Determination of thiamine and riboflavine in meat products by High Performance Liquid Chromatography

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ABSTRACT

A High Performance Liquid Chromatographic method for the quantitative determination of thiamine and riboflavine in meat products is described. Detection is accomplished by use of a flourescence detector, where riboflavine is measured by its native flourescence, while thiamine is detected after a pre-column derivatization into thiochrome. Methods for separate or simultaneous determination of riboflavine and thiamine are discussed, and results are compared to AOAC methods. Limit of detection is approximately 0.1 - 0.2 µg per gram of the meat sample, and the methods gives nearly 100% recovery, when samples are enriched with known amounts of thiamine and riboflavine.

INTRODUCTION

Methods for the quantitative determination of thiamine (B_1) and riboflavine (B_2) by HPLC have been described during the past years. Simultaneous determination of several water-soluble vitamins using UV-detection have been described (4,5,6), however this detection princible is not sensitive enough for determination of B_1 and B_2 in levels normally occuring in meat products. Increased sensitivity and specificity is achieved using flourescence detection, with B_2 to be measured by its native flourescence or after conversion to lumichrome, while B_1 is detected after derivatization into thiochrome (1,2,3). Ang and Moseley (1) describe flourescence detection for the determination of B_1 and B_2 after separate conversion into thiochrome and lumichrome, respectively. Thus the method does not permit simultaneous determination. Hedlund (3) use a post-column derivatization of B_1 into thiochrome, which makes a determination of B_1 and B_2 in the same chromatographic run possible. A major disadvantage is that the chromatograph had to be equipped with two flourescence detectors in addition to a peristaltic pump and a mixing chamber for the derivatization process. Recently, Fellman et al. (2) have described a chromatographic method for the simultaneous determination of B_1 and B_2 in selected foods. The method, in several points, is similar to the here discribed with flourimetric determination of B_2 by its naturally flourescence and B_1 after a pre-column derivatization into thiochrome. A major difference is that Fellman et al. neutralize the extract after thiochrome oxidation followed by a Sep-Pak purification. Furthermore, examination of chromatograms of Fellman et al. would indicate, that there are some difficulties in separating B_1- and B_2-peaks from the sample matrix and from each other.

The methods here described shows excellent separation using either simultaneous or separate determination of B_1 and B_2. Separate determination of B_1 and B_2 is recommended where maximum sensitivity is

required, however, in most cases adequate sensitivity is achieved when determination is performed from a single chromatographic run.

MATERIALS AND METHODS

Equipment

The chromatograph consist of a pump model 6000A, a model U6K sample injector and a model 420 flourescence detector, all parts from Waters Associates. Separations were performed on a Waters µBondapak C18 (3.9 mm id. x 30 cm) steel column. The analytical column was protected by a guard column packed with Bondapak C18/Corasil. Detector responces were recorded and quantitated by a Waters Model 730 Data Module.

Mobile phase

The mobile phase was prepared by mixing 400 ml of reagent grade methanol with 600 ml of a 0.01M sodium phosphate buffer pH 7.0. Prior to use, the mobile phase was degassed by vacuum filtration through a 0.5 µ filter, type FH (Millipore Inc.).
Prior to sample injection, the column was washed with 30 ml of the mobile phase with a flow rate of 1 ml/min. The same flow rate was used for the separations. After each day of work, the LC-system was washed with 30 ml of a degassed mixture of 70% methanol in deionisied water.

Reagents

Clara-Diastase ("Clarase 300") was obtained from Fluka AG, Switzerland. Thiamine and riboflavine (biochemicals) were obtained from Merck, Germany. All other reagents were of reagent grade quality.

Sample preparation

Samples used for this investigation were canned, fully cooked pork luncheon meat.
Five-gram portions of the minced meat sample were weighed into a 100 ml glass beaker, 30 ml of 0.1M HCl were added and samples were autoclaved at 121°C for 20 min. After cooling to room temperature, the pH of the samples were adjusted to 4.0 - 4.5 by adding 2 ml of 2M sodium acetate. After addition of 1 ml 10% diastase in water, the samples were incubated over night (approximately 16 hours) at room temperature in a dark place. Samples were transferred to 50 ml volumetric flasks, brought to volume with deionisied water and then filtered through a Whatmann No 40 filter paper. Filtrates were normally analyzed the same day, but are stable at least one day if stored refrigerated.
Standards (0,5,10 and/or 25 µg of riboflavine and thiamine) were treated in the same way as samples. With the HPLC-methods outlined below, the standard curves were straight-lined with a zero blank reading.

Separate determinations of B_1 and B_2 .

For the separate determination of thiamine and riboflavine, the flourescense detector was equipped with a 365 nm exitation filter and a 455 nm emmision filter. Detections were accomplished with a gain setting 32 or 64, corresponding to respectively 25% and 50% of maximum sensitivity.
For the determination of B_2, 100 µl of the filtrate was directly injected into the LC-system. After 4.4 min, B_2 isocratically elutes separated from the sample matrix.
Determination of B_1 was performed after a pre-column derivatization into thiochrome. 1 ml 4M sodiumhydroxide and 100 µl 0.03M potassium ferricyanide, freshly mixed, was added 2 ml of the sample filtrate. After 5 min oxidation, 25 µl was injected with thiochrome eluting after 5.1 min.

Simultaneous determination of B_1 and B_2

For the determination of B_1 and B_2 in the same chromatographic run, a exitation filter of 365 nm and a emmision filter of 495 nm were used in the flourimeter. The higher emmision filter lowers the sensitivity for thiochrome, but enables the two vitamins to be detected with nearly equal sensitivity. Furthermore, the choice of 495 nm emmision filter gives a more stable baseline, allowing the flourescence detector to be operated at its maximum sensitivity (gain setting 128).
1 ml 4M sodiumhydroxide and 100 µl 0.03M potassium ferricyanide was mixed in a glass tube. 2 ml of the sample filtrate was added and mixed, and after exactly 1.5 min of oxidation, 100 µl was injected.

Quantitation

Chromatograms were digitally integrated by the Data Module. Standards were used for the calibration of signals, and data was quantitated using the method of external standard quantitation.

RESULTS AND DISCUSSION

Fig. 1 shows the effect of the oxidation time on riboflavine and thiochrome responses, when analyzed simultaneously. Following procedure described for the sample, a standard vitamin mixture (10 µg/50 ml each of B_1 and B_2) was oxidized for periods of upto 20 min before chromatographic injection. Results, expressed as percent of the responce for the standard 1.5 min oxidation period, indicates that thiochrome reached its maximum responce after 1 to 1.5 min, hereafter the responce slowly decreased. Riboflavine was consistently destroyed during thiamine oxidation due to the alkaline conditions. However, the loss is limited after short time oxidation. Normally, injection of alkaline sample in liquid chromatography is not recommended due to possible hydrolysis of silica support of the analytical column. It is our experience, however, that direct analysis of the alkaline sample improves peak separation and thiochrome sensitivity compared to neutralizing prior to injection. Small injection volume together with buffered mobile phase and a guard column is here used for protection of the analytical column.

Fig. 2 and 3 show the chromatographic separations of thiochrome and riboflavine for standards and meat samples using separate or simultane determinations respectively. For the separate determinations of B_1 and B_2, the limit of detection is approximately 0.3 ng of thiochrome and 1 ng of riboflavine in the eluting peaks. With the stated method of analysis, this sensitivity corresspond to 0.1 µg of each vitamin per gram of the meat sample, but it is possible to increase sensitivity for thiamine at least four times if using 100 µl injection volume. For the simultaneous determination of B_1 and B_2 the limit of detection is approximately 0.2 µg of each vitamin per gram of the meat sample.

In total 13 single cans of pork luncheon meat was analyzed by the HPLC-methods here described and by the AOAC-methods 43.024-43.030 (vitamin B_1, chemical method) and 43.126-43.133, 43.168-43.176 (vitamin B_2, microbiological method). Results are given in Table 1, where data are grouped in four series. Series I and II, each consisting of data obtained from a single can, differs significally in thiamine level from the rest of the cans and from each other. In series III (data from two cans) the riboflavine content is significally higher than found in the other series. As shown, values obtained for B_1 and B_2 using either of the HPLC-methods is in good agreement with corresponding AOAC values. Standard deviations for the HPLC-methods is similar to the AOAC-methods, and ranged from 2.0% to 5.3%.

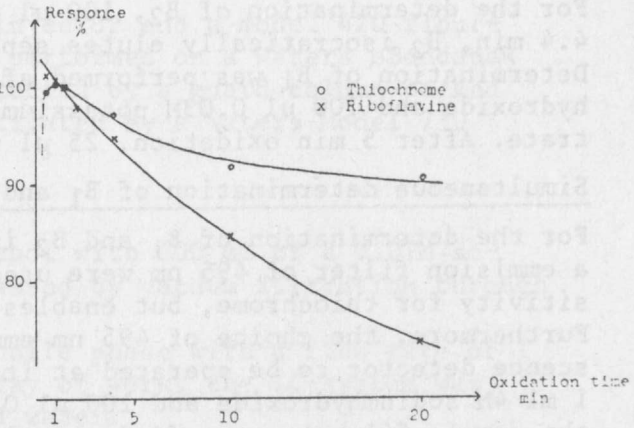

Fig. 1: Effect of the oxidation time on riboflavine and thiochrome responses.

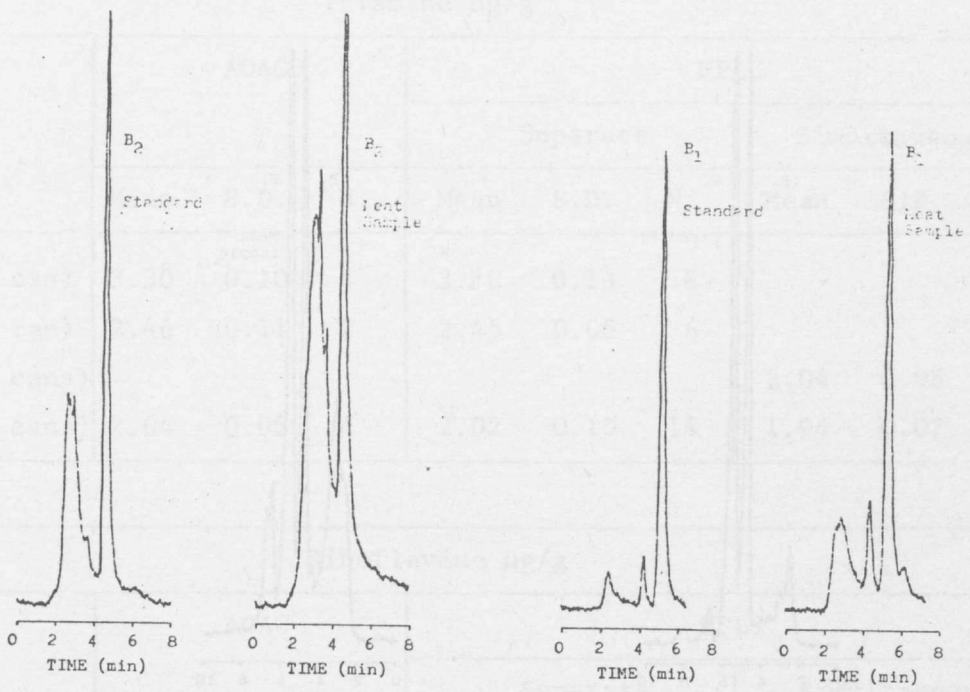

Fig. 2: Chromatograms for the separate determination of riboflavine and thiamine.

Standard	: 10 µg/50 ml each of B_1 and B_2
Sample	: Pork luncheon meat
Injection volume	: 100 µl for B_2 determination 25 µl for B_1 determination
Column	: µBondapak C18
Mobile phase	: 60/40 phosphate buffer/methanol
Flow rate	: 1 ml/min
Detector	: Filter flourescence detector 365 nm exitation, 455 nm emmission GAIN 64

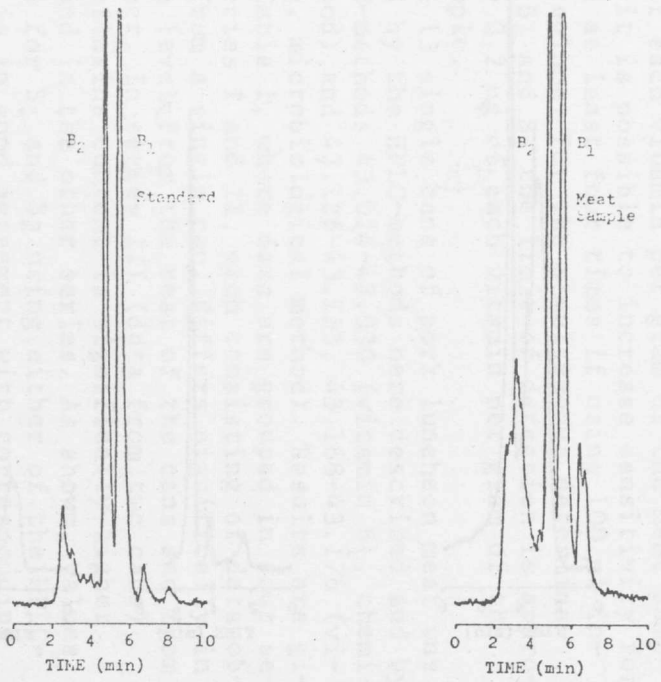

Fig. 3: Chromatograms for the simultaneous determination of riboflavine and thiamine.

Standard	: 10 µg/50 ml each of B_1 and B_2
Sample	: Pork luncheon meat
Injection volume	: 100 µl
Column	: µBondapak C18
Mobile phase	: 60/40 phosphate buffer/methanol
Flow rate	: 1 ml/min
Detector	: Filter flourescence detector 365 nm exitation, 495 nm emmission GAIN 128

Table 1: Results for the determination of thiamine and riboflavine content in canned pork luncheon meat.

Thiamine µg/g									
	AOAC			HPLC					
				Separate			Simultaneous		
Series	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N
I (1 can)	3.30	0.10	4	3.20	0.13	8			
II (1 can)	2.46	0.11	2	2.45	0.05	6			
III (2 cans)							2.04	0.08	12
IV (9 cans)	2.04	0.08	4	2.02	0.10	14	1.94	0.07	18

Riboflavine µg/g									
	AOAC			HPLC					
				Separate			Simultaneous		
Series	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N
I (1 can)	1.87	0.11	2	1.80	0.04	3			
II (1 can)				1.77	0.06	6			
III (2 cans)	2.39	0.05	2				2.44	0.06	12
IV (9 cans)	2.14	0.08	6	1.97	0.06	12	2.09	0.11	18

Table 2: Recovery of 10 µg each of thiamine and riboflavine added to samples before autoclaving.

	HPLC	
	Separate	Simultaneous
µg B_1 recovered	9.94	9.82
S.D.	0.39	0.36
N	10	11
% recovery	99.4	98.2
µg B_2 recovered	9.43	10.20
S.D.	0.85	0.58
N	4	11
% recovery	94.3	102.0

In table 2, results for recovery studies are presented. 10 µg of each of B_1 and B_2 was added to meat samples before autoclaving and vitamin content was determined according to the HPLC-methods here outlined. Recovered amounts of vitamin is given after subtraction of contents in the meat sample and %recovery was consistently nearly 100%.

The HPLC-methods here discribed gives the same average results and precision as the AOAC-methods, but have the advantage of a pronounced decrease in analysis time and manually handling.

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