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Note

Simultaneous determination of thiamine and riboflavin in enriched cereal based products by high-performance liquid chromatography using selective detection*

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Thiamine and riboflavin are two of the major water-soluble vitamins used to enrich cereal based products. Analysis of these vitamins using bioassay, microbiological, and chemical methods has been reviewed[1,2]. Bioassays, although time consuming and with a number of inherent factors that can lead to error, are still the ultimate reference standard for the other methods because they measure the activity of the vitamin rather than the total amount of vitamin present. Because of the time and error factors in these biological methods, the wet chemical methods are the most popular procedures used today.

Semi-automated systems[3,4] based on the wet chemistry of the approved methods[5,6] can run from 40 to 60 samples per hour. Systems such as these can run a number of analyses on the same sample extract, but require that additional channels be added to the sytem to do multiple analyte analyses. These systems have been compared with the approved manual wet chemical methods and have shown good agreement[7,8]. Totally automated and semi-automated systems enjoy great popularity where a large number of the same type of analyses are required.

Simultaneous determination, using high-performance liquid chromatography (HPLC), of a number of the water-soluble vitamins in standard mixtures and multi-vitamin preparations has been described[9,10]. Simultaneous HPLC determination of thiamine and riboflavin, at fortification levels in cereal products, using ion-pairing with UV detection has also been reported[11,12]. However, UV detection techniques lack sensitivity and are frequently subject to serious interference. Work in our laboratory using UV detection has shown the potential for HPLC separation of water-soluble vitamins in fortified cereal products, but the need for gradients and long analysis times makes this methodology impractical[13].

HPLC with fluorescence detection has been used to determine riboflavin in various food products[14,15] giving improved sensitivity and selectivity over UV techniques. Ang and Moseley[16] have determined riboflavin and thiamine in meat extracts by HPLC; however, different chromatographic conditions were required for each vitamin. Fellman *et al.*[17] and Skurray[18] have determined riboflavin and thiamine in

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food extracts simultaneously by HPLC with fluorescence detection, following oxidation and clean-up of the sample extracts. However, some riboflavin may be destroyed during the oxidation step. Van de Weerdhof *et al.*[19] and Kimura *et al.*[20] have applied a post-column thiochrome derivitization technique to the determination of low level thiamine in blood.

The purpose of this work was to develop a method with a simple chemical workup procedure and design a chromatographic system that would allow the simultaneous determination of riboflavin and thiamine from complex matrices.

EXPERIMENTAL

Materials

All reagents used in the extraction were prepared according to AACC Method 86-80[21]. Thiamine and riboflavin (Sigma) were used to prepare standards (0.2 μg/ml and 1 μg/ml, respectively) according to approved methods[5,6]. The liquid chromatographic solvents were of LC grade and the hexanesulfonic acid, sodium salt, was obtained from Regis.

The high-performance liquid chromatograph was composed of the following modules. A Waters Assoc. M6000 high-pressure pump was used with a Rheodyne Model 7120 syringe loading sample injector equipped with a 100-μl loop. The analytical column was a Waters Assoc. μBondapak C_{18} (30 cm × 4.1 mm I.D.), and detection was with a Laboratory Data Control (LDC) fluoro-monitor (Model 1209) and Aminco fluoro-colorimeter (Model J4-7440) connected in series by a reaction tee and delay coil (66 cm × 0.25 mm I.D.). The reaction tee was fabricated from a 1.0-cm segment of 316 stainless-steel hex bar stock drilled axially in a 120 degree configuration to accept three male Swagelok zero dead volume (ZDV) 1/16 in. tube connectors (Fig. 1). A Hewlett-Packard 3385A automation system and a Houston Omniscribe recorder were used in data handling.

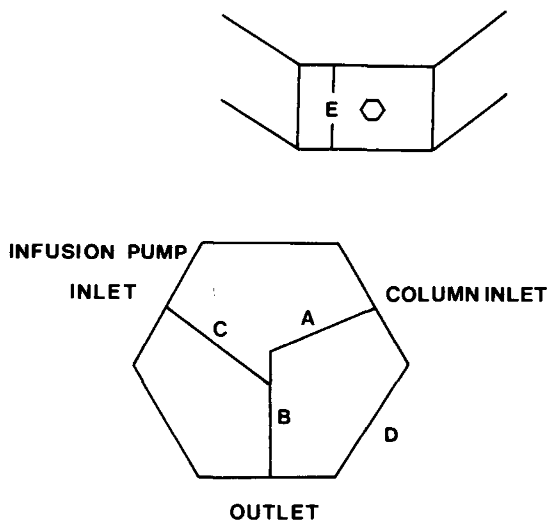

Fig. 1. Reaction tee for post-column production of thiochrome. (A) 13 × 0.51 mm; (B) 15 × 0.51 mm; (C) 15.5 × 0.51 mm; (D) 16 mm, (E) 10 mm.

The Aminco fluoro-colorimeter was adapted to continuous flow with a round 9-μl continuous flow cell (flow cell kit J4-7486). Flow was from bottom to top of the cell to avoid bubble entrapment. A Corning 7-60 primary filter was used in conjunction with a 3-hole aperture plate for excitation and a 415-nm cutoff filter (Wratten 2A). A 1 $\times$ 32 mm aperture plate was placed just after the secondary filter to reduce baseline noise. The LDC fluoro-monitor was equipped with a Corning 7-60 primary filter and a secondary filter which allowed measurement in the 400–700 nm range.

Method

An approximately 2-g sample containing both analytes in the range of 20 μg each was weighed accurately on an analytical balance, placed in a 100-ml volumetric flask, and 50 ml of 0.1 *N* sulfuric acid was added. The sample was mixed well and placed in a boiling water bath for 10 min, removed, and allowed to cool. A volume of 5 ml of Mylase-P solution (2 g in 100 ml of 2.5 *M* sodium acetate buffer) was added and the solution digested at 56°C for 1 h. The sample was allowed to cool and then diluted to volume with distilled water and filtered through Whatman 2V filter paper. A 100-μl aliquot of the filtrate was injected into the liquid chromatographic system (Fig. 2) using the following conditions: eluting solvent, 0.005 *M* hexanesulfonic acid in methanol–water (36:64) with 1% glacial acetic acid, at a flow-rate of

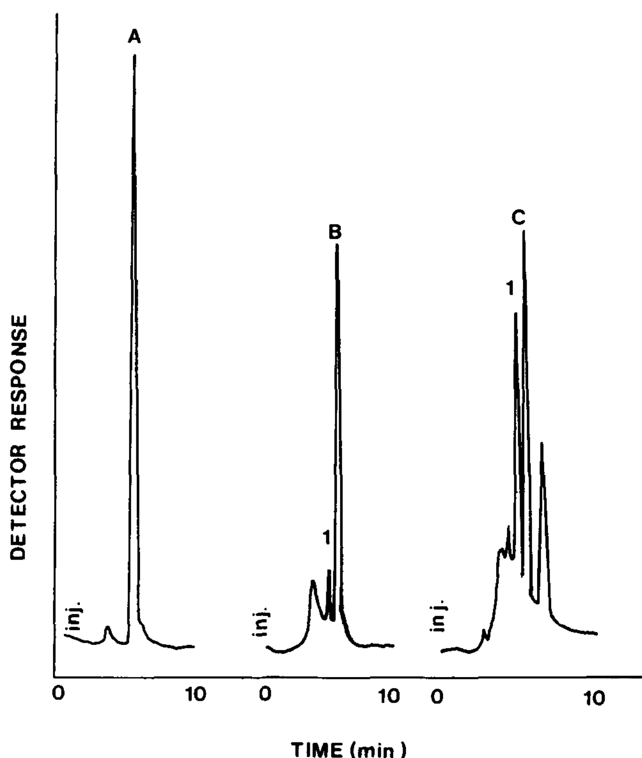

Fig. 2. Chromatographic separation of thiochrome from a standard (A), enriched wheat flour (B), and corn flour (C). 1 = Thiochrome.

0.8 ml/min. Native riboflavin fluorescence was monitored immediately after the column. Thiochrome was produced post column by adding the potassium hexacyanoferrate(III) solution (30 mg/100 ml 3.75 *N* sodium hydroxide) at a flow-rate of 0.25 ml/min through the reaction tee. Thiochrome fluorescence was monitored by a second detector to achieve simultaneous determination.

RESULTS AND DISCUSSION

The classical determination of thiamine, although not a complicated procedure, is long and involves the manual production of thiochrome and its extraction into isobutanol prior to measurement of fluorescence. By increasing the enzyme digestion temperature from 37°C to 56°C, the required digestion time was reduced to one hour. Comparison of 15 corn and wheat flour samples, run under both digesting conditions using the HPLC system here reported, showed excellent agreement with a correlation coefficient of 0.99.

Chromatographic detection of the thiochrome molecule is possible after extraction into isobutanol using a μBondapak C_{18} column and an eluting solvent of methanol–water (60:40) at a flow-rate of 1 ml/min, but use of the liquid chromatograph at this point in the procedure is impractical because manual analysis is faster. HPLC separation of thiochrome without extraction into isobutanol was attempted. A volume of 5 ml of the filtered extract was quantitatively transferred to a 15-ml vial; 3 ml of the alkaline potassium hexacyanoferrate(III) solution were added and the mixture was stirred for 30 sec with a magnetic stirrer (pH 12.3). As anticipated, addition of large amounts of the strongly basic materials rapidly decreased the life of the column.

Adjusting the pH of the solution with dropwise addition of concentrated sulfuric acid while monitoring with a pH meter was attempted, but was too time consuming. One drop of phenolphthalein was added to the solution after the 30-sec reaction time and then the sulfuric acid was added dropwise until the solution was colorless. Injection of this solution onto the column resulted in good separation, but problems arose with the reproducibility of the thiochrome retention time. During seven replicate injections the retention time increased from 4.80 to 5.14 min. This increase was possible due to other material in the extract which affected the packing material and changed the retention characteristics for the thiochrome. This change in the retention made quantitation impossible.

Earlier work using UV detection on vitamin enrichment mixtures showed good chromatographic reproducibility for the thiamine molecule using reversed-phase packings and ion-pairing. Attempts were made to design a system in which thiamine was first separated from the interfering materials by HPLC, and then reacted with the hexacyanoferrate(III) reagent by post-column addition. This was done to aid selectivity and sensitivity. A reaction tee (Fig. 1) was used for the post-column addition of the potassium hexacyanoferrate(III) solution. A flow-rate of 0.25 ml/min gave an optimum response using a delay coil with a 30-sec holdup time.

Following extraction of samples as described in the methodology section, the separation of the thiamine was performed (Fig. 2). Chromatograms of wheat flour yielded one major peak (Fig. 2A) while chromatograms of corn flours yielded multiple, well resolved major peaks (Fig. 2B and C). Chromatograms of the thiochrome

produced by the post-column method showed no significant increase in band broadening due to the addition of the reaction tee and delay coil compared to externally generated thiochrome.

Samples including hard and soft wheat flours, corn flour, durum semolina, cooked spaghetti and white pan bread products were analyzed for thiamine by the approved method and the HPLC procedure described here. The correlation coefficient between the two methods was 0.98. Replicate injections of a single sample resulted in a relative standard deviation of 1.61%. The relative standard deviation between pairs for the overall HPLC procedure was 2.85%. Using the HPLC procedure, the minimum detectable quantity of thiamine was 0.14 μg/g in the products described above. Accuracy for thiamine was ± 0.03 μg/g at a level of 5 μg/g.

Further examination of the sample chromatograms gave indications that one of the major peaks (peak 1, Fig. 2B and C) was actually riboflavin. Addition of riboflavin to the thiamine standard at a level of 0.1 μg/ml showed no peak corresponding to the peak found in the sample chromatograms, but injection of a much larger quantity, 10 μg/ml, did produce a peak which corresponded to the unknown peak. Also, addition of riboflavin to digested samples showed increases in the peak believed to be riboflavin.

The absence of the peak corresponding to riboflavin in the standard mixture is due to the formation of the nonfluorescent anionic form of riboflavin in strongly basic solutions[13,14]. The presence of a riboflavin peak in the corn extracts under the basic condition described, when not found in the standard mixture, may be due to a buffering effect of other nonfluorescent components present in the extract which elute undetected at or near the riboflavin peak. Using the system described, a standard mixture containing 0.1 μg/ml of riboflavin, under acid conditions, produced a peak with a retention time corresponding to that of the suspected riboflavin peak. Addition of riboflavin to that standard and to a sample extract showed corresponding increases in the riboflavin peak heights of each solution.

The samples used for thiamine analysis were also analyzed for riboflavin by the approved method and the HPLC procedure. The correlation between the two methods was 0.996. Replicate injections of a single sample resulted in a relative standard deviation of 1.2%. The relative standard deviation between pairs for the overall HPLC determination was 1.46%. The minimum detectable quantity for the HPLC procedure was 0.013 μg/g. Accuracy was ± 0.1 μg/g at a level of 10 μg/g.

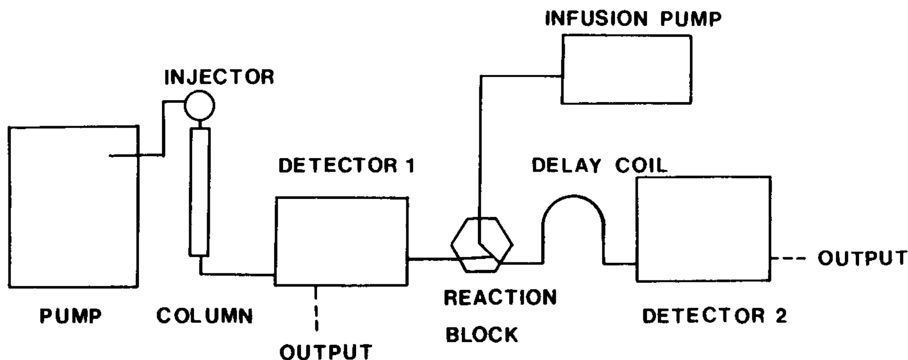

Fig. 3. Liquid chromatographic system for the simultaneous analysis of thiamine and riboflavin.

Modification of the chromatographic system designed for thiamine to include a second fluorometer immediately following the column and prior to the reaction tee allowed for the simultaneous monitoring of riboflavin under acidic conditions, where its natural fluorescence was not inhibited (Fig. 3). Using the first detector to monitor riboflavin and the second detector to monitor thiamine under basic conditions make it possible to detect both the thiamine and the riboflavin simultaneously in 10 min (Fig. 4).

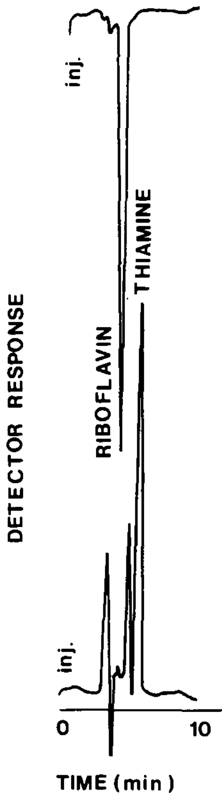

Fig. 4. Simultaneous analysis of riboflavin and thiamine from enriched corn flour.

CONCLUSIONS

Use of the extraction procedure and chromatographic system described shortens the wet chemical workup for both the thiamine and riboflavin. It eliminates the manual production of thiochrome, the use of Decalso or pre-extraction with isobutanol to eliminate interfering materials, and the necessity of running background fluorescence on every sample. In the analysis of riboflavin it eliminates the need for permanganate and hydrogen peroxide oxidation and the necessity of determining background fluorescence readings. This method allows for the determination of a minimum of fifteen riboflavin and thiamine samples in duplicate in one working day.

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