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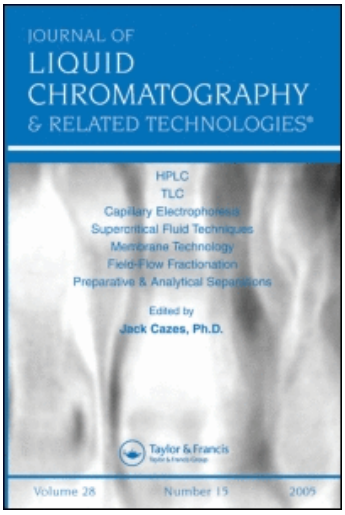

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A PAIRED ION LIQUID CHROMATOGRAPHIC METHOD FOR THIAMINE DETERMINATION IN SELECTED FOODS

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ABSTRACT

A precise and selective method for determining the content of thiamine in dietetic and baby foods by reverse phase liquid chromatography with UV detection is proposed. Enzymatic extracts of food samples were subjeted to purification and preconcentration with a weak ionic exchange column (CBA) and 0.1 M $BaCl_2$ solution as eluent. Ion-pair chromatography using a C_{18} column and a mixture of 5 mM sodium hexanesulphonate as counter-ion, 10^{-2} M potassium dihydrogen orthophosphate/ phosphoric acid buffer solution, pH 2.8, and 0.1% triethylamine was employed. The thiamine was detected at 254 nm.

This method was used to determine the content of thiamine in baby meals, cereals and dietetic cookies. Recovery studies showed good results and the relative standard deviation (n=10) was 2.9%.

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INTRODUCTION

An increasing awareness of the nutritional quality of food has arisen over the last few decades in industry, regulatory agencies and the public in general. This interest has led to an increased demand for rapid and accurate analytical methods for all nutrients, and among these, vitamins.

High Performance Liquid Chromatography has been increasingly used for the separation and determination of vitamins in foods, as, HPLC methods offer an attractive alternative to the more time-consuming chemical and microbiological assays for vitamins, due to their increased specificity, sensitivity and reduced analysis time.[1-3]

The sample treatment for thiamine analysis usually starts with an acid hydrolysis to liberate the thiamine from the food. Either hydrochloric or sulphuric acid is used for this purpose.[4-6] The determination of total thiamine in food products, requires a treatment of the acid extract with an enzyme mixture containing phosphatase activity, such as clarase,[7] β-amylase,[8,9] or takadiastase[4,10] at a pH of about 4-5. Papain has also been used, in addition to takadiastase, for the extraction of thiamine from meat products.[11] The enzyme must hydrolize the starch present, in order to improve sample filtration and release thiamine from its phosphate esters.

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Some methods have described thiamine analysis using ultraviolet detection in food samples that contain sufficient amounts of thiamine, such as multivitamin pharmaceutical preparations[12] and enriched cereal products,[10,13,14] but methods using fluorescence detection appear to be more successful for the detection in unsupplemented food[2,4] that have microgram amounts of thiamine at natural levels, because of the low sensitivity of the UV detector . However, some authors[3,9,12] have pointed out several shortcomings of the fluorimetric method that would affect quantification, for example, the feasible presence of ultraviolet-absorbing compounds can seriously interfere by inducing the quenching of fluorescence.

The low levels of vitamins and high amounts of other interfering materials in many food products, often make chromatographic determination of the direct acid extracts unfeasible. In this case, a solid-liquid extraction step is adequate for the purification and preconcentration of the extracts.

This paper, describes a simple analytical method for thiamine in dietetic and baby foods which, involves extraction with HCl and takadiastase solution, clean-up and preconcentration with a weak exchange-ion column, packed with methylcarboxylate in acid form, followed by HPLC determination.

EXPERIMENTAL

Apparatus and Conditions

The HPLC system consisted of a Hewlett-Packard HP 1090 liquid chromatograph (Waldbronn, Germany), a Rheodyne 7010 injection valve with a 20 µL loop, a Hewlett-Packard(HP) 79881A filter photometric detector, a HP 85B personal computer and a HP 3390A integrator. Column effluent was monitored at 254 nm for thiamine.

The HPLC column used was a Lichrospher 100 RP-18 (125 x 4 mm i.d., 5 µm).

A Visiprep Vacuum Manifold (Supelco, Bellefonte, PA, USA) was used for solid-liquid extraction together with a Vacuum Brand GMBH membrane pump (Wertheim, Germany).

A PW 9422 Philips pH-meter equipped with a combined glass-Ag/AgCl electrode was employed for pH measurements.

The water bath used was from Grant Instruments (Cambridge, England).

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Materials and Reagents

All solvents used were HPLC grade and were employed as supplied by manufacturers. High purity water was obtained through a Millipore Milli-Q system (Milford, MA, USA).

Analytical grade thiamine standard supplied by Sigma (St. Louis, MO, USA) was used. Individual stock solutions of this vitamin were prepared every third day in water to provide a concentration of 1 mg/mL. This solution was degassed with helium and stored in dark glass flasks in order to protect it from light, under -18°C refrigeration.

The working standard was prepared by adding aliquots of individual stock solution and diluting with water.

For acid hidrolysis, HCl (Merck, Darmstadt, Germany) was used and for enzymatic hidrolysis, takadiastase and clarase, both obtained from Fluka (Buchs, Switzerland). Sodium hexanesulphonate(Sigma, St. Louis, MO, USA), methanol HPLC grade (Romil Chemicals, Sps), phosphoric acid, potassium dihydrogen ortophosphate, triethylamine, barium chloride and sodium acetate anhydre (Merck, Darmstadt, Germany) were also employed.

Sample Preparation

Three types of foods were analyzed: baby meal, cereals and dietetic cookies. Finely ground samples containing some micrograms of thiamine, were weighed into a 50 mL volume erlenmeyer flask and 20 mL of HCl was added. The flask was put in a water bath at 100°C for 30 minutes. After cooling, the solution was adjusted to pH 4-4.5 with sodium acetate. Several types and amounts of enzymes were added. The solution was incubated in a water bath, previously heated to 47°C, during 3 h. The cooled samples were filtered through cellulose acetate filter (0.45 μm) and diluted with water in a 50 mL volumetric flask.

Purification of the Extract

An aliquot of this extract (2 mL) was passed through a CBA column, (previously conditioned by passing 1 mL MeOH and 1 mL of phosphate buffer 0.01 M at pH 4) packed with methylcarboxylate in acid form. The interfering substances were removed by washing with 2x500 μL phosphate buffer at pH 4. Thiamine was eluted from the column using 3x200 μL barium chloride 0.1 M.

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Chromatographic Determination of Thiamine

The mobile phase was composed of 5 mM sodium hexanesulphonate (HSA) by adjusting the pH value of the solution to 2.8 through addition of 10^{-2} M potassium dihydrogen orthophosphate/phosphoricacid.

Methanol was selected as organic modifier.[15] The optimum concentration of methanol was selected, in order to get a retention time for the thiamine that avoided its overlap with the substances eluted in the elution front, and so that the run time was shorter in order to avoid the broadening of sample bands that make accurace quantification difficult. As a consequence of the experiences carried out, we arrived at the conclusion that the most adequate percentage of organic modifier was that of 15%.

0.1% of triethylamine was added to mobile phase in order to reduce band tailing, a consequence of the tendency of thiamine to interact with residual silanol groups.

This mobile phase was vacuum-filtered through a 0.45 μm nylon filter and degassed with helium before being used. It was pumped at a flow rate of 1 mL/min. Figure 1 shows the chromatogram obtained with a standard solution of 2.5 μg/mL of thiamine.

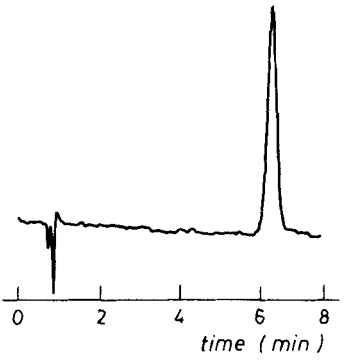

Figure 1. Chromatogram obtained from a standard solution of thiamine by using a Lichrospher 100 RP-18 (125x4 mm i.d., 5 μm). Mobile phase: H_3PO_4/KH_2PO_4 10^{-2}M, pH 2.8; hexanesulphonic acid 5 mM; methanol (15%); triethylamine (0.1%). Flow rate 1 mL/min. Thiamine concentration 2.5 μg/mL.

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Table 1

Recoveries of Thiamine Obtained Using $BaCl_2$ as Eluent at Different Concentrations

Barium chloride (mol/L)	Recovery (%)
0.025	66.4
0.050	70.9
0.10	97.2
1.2	88.3

Table 2

Recovery of Thiamine Depending on Type and Concentration of Enzyme

Enzyme	Amount Added (mg/mL)	Recovery (%)
Clarase	3	79.0
	6	74.7
Takadiastase	3	90.0
	6	95.1

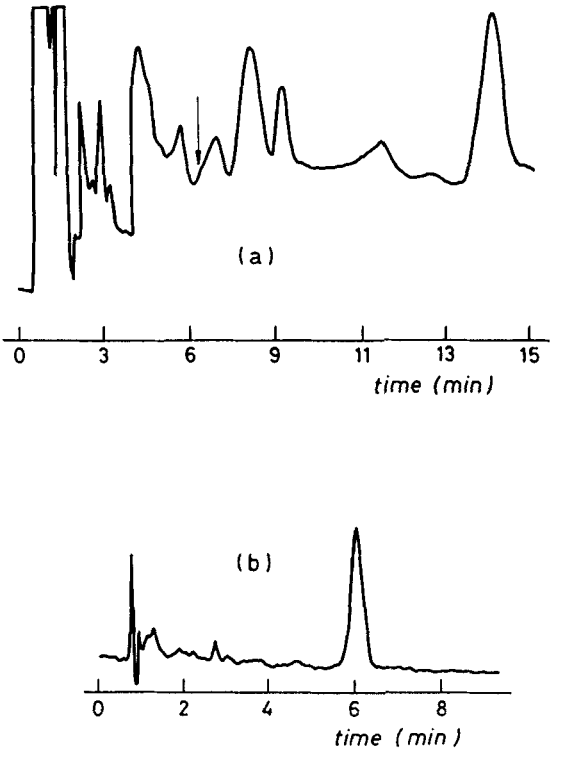

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Figure 2. Chromatograms of enzymatic extract of baby meal: a) Sample directly injected; b) Sample subjected to a solid-liquid extraction step with a weak cation exchanger (CBA) and 10^{-1}M $BaCl_2$ as eluent. Chromatographic conditions as Figure 1.

Table 3

Recovery of Studies of Thiamine in Baby Meals

Amount In Baby Meal (μg/mL(	Amount Added (μg/mL)	Amount Found (μg/mL)	Recovery (%) ± RSD
	0.50	4.34	92.1 ± 5.1
3.88	1.00	4.84	96.0 ± 2.5
	1.50	5.31	95.3 ± 2.8

The quantification of thiamine was achieved by using the external standard method. The calibration curve constructed from the peak area versus thiamine concentration was linear (r=0.9995) from the quantification limit to at least 7 μg/mL of thiamine. Recalibration was performed regulary.

For the recovery test, known amounts of thiamine were added to the samples before the hydrolysis steps and resulting spiked samples were subjected to the entire analytical sequence. The thiamine was spiked at three different concentrations and recoveries were calculated based on the difference between the total amount determined in the spiked samples and the amount observed in the non-spiked samples. All analyses were carried out in triplicate.

RESULTS AND DISCUSSION

Thiamine determination in food products requires a thiamine extraction step. This vitamin occurs in foods in three forms: thiamine mono-, di- and triphosphate. Extraction consists of an acidic hydrolysis step, so as to break the bonds with proteins, followed by enzymatic hydrolysis to convert thiamine phosphates to free thiamine.

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As can be seen in Figure 2, HPLC food analysis without a previous purification step is not advisable. This figure shows a chromatogram corresponding to a sample of baby meal directly injected after enzyme digestion (Fig. 2a) and another corresponding to the same sample after being subjected to a solid-liquid extraction step (Fig. 2b). The great quantity of interfering substances present in the sample, as well as the lower concentration of thiamine, did not allow direct injection of the sample into the chromatographic system.

For the pre-treatment of the samples, in order to isolate and preconcentrate the thiamine, we tested a variety of sorbents and elution solvents, some of which had been assayed previously in our laboratory.[15] In practice, the hydrophobic sorbents such as C_{18} or C_8 bonded silica were not appropiate for thiamine isolation because the vitamin co-eluted with other interfering substances. The strong ion-exchanger with the sulphonic acid functional group (SCX), effectively retain the vitamin but give lower recoveries in the elution process. The best results were obtained with the weak cation exchanger CBA using $BaCl_2$ as eluent.

In order to optimize the $BaCl_2$ concentration, we tested the concentrations over a range of 0.025 to 1.2 M. The results, shown in Table 1, indicate that the best recoveries are obtained with a 10^{-1} M $BaCl_2$ concentration.

The thiamine extraction method was optimised using several samples of baby meal. The recoveries obtained to modify the acid concentration and the type and concentration of the enzyme were studied.

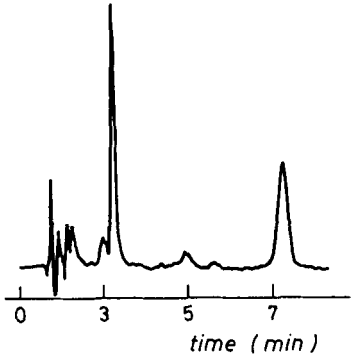

Figure 3. Chromatograms of the thiamine extracted from cereals. Chromatographic conditions as Figure 1. For extraction and preconcentration conditions see text.

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HCl concentrations in the range of 0.1 to 1 M were tested to carry out the acid hydrolysis. The best results were obtained with a 0.1 M HCl solution, as found by other authors[4,16]

Different concentrations of clarase and takadiastase were tested in order to carry out the enzyme digestion. The recoveries obtained are given in Table 2. As can be seen, the best results were obtained employing 6 mg/mL of takadiastase.

In order to determine the accuracy of the method, recovery experiments were performed and the results obtained for baby meals are given in Table 3.

The precision of the method was investigated using different samples. The relative standard deviation (n=10) was always less than 3%. The detection limit, based on a signal-to-noise of 3:1, is 0.408 μg/g.

Several commercial samples (baby meals, cereals and dietetic cookies) were analyzed with this method. Figure 3 shows the typical chromatogram of the thiamine extracted from a cereal sample and the thiamine concentrations determined in these samples are given in Table 4.

CONCLUSIONS

The method described here was applied to the determination of thiamine in three different foods: baby meals, cereals and dietetic cookies. This method includes a clean-up procedure that allows the removal of the major interfering substances present in the samples and thiamine preconcentration which, makes the

Table 4

Thiamine Contents in Baby Meal, Cereals and Dietetic Cookies

Sample	Thiamine (μg/g)
Baby Meal	10.3
Cereals	2.3
Dietetic cookies	8.5

method sufficiently sensitive for these food samples, as indicated by the detection limit achieved. Therefore, this method may be applied to the analysis of thiamine in unfortified foods using an ultraviolet detector.

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