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Determination of thiamine (vitamin B_1) and riboflavin (vitamin B_2) in meat and liver by high-performance liquid chromatography

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Abstract

HPLC methods described so far for the determination of thiamine and riboflavin in meat samples have used mostly fluorescence detection. The aim of this work was to elaborate a suitable method with UV detection for this purpose and to compare the thiamine and riboflavin contents obtained for meat and liver samples from various pig groups. Homogenized spare rib, chop, ham and liver samples were treated with acid and heated (at 121°C, 30 min), followed by enzymatic digestion to release the vitamins. After a clean-up procedure the simultaneous determination of thiamine and riboflavin was performed with a Nucleosil ODS (3 μm) packed column at 45°C. The mobile phase was phosphate buffer (pH 3.0)–acetonitrile (84:16, v/v) containing 5 m*M* sodium heptanesulphonate. The relative standard deviation was 5% for thiamine and 12% for riboflavin in meat and 8% for thiamine and 5% for riboflavin in liver for four parallel determinations.

1. Introduction

During the last 20 years numerous HPLC methods have been published concerning the determination of water-soluble vitamins in foods or enriched foods. UV detection was used successfully with enriched cereals and cereal products for the determination of their thiamine and riboflavin contents [1] and also with rice and rice products for the determination of thiamine, riboflavin and niacin [2], but in meat samples thiamine and riboflavin have been determined mainly by applying fluorescence detection [3–6]. The type of food sample has to be emphasized because the nature and amount of various components other than vitamins may influence the effectiveness of the separation system.

Our aim was to elaborate a suitable HPLC method with UV detection after a clean-up procedure for the parallel determination of thiamine and riboflavin in meat and liver samples because thiamine has no natural fluorescence so it needs a pre- or postcolumn derivatization when applying fluorescence detection. Measuring UV absorbance, the procedure was simpler and the levels of thiamine in meat and liver samples were sufficient for this less sensitive detection. We applied this method to various pig groups in order to establish whether there is a difference in the vitamin contents, besides other important components, between the groups.

2. Experimental

The enzymes used in the sample preparation

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were papain (Cat. No. 7147; Merck, Darmstadt, Germany), taka-diastase (Cat. No. 86250; Fluka, Buchs, Switzerland) and clara-diastase (Cat. No. 27540; Fluka). The clean-up columns (Nucleosil C_{18} cartridge of 500 mg) and the analytical column were purchased from BST (Budapest, Hungary). Acetonitrile and methanol were of chromatographic grade (Merck). Heptanesulphonic acid sodium salt, thiamine and riboflavin were obtained from Fluka.

2.1. Sample preparation

Spare rib, chop, ham and liver were separated from each animal (twelve animals in both groups) and the meat and liver samples were minced twice in a mincer and stored at −18°C before the analysis.

A 5-g amount of homogenized sample was suspended with 35 ml of 0.01 *M* hydrochloric acid and then autoclaved at 121°C for 30 min. After cooling to room temperature, 2.0 ml of taka-diastase suspension (2.5 g per 100 ml of 2.5 *M* sodium acetate solution), 2 ml of clara-diastase suspension (1 g per 100 ml of water) and 2 ml of papain suspension (5 g per 100 ml of water) were added to the meat sample. For liver, the concentration of clara-diastase suspension was increased to 2 g per 100 ml of water because of the higher riboflavin content of the liver sample. The pH was adjusted to 4.5 and the samples were submitted to enzymatic digestion for 16–18 h at 37°C in order to release the vitamins from the bound forms. Then the samples were filtered through paper (Faltenfilter, Macherey–Nagel No. 615), the pH was adjusted to 6.5 and after a second filtration the volume was brought to 50 ml with water.

2.2. Sample clean-up

Purification of the extracts was carried out on the clean-up columns mentioned above. The procedure used was similar to the method published by Wills *et al.* [5], applying another ion-pair reagent and changing the ratio of methanol in the washing mixture and also in the eluent in order to decrease the amount of interfering substances. The columns were preconditioned by washing with methanol (2 ml), then methanol solution (2 ml) containing 5 m*M* sodium heptanesulphonate, an ion-pair reagent, followed by doubly distilled water (twice, 2 ml). Sample solutions of 4 ml were applied to the columns. At the same time thiamine and riboflavin standard solutions were added to one sample of each batch at three levels before the clean-up procedure. The columns were then washed with 2 ml of water–methanol (80:20) containing 0.005 *M* sodium heptanesulphonate. The components of interest were eluted with 2 ml of water–methanol (50:50) containing 0.005 *M* sodium heptanesulphonate. The volumes of the solutions collected were recorded. Samples were protected from light during the whole procedure because of the light sensitivity of riboflavin.

2.3. Chromatographic conditions

Chromatographic separation was performed on a Liquochrom Model 2010 HPLC system (Labor MIM, Budapest, Hungary) equipped with a UV detector operating at 254 nm. Chromatographic separation was achieved on a Nucleosil C_{18} column (150 × 4.6 mm I.D.) with 3-μm diameter particles and a guard column (20 × 4.6 mm I.D.) packed with Nucleosil C_{18}, particle size 10 μm. The eluent used was 0.01 M potassium dihydrogenphosphate buffer (pH 3.0)–acetonitrile (84:16, v/v) containing 5 m*M* sodium heptanesulphonate for meat samples and the same components in a ratio of 85:15 for liver samples. The volume of sample solution injected was 50 μl and the temperature of the column and the eluent was maintained at 45°C.

At the end of the daily work the column was washed with water–acetonitrile (80:20) to remove the water-soluble components and then stored in acetonitrile.

2.4. Calculation

Quantification was based on the peak height of the vitamins in the samples and in spiked samples using a calibration graph obtained at three levels of vitamin addition and the peak height of

the original sample. The peak height was measured from the line connecting the start point and the end point of the peak (valley to valley).

2.5. Microbiological method

The results of HPLC determination were compared with those obtained by microbiological determination of thiamine and riboflavin [7].

3. Results

A combination of sample clean-up and modification of the mobile phase used by Kamman *et al.* [1] gave appropriate chromatograms for meat and liver samples with UV detection. Figs. 1 and 2 show some examples of the chromatographic separation of spare rib and liver samples. For liver a change in sensitivity was necessary between the peaks of thiamine and riboflavin because of the great differences in the absorbances.

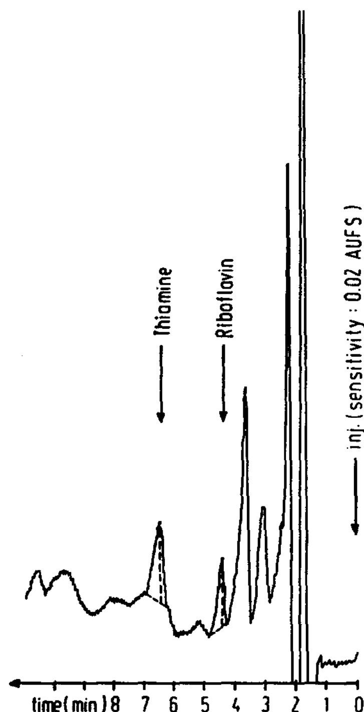

Fig. 1. HPLC of a spare rib sample from group 1. Mobile phase, 0.01 *M* phosphate buffer (pH 3.0)–acetonitrile (84:16, v/v) containing 5 m*M* sodium heptanesulphonate; UV detection at 254 nm, 0.02 AUFS.

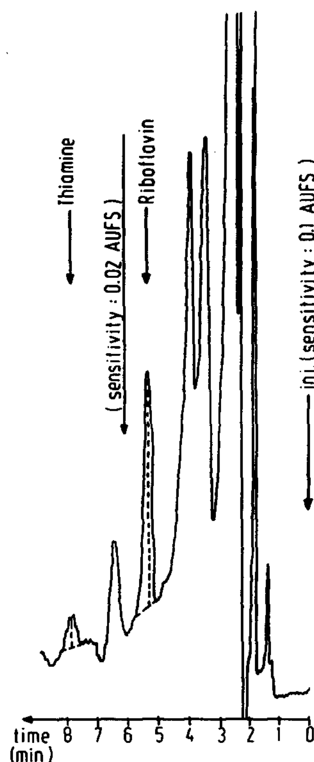

Fig. 2. HPLC of a liver sample from group 1. Mobile phase, 0.01 *M* phosphate buffer (pH 3.0)–acetonitrile (85:15, v/v) containing 5 m*M* sodium heptanesulphonate; UV detection at 254 nm. The sensitivities had to be changed between the peak of thiamine and riboflavin from 0.1 to 0.02 AUFS because of the large differences in the absorbances.

In the HPLC determination of thiamine, the presence of an acidic ion-pair reagent is important. For meat and liver samples heptanesulphonate resulted in the best separation from the interfering substances. The retention time of thiamine was shorter with hexanesulphonate than with heptanesulphonate, but the former resulted in co-elution of the thiamine with another compound. With octanesulphonate the retention time increased, giving shorter and wider peaks.

The concentration ranges for calibration for meat samples were 1.0–4.0 μg/ml of thiamine and 0.2–1.0 μg/ml of riboflavin and for liver

Table 1
Calibration graphs obtained by adding thiamine and riboflavin to the samples before sample clean-up

Sample	Thiamine		Riboflavin	
	Calibration equation[a]	Correlation coefficient (r)	Calibration equation[a]	Correlation coefficient (r)
Spare rib	$y = 5.77x + 3.21$	0.99897	$y = 19.62x + 1.80$	0.99976
Chop	$y = 5.18x + 3.78$	0.99961	$y = 16.14x + 2.23$	0.99963
Ham	$y = 5.04x + 4.17$	0.99891	$y = 18.48x + 4.61$	0.99998
Liver	$y = 5.36x + 1.95$	0.99950	$y = 18.08x + 84.20$	0.99962

[a] y = peak height (cm); x = concentration (μg/ml).

samples 0.4–2.0 μg/ml of thiamine and 2.0–10.0 μg/ml of riboflavin according to their vitamin contents. Calibration graphs obtained with spiked samples (spiking before clean-up) are presented in Table 1. The slopes of the lines are similar in the various samples in spite of the differences in disturbing compounds. The intercepts on the ordinate depend on the original vitamin contents of the samples.

The limits of detection were 0.1 μg/ml of thiamine and 0.03 μg/ml of riboflavin using a signal-to-noise ratio of 3. This means that 50 μg of thiamine and 16 μg of riboflavin in a 100-g sample can be determined.

The average recoveries of thiamine and riboflavin were 85% and 71%, respectively, in meat samples and 83% and 89%, respectively, in liver samples with addition of vitamin standard solutions before the extraction at the same level as present in the sample originally. The relative standard deviation was 6% for thiamine and 12% for riboflavin in meat and 8% for thiamine and 5% for riboflavin in liver for four parallel determinations.

A comparison between the HPLC and microbiological methods is given in Table 2. The concentrations of thiamine and riboflavin in meat samples measured by HPLC were lower than those determined by the microbiological method (10% and 40%, respectively). For liver the two methods gave approximately identical results.

The results obtained for the various samples are given in Table 3.

4. Conclusions

In the determination of B-group vitamins in biological materials the extraction and release of the free vitamins from the coenzymes are very

Table 2
Comparison of the HPLC and microbiological methods

Parameter	Vitamin	HPLC method		Microbiological method	
		Meat	Liver	Meat	Liver
Recovery (%)[a]	Thiamine	85	83	94	97
	Riboflavin	71	89	95	98
Reproducibility (%)[a]	Thiamine	6	8	17	18
	Riboflavin	12	5	16	15

[a] $n = 4$.

Table 3
Thiamine and riboflavin contents of the samples

Sample	Group 1		Group 2	
	Thiamine (μg per 100 g)	Riboflavin (μg per 100 g)	Thiamine (μg per 100 g)	Riboflavin (μg per 100 g)
Spare rib	382 ± 154	68 ± 18	312 ± 45	145 ± 21
Chop	395 ± 143	86 ± 10	404 ± 146	80 ± 21
Ham	507 ± 171	99 ± 21	482 ± 186	96 ± 21
Liver	210 ± 90	2347 ± 258	121 ± 24	1630 ± 231

Mean results ± S.D. ($n = 12$).

important steps in the method. A combination of acid and heat treatment and also enzyme digestion is necessary for this purpose. The most frequently used dephosphorylating enzyme preparations are taka-diastase and clara-diastase; the former proved more effective for thiamine and the latter for riboflavin. Digestion with papain resulted in chromatograms that were clearer and easier to evaluate with UV detection than that without a proteolytic treatment. The incomplete decomposition of the compounds related to the vitamin molecules may cause some discrepancy between the results of the HPLC and microbiological methods. This is a possible explanation of why the vitamin levels in meat samples were lower than would be expected.

The characteristics of the clean-up columns for the sample may change when they are used several times in the sample series. It cannot be determined in advance how many times they can be used with a selected sample type without considerable changes in retention.

By applying samples with vitamins added at the same levels in each batch the slight variations in the charge of the clean-up columns can be eliminated.

The purity of the peaks was confirmed by measuring the thiamine and riboflavin peaks of the standard and sample solutions at a different wavelength (268 nm) and was found to be acceptable.

5. References

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