integrated with a Gelman planimeter. Each curve was measured twice and the values were averaged.

Within a range of 0.033 to 0.167 μmole of standard thioctic acid, a plot of concentration vs. peak area (square inch) obtained from densitometry gave a linear relationship.

RESULTS AND DISCUSSION

Developing TLC for Thioctic Acid Determination

Preliminary study was done by following the procedure of Dahle and Sullivan (1). The chloroform extract of the acid-hydrolyzed wheat flour was spotted on Whatman No. 1 paper and chromatographed (ascending) with 1% acetic acid as the solvent. After immersion in 0.1N potassium permanganate, a thioctic acid spot was developed with an R_f value of 0.7, the same as identified by Dahle and Sullivan (1).

TLC is a simple, inexpensive, and sensitive way to separate lipids. The extent to which a lipid is charred is related to its carbon content, and under suitable conditions the charring reaction can be used for determining the carbon content as well as the content of a particular lipid separated by TLC (8,9). We attempted, therefore, to use TLC (instead of paper chromatography) to separate thioctic acid from fatty acids and other impurities.

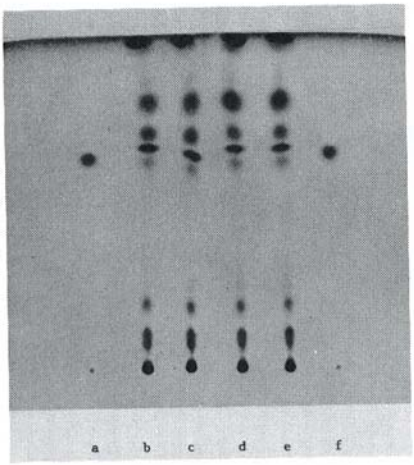
For one-dimensional ascending TLC of thioctic acid, a solvent system of ethyl ether:petroleum ether:acetic acid (60:40:2, v./v./v.) was found best for the separation.

Different extracting solvents could affect the chromatographic separation of thioctic acid. When free lipids, extracted with petroleum ether (b.p. 30° to 65°C.), and bound lipids, extracted with water-saturated butanol from wheat flour, were spotted and developed on TLC plates, the separation was not clear enough for quantitative determination of thioctic acid. However, when the total lipids extracted by the rapid extracting method were used for the chromatography, the thioctic acid spot was clearly separated from other spots of lipid impurities; its R_f value was 0.60. Figures 1 and 2 show the typical chromatograms of thioctic acid in the total lipids extracted from wheat flours and germs. Why thioctic acid can be separated better from the total lipids extracted by using the ternary mixture of chloroform, methanol, and water than by using the other extracts is not clear. Perhaps, in the ternary mixture, methanol and water increase the polarity of the solvent system so as to dissociate bonds (mainly hydrogen bonds) readily between thioctic acid and wheat proteins, and the dissociated thioctic acid is then extracted and purified by chloroform through phase partition (7). As a result, thioctic acid would be in a more purified form by the rapid-extracting method than by the other extracting methods for the chromatography.

Dahle and Pinke (4) suggested a strong covalent linkage of thioctic acid to another moiety on the ground that endogenous thioctic acid recovered from flour required vigorous hydrolysis prior to separation. However, the present finding that thioctic acid can be readily extracted by the ternary mixture makes it seem that wheat thioctic acid is more likely bound to protein through hydrogen bonds than covalent bonds.

Loss of Thioctic Acid by Acid Hydrolysis

Standard thioctic acid (100 μmoles) was used to check the efficiency of



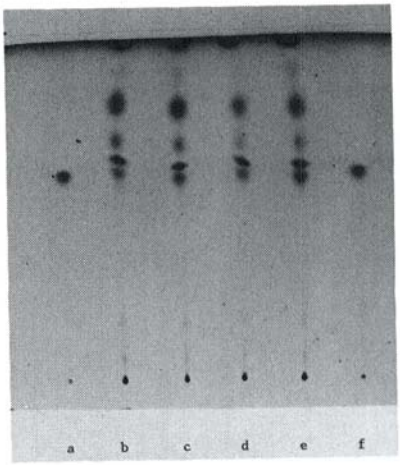


Fig. 1 (left). Thin-layer chromatogram of total lipids from wheat flour samples. Spots a and f, standard thioctic acid; b, total lipids from Eagle; c, Bison; d, Arthur; and e, Logan.
Fig. 2 (right). Thin-layer chromatogram of total lipids from wheat germ samples. Spots a and f, standard thioctic acid; b, total lipids from Eagle; c, Bison; d, Arthur; and e, Logan.

extraction methods. By the rapid-extracting method, 91% thioctic acid was recovered. By the acid-hydrolysis method (as a check), with 1N HCl, 6N HCl, and 6N H_2SO_4, recovery was 77, 42, and 26%, respectively. Obviously the rapid-extracting method was superior. In the acid-hydrolysis method, thioctic acid loss increased when acid normality was increased; at the same normality, sulfuric acid could cause a greater loss than hydrochloric acid.

To investigate further recovery of thioctic acid, we extracted increasing amounts of standard thioctic acid (50,100,150, and 200 μmoles) by the rapid-extracting method; the linear relationships of amounts of thioctic acid added and recovered were obtained (Fig. 3). The average recovered thioctic acid was 91%.

Mixing increasing amounts of thioctic acid with 4 g. flour (Eagle) and then extracting each mixture by the rapid extracting method showed a linear relationship, with a slope paralleling that plotted for no added flour. Adding 50 μmoles thioctic acid per g. flour also produced a linear response, but the slope was greater than that for thioctic acid only. All thioctic acid added to the flour was recovered.

In addition to the loss, thioctic acid extracted by acid hydrolysis of HRS wheat flours (though not of the other flours), spotted on TLC, could not be measured quantitatively because the tailing of the lipid material covered the spot of thioctic acid.

Confirming Thioctic Acid by Two-Dimensional TLC

To confirm the identified spot as thioctic acid, two-dimensional TLC was used to separate thioctic acid from total acid from total lipids from Eagle flour extracted by the rapid method, as compared to standard thioctic acid chromatographed under the same conditions. Ethyl ether:petroleum ether:acetic acid (60:40:2) was the solvent in the first dimension, chloroform in the second. The TLC chromatograms

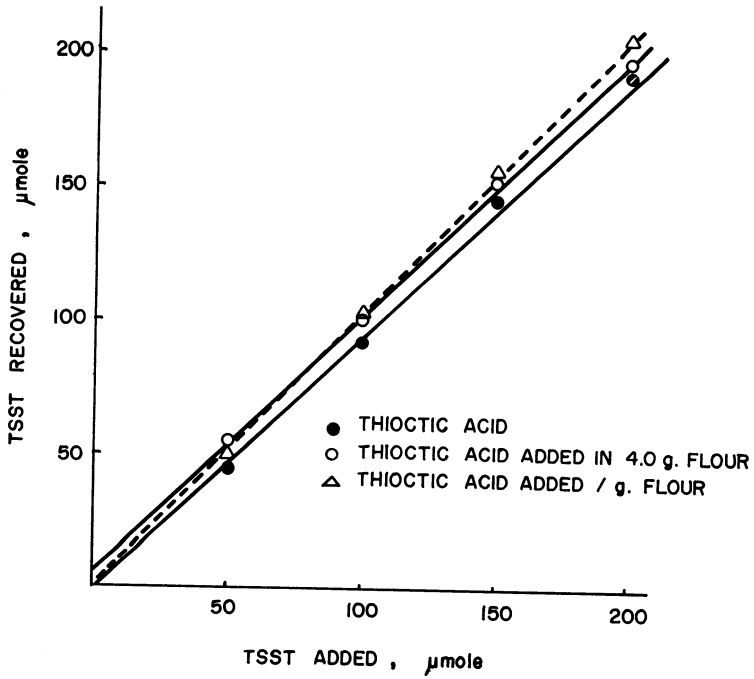


Fig. 3. The content of thioctic acid recovered, extracted by rapid method. ●, Thioctic acid, 50, 100, 150, and 200 μmoles; ○, thioctic acid, 50, 100, 150, and 200 μmoles added in 4 g. flour; ▲, thioctic acid, 50, 100, 150, and 200 μmoles added per g. flour.

(Fig. 4) showed that standard thioctic acid and the identifed spot migrated in the same direction with the same R_f value. This confirmed the identified spot as thioctic acid. However, additional studies with other techniques to ascertain the purity of the thioctic acid spot seemed required to substantiate the confirmation by the TLC techniques employed in this study.

Absence of Reduced Thioctic Acid in Flour

As presented in the previous sections that thioctic acid could be extracted by the rapid method, separated and identified by the present TLC method, further study was undertaken to examine in which form thioctic acid was present in wheat flour.

Preliminary work on the reduction of thioctic acid showed that more thioctic acid was reduced in 1N HCl (10) than in 80% acetic acid by zinc. Total lipid containing thioctic acid extracted by the rapid method from 6 g. Eagle flour was then reduced in 1N HCl in the presence of zinc for 1 hr. Upon adding an aliquot of the reduced solution to 10^{-3}M 2,2′-dithiobis-(5-nitropyridine) (DTNP) in acetone solution (11), the mixture appeared yellow, indicating the presence of reduced thioctic acid. However, without the zinc reduction, no reduced thioctic acid was detected in the total lipids. This suggests that the thioctic acid present in wheat flour is in oxidized form, providing that no oxidation of thioctic acid takes place during extraction.

Thioctic Acid Content in Flours

Table II shows that the thioctic acid content of wheat flours tested ranged from 1.38 to 2.85 μmoles per g. (dry basis). The thioctic acid content seemed to increase with increasing protein content based on the data listed in Table II. The content was higher in total lipids extracted by the rapid method than in those extracted by acid hydrolysis.

Sullivan et al. (2) estimated the thioctic acid content in flour to be approximately 1 to 10 p.p.m. (4.85×10^{-3} to 4.85×10^{-2} μmole per g. of flour). The thioctic acid contents, determined by our method (Table II), were much higher.

Thioctic Acid Content in Wheat Germs

The thioctic content of germ (Table II), about 24 times that of flour, ranged from 36.59 to 41.04 μmoles per g. Sullivan et al. (2) reported the estimated value

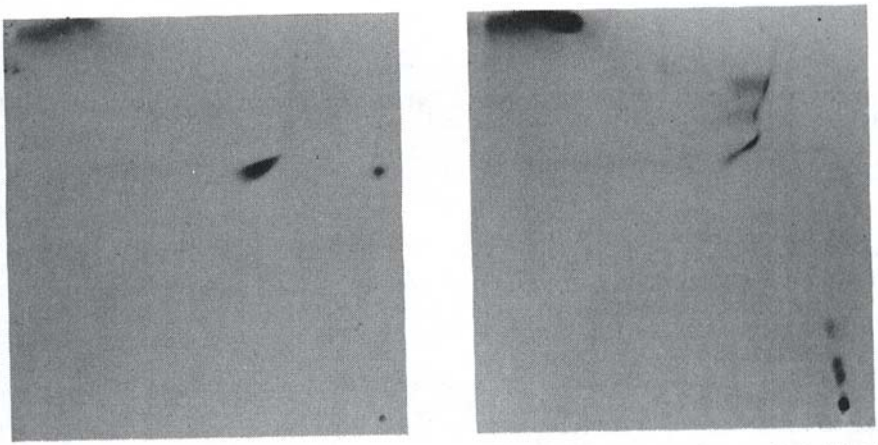


Fig. 4. Thin-layer chromatograms showing two-dimensional separation. Left, standard thioctic acid; right, total lipid from Eagle flour (extracted by rapid method).

TABLE II. THIOCTIC ACID CONTENTS OF WHEAT FLOURS AND GERMS (DRY BASIS)[a]

	Flour		Germ
	Rapid method μmoles/g.	Acid hydrolysis μmoles/g.	Rapid method μmoles/g.
HRW			
Eagle	1.48 ± 0.35 (4)	1.40 ± 0.09 (5)	41.04 ± 6.80 (4)
Bison	1.68 ± 0.24 (4)	1.56 ± 0.13 (6)	36.59 ± 1.70 (4)
HRS			
Waldron	2.28 ± 0.32 (4)	...	...
Chris	2.85 ± 0.21 (4)	...	...
SW			
Arthur	1.38 ± 0.11 (4)	1.38 ± 0.18 (2)	39.62 ± 1.99 (4)
Logan	1.39 ± 0.01 (4)	1.08 ± 0.01 (4)	37.82 ± 1.29 (4)

[a]Each entry shows the mean value and its standard error, and, in parentheses, the number of determinations.

of the thioctic acid content in germ at 200 to 300 p.p.m. (0.97 to 1.45 μmoles per g.). Our figures were much higher. There was no substantial difference in thioctic acid content in germs among different classes of wheat.

The contents of thioctic acid determined by the TLC method are much higher than those estimated by others in wheat flour and germ. One of the reasons for the low thioctic acid reported by Sullivan et al. (2) is that the acid hydrolysis conditions that they employed could cause significant losses in thioctic acid, as suspected by them and demonstrated in the present study.

Tsen and Anderson (12) have reported that wheat (hard and soft) flours contain 10.79 to 16.35 μmoles disulfide groups per g. flour. Of the total disulfide content in flour, thioctic acid is estimated to contribute about 10%, according to the disulfide figures listed in Table II. This high thioctic acid content itself seems enough to provide incentive for expanding the work of Dahle et al. (1,2,4,13) on the effect of thioctic acid on dough properties. Further work along this line is in progress.

Acknowledgments

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