

## Optimization of Oxidation Steps Used in Fluorometric Determination of Thiamin in Soft Wheat Flour

J. C. Moore<sup>1</sup> and K. D. Dolan<sup>1-3</sup>

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Among the standard fluorometric methods and published research to measure thiamin in soft wheat flour, several differences exist in the oxidation steps presented. Therefore, the oxidation steps for a specific product were investigated in this work using a modified version of a rapid method of AOAC (2000). The results may have implications for the many grain products analyzed for thiamin using the fluorometric method.

The oxidation of thiamin to thiochrome involves the addition of an oxidizing agent such as potassium ferricyanide ( $K_3Fe(CN)_6$ ) to an aqueous thiamin solution under alkaline conditions. Jansen (1936) noted that the amount of oxidizing agent used was critical. Hoffer et al (1943) and Andrews (1944) noted how an excess of  $K_3Fe(CN)_6$  could lower fluorescence. Ellinger and Holden (1944) studied the quenching effects of various electrolytes on thiochrome fluorescence and found that a 10 mg/L isobutanol thiochrome solution could lose 18% fluorescence in the presence of 0.1N  $K_3Fe(CN)_6$ .

Many researchers have studied how to optimize this oxidation reaction and have recommended using certain levels or range of levels of oxidizing agent. However, different grain products contain different concentrations of organic materials that will be preferentially oxidized instead of thiamin, consequently making the optimal amount of oxidizing agent unique to each grain product and extraction procedure (McFarlane and Chapman 1941). For convenience of comparison, we reported levels of oxidizing agent as  $\mu g$  of  $K_3Fe(CN)_6$  recommended to react with 1  $\mu g$  of thiamin-HCl. The  $K_3Fe(CN)_6$  levels recommended for each thiamin analysis method are 0.94 (AVC 1966), 500 (AACC 2000), 1,226 (AOAC 2000), and 21,670 (Brubacher et al 1985). Optimal ranges have been reported as 3–300 (Jansen 1936), 33–2,400 (Hoffer et al 1943), 1,000–1,500 (Conner and Straub 1941), 1,111–4,444 (McFarlane and Chapman 1941), and 100–10,000 (Watson 1946).

A second issue that most published methods have stressed is the importance of consistent timing during the oxidation steps. However, the methods have not addressed how these timings affect errors. Adding NaOH and oxidizing agent separately, as discussed below, has already shown potential for error arising from the contact time of thiamin and NaOH. Other timings should be investigated to show their potential for error.

The next critical point during the oxidation step involves whether to use a mixed or separated oxidizing solution. Methods by the Association of Official Analytical Chemists (AOAC 2000) and the Association of Vitamin Chemists (AVC 1966) require adding a sodium hydroxide (NaOH) and  $K_3Fe(CN)_6$  solution (mixed oxidation solution) to the aqueous thiamin extract. Approved Method 86-80 (AACC 2000) and Brubacher et al (1985) allow for the use of an aqueous NaOH solution followed by an aqueous  $K_3Fe(CN)_6$  solution to the aqueous thiamin extract (separated oxidation solu-

tion). Brubacher et al (1985) further explained that using a separated oxidation solution resulted in 6–8% higher thiochrome yields compared with using the mixed solution, and that if using the latter, 30% lower fluorescence would result if the addition took 20 sec or more. Both Andrews (1944) and Watson (1946) found the highest fluorescence values when the alkali was added first and then followed immediately by  $K_3Fe(CN)_6$ , the lowest values when the order was reversed, and intermediate results when the mixed solution was used.

Further complicating this issue is the fact that thiamin is very unstable in alkaline solutions (Jansen 1936; Watson 1946), yet thiochrome is very stable under identical conditions (Jansen 1936). Watson (1946) also studied the effect of exposure of pure thiamin to different amounts of NaOH over time on the fluorescence of thiochrome. He found the highest rate of fluorescence loss when using the lowest amount of NaOH (1 drop of 0.1N), followed by 3 mL of 30% (w/w) NaOH, and 3 mL of 15% (w/w) NaOH.

Lastly, the addition of sodium chloride as a clarifying agent for the isobutanol layer, as recommended by standard methods such as AOAC (2000), is also an unclear part of the oxidation step. This use of NaCl was first reported by McRoberts (1957) to be very effective at drying any water in the isobutanol layer, thereby preventing clouding, which can interfere with fluorescence measurement. Before the use of NaCl, other clarifying agents such as anhydrous sodium sulfate were used, but findings by Watson (1946) indicated contamination in this drying agent could lead to fluorescence. Thereafter, many researchers discontinued its use. Other methods such as AACC 86-80 use ethanol as an alternative to NaCl and sodium sulfate. Findings by Ellinger and Holden (1944) indicated that the presence of 0.1N NaCl in an aqueous thiochrome solution could cause a 5% loss of fluorescence over 3 min. Brubacher et al (1985) noted the “salting-out effect,” whereby thiochrome yields could be reduced by 15–19%. This effect was demonstrated by Fukuda and Kobayashi (1981), who showed that NaCl caused the decomposition of thiamin and that the loss was dependent on the initial concentration of thiamin.

Because of the differences among various standard methods and research studies, the objectives of this study were to investigate and clarify these issues, and determine for a specific product (0.3% [w/w] thiamin-HCl soft wheat flour): 1) effect of the amount of  $K_3Fe(CN)_6$  used on thiochrome fluorescence; 2) effect of timing before and after adding isobutanol on fluorescence; 3) whether it is beneficial to use a separated oxidizing solution, as opposed to a mixed one; 4) effect of using NaCl during oxidation on thiochrome fluorescence.

### MATERIALS AND METHODS

#### Sample Preparation

Soft wheat flour (Star of the West Milling, Frankenmuth, MI) was thoroughly mixed with food-grade thiamin-hydrochloride (Spectrum Laboratory Products, Gardena, CA) in a twin-shell mixer (Patterson-Kelly, East Stroudsburg, PA) for 40 min, resulting in a 0.3% thiamin-HCl enriched wheat flour. The thiamin concentration of this enriched flour was confirmed to be  $\approx 0.3\%$  with a coefficient of variance  $< 5\%$  for nine random samples.

<sup>1</sup> Dept. Food Sci. and Human Nutrition, Michigan State Univ., East Lansing, MI 48824.

<sup>2</sup> Dept. Agric. Engineering, Michigan State Univ., East Lansing, MI 48824.

<sup>3</sup> Corresponding author. Phone: 517-355-8474, ext 119. Fax: 517-353-8963. E-mail: dolank@msu.edu.

The procedures for extraction and oxidation were modified from a rapid method (AOAC 2000) designed for flour and other products containing only unbound and unesterified thiamin and no substances that can interfere with the fluorescence of thiamin. These conditions, eliminate the use of the enzymatic hydrolysis and purification steps prescribed by the AACC Method for a variety of grain products.

### Sample Extraction

An  $\approx 1$ -g sample of the enriched flour with 10 g of salt was diluted to 200 mL with 0.1N HCl, transferred to a 250-mL centrifuge tube, heated, and shaken at 95°C for 30 min using an Orbital shaker bath (Precision Scientific, Chicago, IL), cooled to room temperature for 20 min in an ice-water bath, and centrifuged using a RC-5B centrifuge (Sorval Instruments, Newton, CT) at 5,000 rpm ( $4,066 \times g$ ) for 15 min. The resulting clear aqueous thiamin solution was decanted from the solid pellet. This procedure was done with four enriched flour samples, resulting in 800 mL of an  $\approx 15 \mu\text{g/mL}$  thiamin extract solution. This solution was stored in the absence of light at 5°C for the entirety of the experimental time.

### Sample Extract Oxidation Procedure

For each sample, two 2.5-mL aliquots of thiamin extract (each containing 37.5  $\mu\text{g}$  of thiamin-HCl) were pipetted into two 50-mL centrifuge tubes. One tube was oxidized with 10 mL of a mixed solution of 0.01398% (w/w)  $\text{K}_3\text{Fe}(\text{CN})_6$  (Sigma, St. Louis, MO) and 15% (w/w) NaOH, resulting in an oxidation level of 37.28. To the other tube, 10 mL of 15% (w/w) NaOH was added, representing the blank. Anhydrous isobutanol (13 mL) (Sigma) was then immediately added and the tube shaken by hand for 15 sec. Then all samples together were shaken in a mechanical shaker (Mexi-Mix III, Termolyne, Dubuque, IA) for 2 min, and centrifuged for 5 min at 10,000 rpm ( $11,950 \times g$ ). A 3.3-mL isobutanol extract was added to a methylacrylate cuvette and fluorescence was measured using a fluorometer (Varian SF-330, Palo Alto, CA).

### Fluorometer Setup

Excitation slit, 5nm; emission slit, 10 nm; sensitivity setting,  $\times 1/10$  for samples,  $\times 10$  for blanks. The fluorometer was calibrated daily with 0.25 mg/L of quinine sulfate solution at excitation wavelength of 343 nm and emission wavelength of 459 nm.

Isobutyl thiochrome extracts were measured in the fluorometer at excitation wavelength of 373 nm and emission wavelength of 410 nm.

### Changes in Oxidation Procedure

*Effect of oxidation agent level on thiochrome fluorescence.* Thiamin extract was oxidized with 10 mL of mixed oxidizing solution containing 15% (w/w) NaOH and  $\text{K}_3\text{Fe}(\text{CN})_6$  at varying  $\text{K}_3\text{Fe}(\text{CN})_6$  concentrations (3.814, 7.627, 11.44, 18.16, 36.32, 54.48, 70.02, 140.0, 377.8, 4,520, and 81,250  $\mu\text{g/mL}$ )

*Timing after thiamin oxidized but before isobutanol was added.* Thiamin extract was oxidized. To one sample, isobutanol was immediately added, while the other sample remained for 30 min at room temperature before addition of isobutanol.

Timing after addition of isobutanol but before aliquot was removed for fluorescence measurement. Thiamin extract was oxidized and isobutanol added immediately. After shaking and centrifuging, one sample was immediately analyzed on the fluorometer, while the other sample remained at room temperature for 30 min before being analyzed on fluorometer.

*Effect of contact time between NaOH and thiamin.* Thiamin extract and 10 mL 15% (w/w) NaOH remained in contact for varying times (0, 20, 60, and 120 sec) before oxidation with 1 mL of 0.1398%  $\text{K}_3\text{Fe}(\text{CN})_6$  and 15% NaOH, resulting in an oxidation level of 37.28.

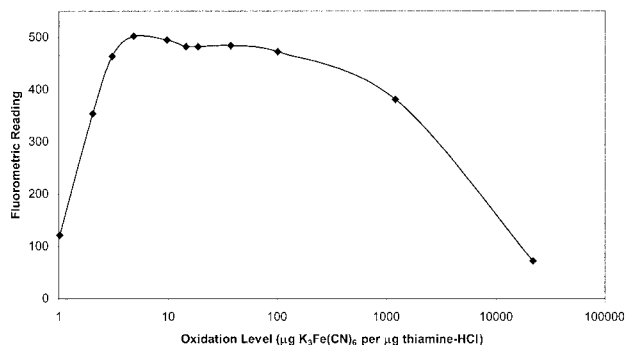
*Effect of sodium chloride on thiochrome fluorescence.* Thiamin extract and varying amounts of reagent-grade NaCl (0, 1.25, 2.5, and 4.0 g) were poured into centrifuge tubes and immediately oxidized. Oxidization was performed using 10 mL of 0.001850 or 0.4600% (w/w)  $\text{K}_3\text{Fe}(\text{CN})_6$  solutions resulting in oxidation levels of 4.94 and 1,200 respectively.

For all experiments, samples were run in triplicate. Fluorometer readings were reported with blanks subtracted. Statistical analysis of data was performed with Microsoft Excel software.

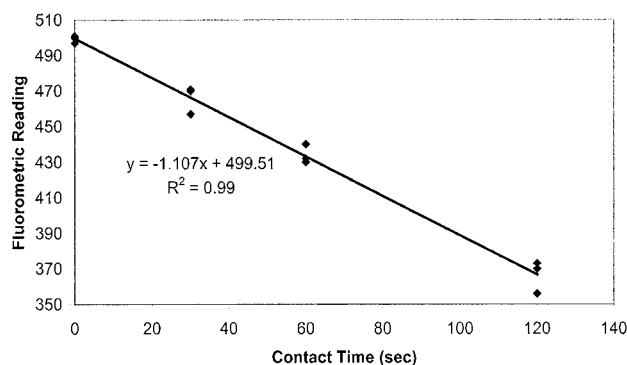
## RESULTS AND DISCUSSION

### Effect of Oxidation Agent Level on Thiochrome Fluorescence

A wide range of oxidation levels was covered (from 1 to 21,666  $\mu\text{g}$  of  $\text{K}_3\text{Fe}(\text{CN})_6$  / 1  $\mu\text{g}$  of thiamine-HCl) including those recommended by other researchers and by standard methods. Results show increasing fluorescence from a level of 1 to a maximum at 4.84 (Fig. 1). Interestingly, the stoichiometry of the redox reaction between thiamin and  $\text{K}_3\text{Fe}(\text{CN})_6$  as proposed by Rosenberg (1942), predicts an oxidation level of  $\approx 3.46$  which is very close to our optimal results. Beyond the 4.84 level, a plateauing effect was seen up to a level of 100, followed by an 86% decrease in fluorescence as the oxidation level increased to 21,666. A decrease in fluorescence is typically seen due to analyte self-quenching when the analyte reaches a concentration of 0.05M (Skoog et al 1998). Because our analyte concentration was significantly lower than this value ( $\approx 8.5 \times 10^{-6} M$ ), our loss of fluorescence cannot be attributed to analyte self-quenching but to an excess of the oxidizing agent ( $\text{K}_3\text{Fe}(\text{CN})_6$ ) itself as reported by other researchers (Jansen 1936; Hoffer et al 1943; Andrews 1944), as well as Ellinger and Holden (1944), who attributed this loss of fluorescence to the quenching action of the  $\text{K}_3\text{Fe}(\text{CN})_6$ . Our results show that for the particular product analyzed, a distinct range (much lower than recommended by other methods and researchers) exists where the concentration of  $\text{K}_3\text{Fe}(\text{CN})_6$  causes an optimal oxidation of thiamin to thiochrome and optimal thiochrome fluorescence. We therefore recommend that for analysis of soft wheat flour using this method, the oxidation level be lowered to a value within optimal range such as 37.28 (used in this study), ensuring optimal oxidation and fluorescence for samples of different thiamine concentrations. For



**Fig. 1.** Optimization of thiamin to thiochrome conversion during oxidation. Each point represents the average of triplicate readings with blanks subtracted. All data had coefficient of variations  $< 1.5\%$ .



**Fig. 2.** Effect of contact time between NaOH and wheat flour thiamin extract on thiochrome fluorescence.

other products and methods, researchers can perform one-time tests to determine whether their optimal oxidation ranges should be adjusted for individual products, thereby improving accuracy. In addition, we recommend further research be performed to better understand the fluorescence quenching mechanism of  $K_3Fe(CN)_6$ .

### Effect of Timing Before and After Addition of Isobutanol

Preliminary tests for the time allowed between oxidation and isobutanol addition, and the time after shaking before analyzing the sample showed no significant losses of fluorescence over 30 min. These findings agree with similar studies by Andrews (1944).

### Effect of Contact Time Between NaOH and Thiamin on Thiochrome Fluorescence

Once the timing of all other steps had been eliminated as sources of fluorescence change, the effects of a 15% NaOH solution in contact with the flour thiamin extract were tested. Results indicate that the fluorescence of thiochrome was reduced by 26% when  $\approx 37.5 \mu\text{g}$  of thiamin from flour extract was exposed to 10 mL of 15% (w/w) NaOH for 2 min (Fig. 2). The findings of Watson (1946) showed a loss of  $\approx 32\%$  over 7.5 min when  $1 \mu\text{g}$  of thiamin was exposed to 3 mL of 30% (w/w) NaOH.

Applying our results to soft wheat flour and the rapid fluorometric method, the use of a separated oxidizing solution [NaOH solution added to thiamin extract followed by  $K_3Fe(CN)_6$ ] according to Fig. 2, could lead to an experimental error as a result of inconsistent timing between addition of NaOH and  $K_3Fe(CN)_6$ . For example, a timing difference of 20 sec between two identical samples would lead to a 4.43% difference in measured thiamin concentration. While some researchers have reported slightly higher fluorescence using this separated method, one must question whether the higher fluorescence is worth the possibility of higher experimental error. Therefore, we recommend the use of the mixed oxidizing solution over the separated one for soft wheat flour and this method. For other products and methods, research will need to be done to confirm these results.

### Effect of Sodium Chloride on Thiochrome Fluorescence

Results show that the effect of sodium chloride was dependent on the amount of oxidizing agent used during oxidation (Fig. 3). The  $P$  value, which is the probability that the slope equals zero, was  $3.71 \times 10^{-7}$  and  $3.70 \times 10^{-6}$  for oxidation levels of 4.94:1 and 1,226:1, respectively. The low  $P$  values show that the slopes and represented trends were highly significant. We can be 95% confident that the slopes are within the intervals 7.2 to 10.6 and  $-10.7$  to  $-6.5$  for oxidation levels 4.94:1 and 1,226:1, respectively. At near the optimal oxidation level as determined above (4.94), the effect of the addition of 4 g of NaCl was to increase linearly the fluorescence of the isobutanol thiochrome by 7.5%. At 1,226, the oxidation level recommended by AOAC (2000), the opposite effect was seen, and addition of 4 g of NaCl decreased fluorescence by

9.3%. As noted earlier, researchers investigating this effect (Ellinger and Holden 1944; McRoberts 1957; Fukuda and Kobayashi 1981) found differing results, but these differences were probably due to their using a single oxidation level. The first implication of these results is that researchers using NaCl as a clarifying agent should determine for their specific product and oxidizing conditions, whether or not the addition of NaCl is beneficial, and possibly explore the use of other drying agents such as ethanol. Secondly, our results indicate that if NaCl is used, the level in samples (both in the product and added during analysis) must be consistent to minimize experimental error. Our results also suggest that further research needs to be done on these effects at other oxidation levels.

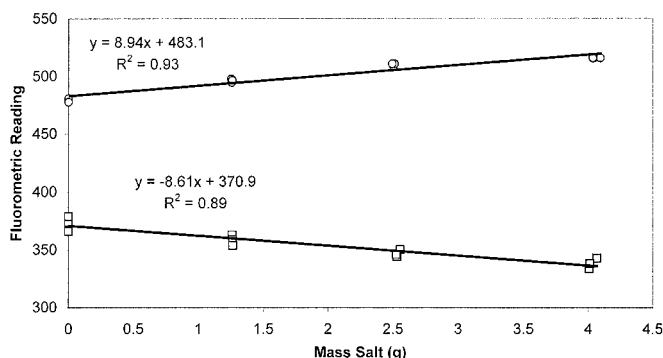
## CONCLUSIONS

Results indicate that for the determination of thiamine in soft wheat flour using a rapid fluorometric method: a) an optimal oxidation and fluorescence range exists between 4.84 and  $100 \mu\text{g}$  of  $K_3Fe(CN)_6$  /  $\mu\text{g}$  of thiamin hydrochloride, a range much lower than recommended by many methods and researchers; b) the potential for experimental error is greater when using a separated oxidizing solution compared to using a mixed oxidizing solution; c) the use of sodium chloride as a drying agent was beneficial for fluorescence at an optimal oxidation level, and detrimental at higher oxidation levels; and d) the time allowed between oxidation and isobutanol addition and time after shaking have no effect on fluorescence. Researchers and commercial testing laboratories using a similar rapid method for thiamin determination of soft wheat flour should be able to directly apply our results and should find improved accuracy with our proposed changes. While this study has specific significant findings for the material studied, there are implications for other products and variations on the fluorometric method because they all involve the steps discussed in this study. For these, further research should be done to determine the contribution that these factors play in accuracy and precision.

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**Fig. 3.** Effect of salt on fluorescence of thiochrome at different oxidation levels: 1,226  $\mu\text{g}$  of  $K_3Fe(CN)_6$  to 1  $\mu\text{g}$  of thiamin and HCl ( $\square$ ); 4.94  $\mu\text{g}$  of  $K_3Fe(CN)_6$  to 1  $\mu\text{g}$  of thiamin and HCl ( $\circ$ ).