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Determination of Thiamin and Riboflavin in Meat and Meat Products by High-Pressure Liquid Chromatography

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A sensitive method for the determination of thiamin and riboflavin in meat and meat products is described that involves high-pressure liquid chromatography and fluorometric detection. Riboflavin is converted to lumiflavin by UV irradiation and thiamin is oxidized to thiochrome prior to the chromatographic separation. These conversions enhance the detectability of trace amounts of vitamins. The lowest detection limit for thiamin was 0.05 ng and for riboflavin, 0.02 ng. Analyses of five samples of meats or meat products and the recovery data for added vitamins are given. Mean recovery values ranged from 84.4 to 94.2% for thiamin and from 88.1 to 99.9% for riboflavin.

High-pressure liquid chromatography (LC) has been used increasingly for the separation and determination of nonvolatile compounds. It has an advantage over some chemical methods in its specific resolution characteristics to differentiate some closely related chemicals. The use of LC for thiamin and riboflavin analysis is not new. However, most of the methods have been developed for pharmaceutical products and vitamin premixes that contain high levels of vitamins in pure form (Williams et al., 1973; Callmer and Davies, 1974; and Wills et al., 1977).

Recently, a few studies have been reported on the application of LC in the determination of B vitamins in food products. Van de Weerdhof et al. (1973) described a procedure using a silica gel column to separate riboflavin and thiamin, to oxidize thiamin to thiochrome after the separation, and to measure the quantity of riboflavin and thiochrome with a fluorometric detector. Mauro and Wetzel (1978) used a post column for the production of thiochrome after the chromatographic column separation and also suggested the use of a second fluorometer to determine riboflavin simultaneously. Toma and Tabekhia (1979) used paired-ion reagents in mobile phase to separate thiamin, riboflavin, and niacin from aqueous extract of rice and rice products with a reverse-phase column (Bondapak C_{18}) and an ultraviolet light (UV) detector.

The low levels of vitamins and high amounts of other interfering materials in many food products often make the chromatographic determination of the direct extracts unfeasible. This paper describes a procedure for conversion of riboflavin to lumiflavin and thiamin to thiochrome before the chromatographic separation and fluorometric determinations of the products. Natural levels of these two vitamins in meats were measured without difficulty.

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EXPERIMENTAL SECTION

Apparatus and Liquid Chromatograph Conditions. A Waters Associates Model ALC/GPC 204 liquid chromatograph with a Wisp 710 automatic sampler (Waters Associates, Milford, MA) was used. The chromatographic column was a 2.1 mm × 50 cm stainless steel column packed with spherisorb silica 20 μm. The mobile phase was a solvent mixture of 90% chloroform and 10% methanol at a flow rate of 1.0 mL/min for thiamin and 0.8 mL/min for riboflavin.

The following settings of a Schoeffel Model 970 fluorescence detector (Schoeffel Instrument Corp., Westwood, NJ) were used for measuring thiamin: wavelength, 367 nm; entrance filter, No. 7-51; exit filter, KV 418; sensitivity, 7.0; range, 0.05; and time constant, 6 s. For measuring riboflavin, the following settings were used: wavelength, 270 nm; entrance filter, No. 7-54; exit filter, KV 418; sensitivity, 6.0; range, 0.05; time constant, 4 s. (Settings for sensitivity, range, and time constant must be determined on each instrument for best results.)

For UV irradiation of riboflavin, a chromato-VUE cabinet, model C-5 (Ultra-Violet Products, Inc., San Gabriel, CA), with both long and short UV lamps was used.

Extraction of Meat Samples. Five types of meats and meat products were analyzed: chicken thigh meat without skin, ground beef, cooked wieners, bologna, and canned pork shoulder picnic. Samples were prepared and extracted primarily according to AOAC procedures (AOAC, 1970) with slight modifications. Sufficient ground material (5 g) weighed to the nearest hundredth gram was hydrolyzed with 0.1 N HCl (60 mL) at 121 °C in an autoclave for 30 min. After the pH of the solution was adjusted to 4.0–4.5 with about 5 mL of sodium acetate (2 M), 4 mL of each freshly prepared aqueous enzyme solution (5% Takadiastase and 10% papain) were added and the samples were incubated at 42–45 °C for 2.5–3 h. To precipitate the proteins, 2 mL of 50% trichloroacetic acid was added, and the solution was heated on a steam bath for 5 min (Van de Weerdhof, 1973). The sample solution was then diluted to 100 mL, filtered through Whatman No. 40 filter paper, and refrigerated until use the following day. At least five replicates were prepared for each meat sample. All solutions containing thiamin, riboflavin, lumiflavin, and thiochrome were handled under subdued light.

Preparation of Standard Solutions and Samples for Recovery Test. Standard stock solutions of riboflavin (25 μg/mL) and thiamin (100 μg/mL) were prepared from USP Reference Standards in 0.01 N HCl. Working standard solutions (0.5 μg/mL) and intermediate standards (5 μg/mL) were made on the day of use from the stock solutions by suitable dilutions.

For recovery studies, 1 mL of the intermediate standard solution was added to the sample solution before the enzyme hydrolysis step. For pork shoulder picnic, 2- to 5-mL aliquots of the thiamin intermediate solution were added because this product has a high amount of thiamin and further dilutions are necessary. At least five replicates were prepared for each meat sample for the recovery test.

Determination of Riboflavin. The procedure for converting riboflavin to lumiflavin was adapted from Strohecker and Henning (1966). Ten milliliters of the sample filtrate was pipetted into a 50-mL beaker. The pH of the solution was adjusted to 10–12 with 15% NaOH, and the beaker was placed 10 cm below the UV lamp for 30 min. Immediately after the irradiation, 1 mL of glacial acetic acid was added to the beaker, and the contents were transferred into a 60-mL separatory funnel and extracted with 10 mL of chloroform. The chloroform extract was dried with anhydrous sodium sulfate and was then ready for chromatography. Aliquots of 5 or 10 μL, depending on the final riboflavin concentrations, were injected into the LC system. Variable injection volumes were used to eliminate any further dilution or concentration steps which might be required. Heights of the chromatogram peaks were recorded for quantitative calculations.

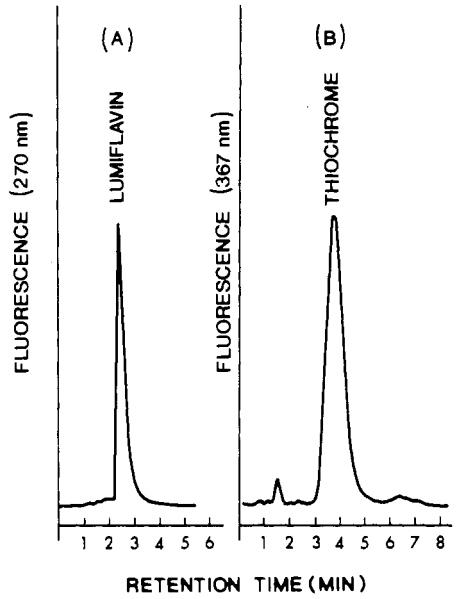

Figure 1. Chromatograms of lumiflavin and thiochrome. Column, 2.1 mm × 50 cm, spherisorb silica 20 μm; mobile phase, 90% chloroform/10% methanol; detector, fluorescence.

For generation of a riboflavin standard curve, 1- to 4-mL aliquots of the intermediate standard solution were added to a series of 100-mL volumetric flasks, which were prepared with 0.1 N HCl, buffers, and enzyme solutions, and were handled in the same manner as for the samples. The concentrations of riboflavin in the final chloroform extract from this series of flasks were 0.05–0.20 μg/mL. Standard curves were plotted with peak height in units vs. nanograms of vitamin per injection.

Determination of Thiamin. Thiamin was oxidized to thiochrome with the techniques described in AOAC (1970). No Decalso ion-exchange column clarification was necessary. Ten milliliters of the sample filtrate was oxidized with 5 mL of 1% alkaline potassium ferricyanide and the resulting thiochrome was extracted with 10 mL of isobutyl alcohol. Aliquots of 10 or 20 μL of isobutyl alcohol extract, depending on the final thiamin concentrations, were injected into the LC system.

For the construction of a standard curve for thiamin, 1- to 4-mL aliquots of the working standard solution were oxidized in the same manner as were the sample solutions. The final concentrations of the standard in the isobutyl alcohol extracts ranged from 0.05 to 0.2 μg/mL.

Calculations. The vitamin content of the sample extracts was obtained by interpolation on the corresponding standard curve, and the final concentration in meat samples was calculated in units of milligrams per 100 grams by applying the proper dilution factors.

RESULTS AND DISCUSSION

Typical chromatograms of lumiflavin and thiochrome in meat products are shown in Figures 1A and 1B. Retention times under the chromatographic conditions in this experiment were 2.5 min for lumiflavin and 3.6 min for thiochrome. The backgrounds of the chromatograms were free of interference, except that one small peak was present

Table I. Thiamin and Riboflavin Content (mg/100 g, Wet Weight) of Meat and Meat Products as Determined by High-Pressure Liquid Chromatographic Method[a]

meat	thiamin	riboflavin
chicken thigh meat	0.085 ± 0.003	0.215 ± 0.005
bologna	0.094 ± 0.003	0.199 ± 0.007
ground beef	0.067 ± 0.003	0.207 ± 0.008
weiners	0.062 ± 0.003	0.138 ± 0.007
pork shoulder picnic	0.486 ± 0.009	0.144 ± 0.006

[a] Mean and standard deviation of five separate determinations.

just before the thiochrome peak. This peak was found to originate in the anhydrous sodium sulfate and could represent a contaminant. Because it did not interfere with the thiochrome peak, no further identification was attempted.

Linear regression analysis of the relationship between the peak height and amount of standard from eight separate determinations at concentrations of 0.5–2 ng per injection resulted in the following values for intercept (I), slope (S), and correlation coefficient (r):

	thiamin	riboflavin
I	0.5-2.5	−2.0-1.0
S	19.2-23.8	19.5-22.5
r	0.9947-0.9999	0.9881-1.0000

The data indicate that standard curves are practically linear within the range tested. Linear relationships were also found for riboflavin at 0.2–1 ng and 2–8 ng and for thiamin at 2–8 ng per injection. However, the instrumental conditions had to be set at different sensitivities and the slopes of the curves changed accordingly. Thus, it is important to generate a new standard curve with every batch when samples are analyzed.

For riboflavin, it was necessary to run standards in the same manner as for the samples, i.e., with the addition of enzyme preparations, because the slight amount of soluble protein solids in the filtrate had some effect on the UV irradiation characteristics. Preliminary tests showed that the conversion of riboflavin to lumiflavin in pure riboflavin solutions was less than that in the enzyme solutions.

The results of analysis of five meats are presented in Table I. The standard deviations of the means were relatively small and the coefficients of variation were below 5%. The recovery studies of vitamins added to replicate samples are shown in Table II. The average values were 84.4–94.2% for thiamin and 88.1–99.9% for riboflavin. The standard deviations of the mean percent recovery were below 6%. These data suggest that the method described here is suitable for analysis of thiamin and riboflavin in meat and meat products.

Experimental errors may be encountered at the oxidizing step of thiamin to thiochrome and at the UV irradiation step of riboflavin. At least two aliquots from the same sample filtrate should be analyzed at these steps. Another place where variations could occur is the injection volume. Duplicate injections are recommended for each sample extract.

We have attempted to determine the vitamin concentrations with the method of Toma and Tabekhia (1979). However, the UV detector was not sensitive enough to detect the natural levels of vitamins in meats and there were many overlapping peaks if the sample extracts were prepared in high concentrations.

The conversion of riboflavin to lumiflavin and thiamin to thiochrome described here enhanced their detectability by the fluorometric detector and also resulted in a much cleaner sample for injection on the liquid chromatograph. The lowest detection limit was 0.05 ng for thiamin and 0.02

Table II. Recoveries of Vitamins Added to Meat Samples by High-Pressure Liquid Chromatographic Method

	thiamin			riboflavin		
meat	amount added, mg/100 g	total found, mg/100 g	% recov[a]	amount added, mg/100 g	total found, mg/100 g	% recov
chicken thigh meat	0.100	0.175	89.8	0.100	0.297	82.0
	0.100	0.177	91.8	0.100	0.300	85.0
	0.100	0.174	88.8	0.100	0.304	89.0
	0.098	0.184	100.8	0.094	0.302	92.6
	0.100	0.185	99.8	0.098	0.305	91.8
mean ± SD			94.2 ± 5.7			88.1 ± 4.5
bologna	0.092	0.181	94.6	0.100	0.298	99.0
	0.100	0.181	86.7	0.094	0.292	98.9
	0.100	0.181	86.6	0.097	0.297	101.0
	0.094	0.176	86.8	0.092	0.279	87.0
	0.099	0.177	83.4	0.099	0.293	94.9
mean ± SD			87.6 ± 4.2			96.2 ± 5.6
ground beef	0.100	0.153	86.4	0.100	0.298	91.0
	0.098	0.148	83.1	0.099	0.290	83.8
	0.099	0.154	88.3	0.093	0.287	86.0
	0.096	0.142	78.9	0.100	0.306	99.0
	0.096	0.152	89.1	0.096	0.295	91.7
mean ± SD			85.2 ± 4.2			90.3 ± 5.9
wieners	0.099	0.145	83.6	0.099	0.236	98.9
	0.092	0.143	87.8	0.098	0.237	101.0
	0.092	0.136	80.2	0.097	0.236	101.0
	0.098	0.145	84.5	0.093	0.228	96.8
	0.098	0.146	85.7	0.098	0.238	102.0
mean ± SD			84.4 ± 2.8			99.9 ± 2.1
pork shoulder picnic	0.196	0.669	93.4	0.098	0.231	89.3
	0.198	0.688	102.0	0.093	0.228	90.4
	0.294	0.755	91.5	0.097	0.229	87.6
	0.483	0.933	92.5	0.097	0.237	95.9
	0.496	0.939	91.3	0.097	0.236	94.8
mean ± SD			94.1 ± 4.5			91.6 ± 3.6

[a] Percent recovery = [total found − mean value (Table I)]/amount added × 100%.

ng for riboflavin. For some samples, if further clarifications of the extracts are needed, one can use isobutyl alcohol (for thiamin) or chloroform (for riboflavin) to extract the sample filtrate prior to the oxidation step or the UV irradiation step to remove the interfering substances.

With automatic sampler and data processor, the analyst's time can be saved markedly. One operator should be able to analyze at least 20 samples, in duplicate, for thiamin or riboflavin in 1 day provided that samples are prepared and hydrolyzed the day before.

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Comparison of High-Performance Liquid Chromatographic and *Saccharomyces uvarum* Methods for the Determination of Vitamin B_6 in Fortified Breakfast Cereals

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A previously developed high-performance liquid chromatographic (LC) method was modified to permit its use for the determination of vitamin B_6 in fortified breakfast cereals. The LC method employed an acidic potassium phosphate mobile phase, an octadecylsilica column packing, and detection of the natural fluorescence of the eluted B_6 vitamers. The LC assay of vitamin B_6 in five selected cereals provided consistently high recovery (93.9 ± 7.6%) and precision (coefficient of variability = 4.5 ± 2.7%) and was found to be free of interfering fluorophores. Microbiological assays for total vitamin B_6 using *Saccharomyces uvarum* showed evidence of growth inhibition in four of the five cereal samples. Microbiological assay results based on the mean response for three levels of extract addition to the assay tubes correlated well with the LC results. However, the validity of this comparison must be questioned because of the interference encountered in the microbiological analysis. The LC method was more satisfactory than the microbiological procedure for cereal analysis because of its simplicity, potentially large number of samples which may be analyzed, demonstrated accuracy, and high precision.

Extensive research has been directed toward the development of rapid, sensitive methods for the determination of vitamin B_6 in foods. Because of the limitations of time and precision often associated with conventional microbiological assay methods, recent research has dealt mainly with direct analysis using chemical and physical techniques.

Many complex fluorometric methods have been developed for the determination of vitamin B_6 in foods and biological materials. These have been based mainly on preparative electrophoresis or ion-exchange chromatography, followed by conversion of the B_6 vitamers to either 4-pyridoxic acid or its lactone for fluorometric quantitation (Fujita et al., 1955; Hennessy et al., 1960; Kraut and Imhoff, 1967; Contractor and Shane, 1968; Loo and Badger, 1969; Columbini and McCoy, 1970; Takanashi et al., 1970; Masukawa et al., 1971; Fiedlerova and Davidek, 1974; Chin, 1975; and Gregory and Kirk, 1977). Although the accuracy of these methods has not been extensively examined, the lack of close agreement between fluorometric and microbiological assay results indicates that the fluorometric methods are subject to interference (Kraut and Imhoff, 1967; Chin, 1975; Gregory and Kirk, 1978a,b).

High-performance liquid chromatography (LC) was first applied to the determination of vitamin B_6 in foods by Yasumoto et al. (1975), although application of their procedure is limited by cumbersome gradient elution and post-column derivitization instrumentation. Several simpler LC methods for the separation and ultraviolet absorption detection of the B_6 vitamers have been reported (Williams and Cole, 1975; Wong, 1978; Williams, 1979). However, a lack of chromatographic efficiency and detection sensitivity has precluded the application of these methods to vitamin B_6 assay in foods. Gregory and Kirk (1978b) reported an LC method for the determination of the B_6 vitamers in dehydrated model food systems which was based on an isocratic reverse-phase separation and fluorescence detection. Although this method has been adapted to the determination of 4-pyridoxic acid in urine (Gregory and Kirk, 1979), it has not yet been employed for food analysis. Recently, Vanderslice et al. (1979) reported a method based on anion-exchange LC separation

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