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An improved high performance liquid chromatographic method for thiamin analysis in foods

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Eine verbesserte HPLC-Methode für die Thiamin-Analyse in Lebensmitteln

Zusammenfassung. Es wurde eine HPLC-Methode zur quantitativen Bestimmung von Thiamin in Lebensmitteln ausgearbeitet. Nach der Reinigung der angesäuerten und enzymatischen Extrakte der Proben wurden niedrige Gehalte von Thiamin unter den angegebenen chromatographischen Bedingungen bestimmt. Die Thiaminbestimmung wurde mit Umkehrphasen-Ionenpaachromatographie unter Anwendung verschiedener Mischungen von Natriumhexansulfonat und Natriumheptansulfonat als Gegenion durchgeführt. Für den Nachweis verwendet man einen UV-Detektor (Wellenlänge 254 nm). Die Nachweisgrenze für Thiamin lag bei 0,5 ng/Einspritzung. Es werden die Ergebnisse der Analysen von vier Leguminosenproben, einer lyophilisierten Fleischprobe und einer lyophilisierten Milchprobe sowie die Wiederfindungsrate für Standardthiamin angegeben. Der Mittelwert der Wiederfindung für die Extraktion und die Reinigung lag bei etwa 97–98%.

Summary. A high performance liquid chromatography (HPLC) procedure for the determination of thiamin in foods has been developed. Acid and enzymatic extracts of food samples were subjected to purification and chromatographic conditions which allowed the quantification of thiamin in foods with only a low content. Reverse-phase ion pair chromatography, using mixtures of sodium hexanesulfonate and sodium heptanesulfonate as counterions and detection at 254 nm, was employed. The lowest detection limit for thiamin was 0.5 ng/injection. Analyses of four samples of legumes and lyophilized meat and milk and the recovery for standard thiamin are given. Mean recovery values of extraction and purification procedure ranged over 97–98%.

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Introduction

In a recent cooperative work Nicolson et al. [1] concluded that HPLC methods of determination of thiamin in foods were not ready for routine analyses. Most of the methods employed in this cooperative work used reverse-phase columns and ultraviolet detection. Methods using fluorescence detection appear to be more successful [2–10]. However, Finglas and Faulks [11] have pointed out some short comings of the fluorimetric method, such as the quenching of fluorescence by co-eluted ultraviolet-absorbing species that would affect quantification; in the case of legumes, the presence of naturally occurring compounds, such as polyphenols, causes most concern. The analysis of thiamin in foods by fluorescence detection requires reacting thiamin and its phosphate esters with alkaline ferricyanide to form thiochrome. This derivatization can be done either pre-column or post-column. The presence of polyphenols and other ultraviolet-absorbing compounds can seriously interfere with both approaches [12, 13]. In fact most of the published applications of this method refer to processed cereal products, meat and milk. Other authors [6], who have extended the method to a wide range of foods, showed that a sample clean-up was required in the analysis of fresh fruits and vegetables to obtain resolved chromatograms.

A few reports have described thiamin analysis of a food item using ultraviolet detection [11]. Most of these HPLC methods have been developed for products which contain high levels of thiamin in a pure form, such as fortified or enriched food products [13–16]. Low levels of vitamins and high amounts of interfering materials, in legumes and other foods, make the direct chromatographic determination of the extract difficult. Wimalasary and Wills [6] could not resolve thiamin satisfactorily by ultraviolet detection with most of the foods they analyzed. We have also failed to replicate these methods [13–16] because of the presence of an array of overlapping peaks. In fact, no satisfactory HPLC method has been published for the analysis of thiamin in legumes. Con-

sequently, it was considered necessary to modify both the clean-up and chromatographic procedures.

It is known [17] that with non-polar stationary phases the capacity factors can be modified by changing either the pH or the methanol content of the eluent. Our purpose has been to carry out a study of the various factors which can alter the retention time of thiamin. A knowledge of these factors, combined with an effective purification of the sample extracts of legumes, has enabled us to discover satisfactory conditions to carry out the HPLC analysis of thiamin in various legumes. We have also extended the method to simpler food matrices, such as those derived from meat and milk.

Experimental

Apparatus and liquid chromatograph conditions

A Waters Associated liquid chromatograph equipped with an M-510 pump, a U6K injector and a model 440 absorbance detector, was provided with a filter at 254 nm. The detector signal was recorded on a Houston Instruments Omniscribe recorder. A precolumn 3.2 mm i.d. $\times$4.0 cm packed with C_{18} Porasil B (Waters Associates) was also used. The chromatographic column was a stainless steel column (3.9 mm i.d. $\times$30 cm) packed with μBondapack C_{18} (10 μm) (Waters Associates). Exact chromatographic conditions (mobile phase, temperature) used for each food are indicated in Table 4 (see below).

Extraction of legume samples

Sufficient ground materials (5–10 g, which contain 10–80 μg thiamin) were hydrolyzed with 0.1 mol/L HCl (30 ml) and 6 mol/L HCl (1 ml) in an autoclave at 121° C for 15 min. The pH of the solution was then adjusted to 4.0–4.5 with sodium acetate (2 mol/L); 5 ml freshly prepared aqueous enzyme solution (6% Takadiastase) was added and the sample was incubated at 48° C for 3 h. The sample solution, once cooled, was filtered through Whatman no. 40 filter paper and diluted to 100 ml with distilled water.

Purification of the extract

The pH of the extract was adjusted to 5.0–5.5 with 2 mol/L sodium acetate and kept in an ice bath. An aliquot of this extract (10 ml) was passed through a column (1.0 i.d. $\times$2.0 cm) packed with Amberlite CG-50 resin in acid form. The column was washed with 25 ml distilled water, followed by 30 ml HCl 0.15 mol/L. This final eluent was collected and evaporated to dryness. The residue was dissolved in 10 ml distilled water and adjusted with 2 mol/L sodium acetate to pH 5.5–6.0. The solution (1.0 ml) was passed through a C_{18} Sep-Pak cartridge (Waters Associates; previously conditioned by passing 10 ml 5 mmol sodium hexanesulfonate in methanol and 10 ml 5 mmol sodium hexanesulfonate in distilled water). The interfering substances were removed by washing with 20 ml aqueous 5 mmol sodium hexanesulfonate. Thiamin was eluted from the cartridge using 1.0 ml methanolic 5 mmol sodium hexanesulfonate. This solution was filtered through a Millipore filter (0.45 μm), and kept in an ice bath. At least three replicates for each sample were made.

Preparation of standard solutions and recovery test

Standard stock solutions of thiamin (80 μg/ml) were prepared. An aliquot of the standard solution (160 μg) was subjected to the extraction and purification procedures described above. Several replicates were carried out simultaneously.

HPLC determination of thiamin

Using standard solutions, calibration curves were constructed by plotting peak areas vs concentrations for each of the mobile phases. Linear least-squares analyses were performed to determine the best-fitting line. Aliquots of 20–50 μl of purified extract solution, depending on the final thiamin concentrations, were injected. The thiamin content of the sample extracts was obtained by interpolation on the corresponding standard curve.

Results and discussion

Preliminary trials for the HPLC separation of thiamin were made with standard solutions of this vitamin on a μBondapak C_{18} column. Methanol/water/acetic acid mixtures were the mobile phases assayed and various concentrations of sodium hexanesulfonate and/or sodium heptanesulfonate salts were added as counterions.

Considering the problem posed by the interfering peaks derived from the food matrices, we tried to discover which chromatographic factors affect the retention time of thiamin, hoping that this would allow us to predict the optimum composition of the mobile phase in order to separate the thiamin peak from interfering food matrix peaks as much as possible.

The capacity factor (K') and number of theoretical plates (N) obtained with different mobile phases using μBondapak C_{18} columns are shown in Tables 1–3. According to the results obtained using standards solutions of thiamin (see Tables 1–3), increasing the percentage of either methanol or acetic acid lowered the K' of thiamin. The opposite effect could be achieved by changing from sodium hexanesulfonate to sodium heptanesulfonate. In this manner, we could modulate the rentention time of thiamin in order to avoid the interference of any matrix peaks. Consequently we proceeded to the analysis of legume extracts purified as indicated in the Experimental Procedure. Table 4 shows the results obtained with four legumes (chick peas, beans, green beans and lentils), milk and meat samples, while Figs. 1–3 show the chromatograms obtained with some of these food samples.

A linear regression analysis of the relationship between peak area and the standard amounts of five separate determinations, at several concentrations, was carried out using a PC computer. The regression lines ($y=a+bx$) obtained with mobile phases h,l,k,c, and g and with concentrations ranging over 6–80 ng standard thiamin, gave correlation coefficients between 0.993–0.999. The detection limits ($3s/b$) [18], ranged between 5.0–0.5 ng/injection, where (b) ist the slope and (s) the standard error of estimation, depending on the correlation coefficient and mobile phase used.

The extraction method usually employed for the determination of thiamin in foods begins with an acid hydrolysis followed by an enzymatic hydrolysis. The use of sulfuric acid to hydrolyze the sample [14] may alter the thiamin content [19]. The use of HCl is according to [4], pre-

Table 1. Effect of the methanol concentration in the mobile phase on the chromatographic constants of thiamin

Mobile phase	Methanol (Vol., %)	Acetic acid (Vol., %)	Sodium hexane sulfonate 5 mmol (%)	Sodium heptane sulfonate 5 mmol (%)	N	K'
a	27	1	75	25	1936	5.9
b	28	1	75	25	1740	5.0
c	31	1	75	25	1785	3.7
d	31	1	100	–	1609	3.4
e	39	1	100	–	1407	2.0
f	28	0.1	100	–	1995	5.2
g	32	0.1	100	–	1328	4.6
h	28	0.1	75	25	2317	5.9
i	31	0.1	75	25	1760	4.5
j	33	0.1	75	25	1896	3.9

Table 2. Effect of the acetic acid concentration in the mobile phase on chromatographic constants of thiamin

Mobile phase	Methanol (Vol., %)	Acetic acid (Vol., %)	Sodium hexane sulfonate 5 mmol (%)	Sodium heptane sulfonate 5 mmol (%)	N	K'
b	28	1	75	25	1740	5.0
h	28	0.1	75	25	2317	5.9
i	31	0.1	75	25	1760	4.5
k	31	0.5	75	25	1795	3.8
c	31	1	75	25	1785	3.7

Table 3. Effect of the counterion in the mobile phase on chromatographic constants of thiamin

Mobile phase	Methanol (Vol., %)	Acetic acid (Vol., %)	Sodium		N	K'
			hexane sulfonate 5 mmol (%)	heptane sulfonate 5 mmol (%)		
f	28	0.1	100	–	1995	5.2
h	28	0.1	75	25	2317	5.9
l	28	0.1	75	25 (8 mmol)	2584	7.3
d	31	1	100	–	1609	3.4
c	31	1	75	25	1785	3.7
e	39	1	100	–	1407	2.0
m	39	1	–	100	1810	4.2

Table 4. Thiamin content and chromatographic conditions of food samples analyzed by HPLC

Food	Thiamin		Temperature of column (°C)	Mobile phases
	(mg/100 g wet matter)[a]	(mg/100 g dry matter)		
Chick peas	0.20±0.01	0.20	30	h, k, l
Beans	0.19±0.01	0.19	30	h, k, l
Lentils	0.17±0.01	0.17	30	h, k, l
Green beans: lot 1	0.09±0.01	0.09	39	k
lot 2	0.09±0.01	0.09	39	k
Pork muscle lot 1	1.53±0.15	1.55	50	k
lot 2	1.60±0.16	1.63	50	k
Full-Cream milk power: lot 1	0.17±0.01	0.17	50	k
lot 2	0.20±0.01	0.20	50	k

[a] Means value±standard deviation of at least six replicates

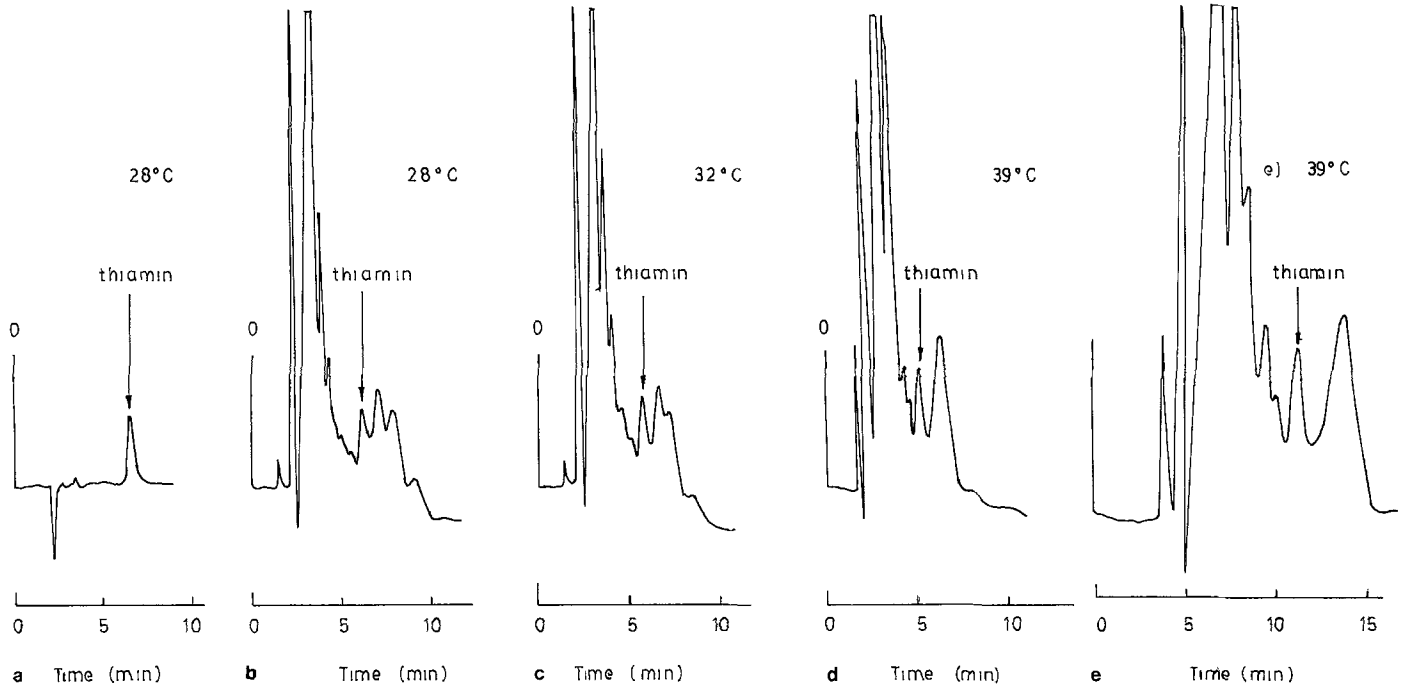

Fig. 1a–e. Effect of column temperature on the separation of thiamin peak. **a** Standard thiamin; **b–e** lyophilized green bean sample. Absorbance range 0.005. Mobile phase k. Detection at 254 nm. Flow rate 1.5 ml/min for **a–d** and 0.7 ml/min for **e**

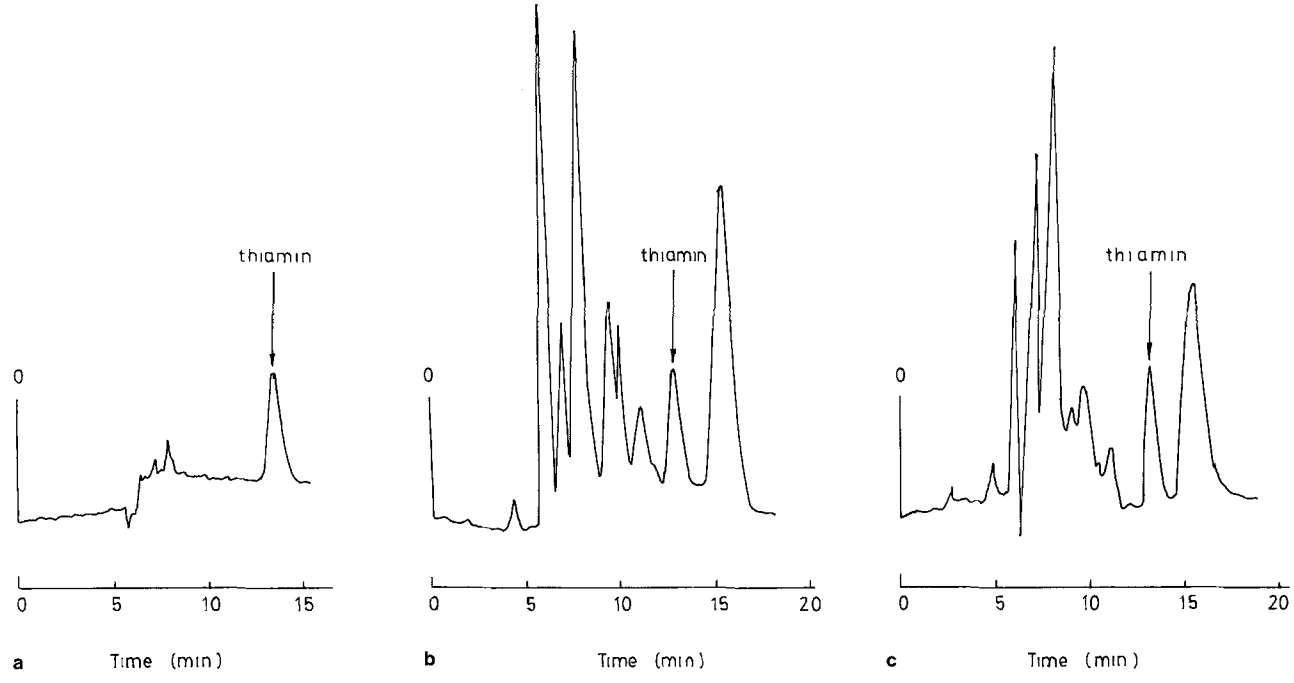

Fig. 2 a–c. Chromatogram obtained with standard thiamin (**a**), full cream milk powder sample (**b**) and lyophilized pork muscle sample (**c**). Mobile phase k. Absorbance range: 0.005 (**a**, **b**) and 0.01 (**c**). Flow rate 0.5 ml/min. Detection at 254 nm

ferable. The determination of total thiamin in food products requires treatment of the acid extract with an enzyme preparation [20]. The enzyme must hydrolyze the starch present and release thiamin from its phosphate esters. The enzymatic hydrolysis was carried out with Takadiastase.

In the case of legumes, HPLC analysis without a previous purification step is not advisable. The use of filtration, through membrane filters (0.22–0.45 μm) [14], in order to obtain clear extracts for injection onto the analytical column, or the use of Sep-Pak (Millipore Waters) disposible cartridges was inadequate for removing interfering substances present in the hydrolyzed samples [16]. Fellman et al. [4] have recommended the use of trichloroacetic acid to purify the extract but, in our experience, this procedure was detrimental to the HPLC col-

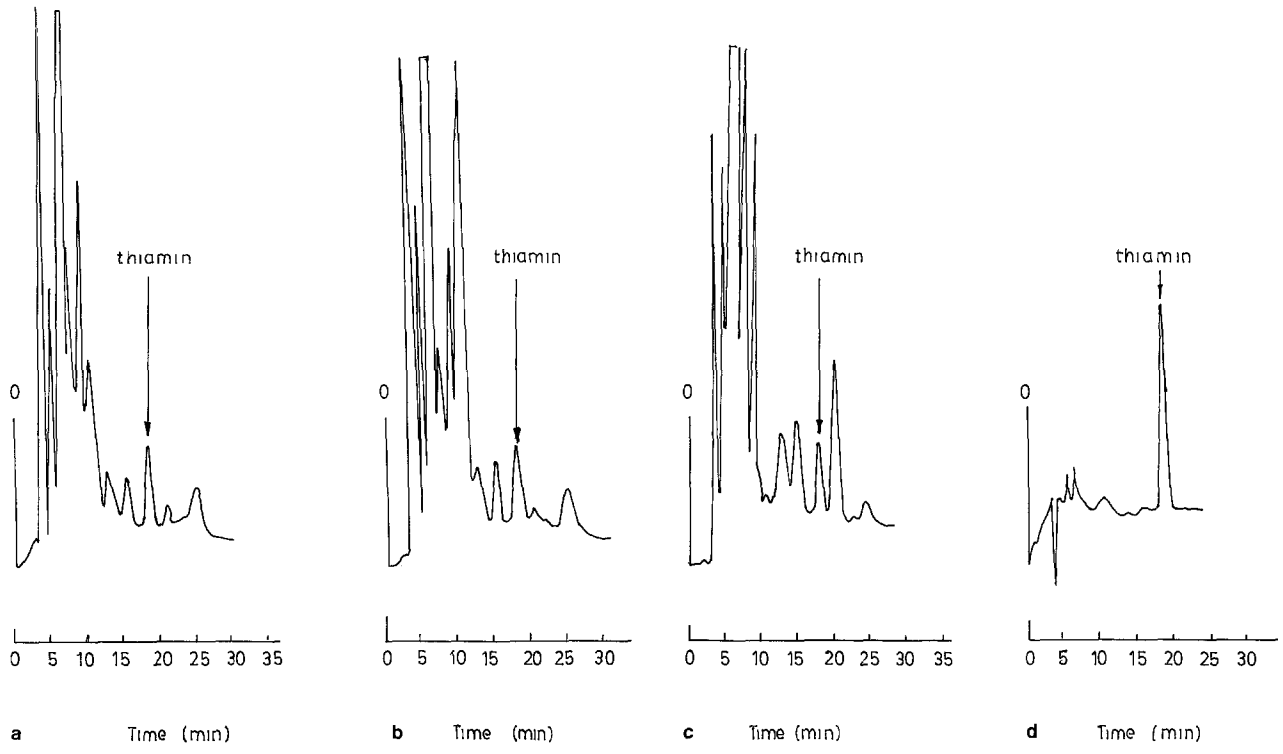

Fig. 3 a–d. Chromatogram of thiamin from a chick pea sample (**a**), bean sample (**b**), lentil sample (**c**), and standard thiamin (**d**). Mobile phase h. Absorbance range 0.005. Flow rate 1.0 ml/min. Detection at 254 nm

Table 5. Recovery of standard solutions of thiamin subjected to extraction and/or purification procedures

Step	Recovery (%)[a]
a. Acid and enzymatic hydrolysis	99.1±1.5
b. CG_{50} resin	97.2±2.1
c. Sep-Pak Cartridge	98.8±0.9
d. Step (a) followed by step (b)	97.5±3.6
e. Step (d) followed by step (b) and (c)	97.2±2.6

[a] Mean value ± standard deviation of six different recoveries of thiamin estimated by peak area

umn life and to certain parts of the chromatographic system, probably due to the low pH of the sample. Purification of the sample extract by alcohol precipitation [21] was also tried, a standard solution of thiamin being assayed as a first trial, but the recovery was low. Sebrell and Harris [22] have pointed out that thiamin hydrochloride crystallizes from alcoholic aqueous solution as the hemihydrate. Despite some reported recovery problems from ion-exchange columns [20], purification through Amberlite CG-50 [21] was chosen. This resin provided a fairly clean sample and the best thiamin recovery >97% (see Table 5). The recovery was excellent (>98%), in the purification step through a Sep-Pak cartridge. The overall recovery of thiamin throughout the extraction and clean-up procedure was essentially quantitative, as can be observed from the results in Table 5.

The effect of temperature on the separation of thiamin was studied for each food extract; we have used in each case the temperature giving the best resolution of thiamin (see Table 4). The corresponding calibration curve was obtained at the optimum temperature. In Fig. 1 we represent the effect of the temperature on the peak shape of thiamin from a green bean extract.

The simultaneous determinations of thiamin, riboflavin and niacin can be easily achieved using a mixture of pure standards. However, in our work with legume samples, each of these vitamins requires a specific purification procedure.

In conclusion, we have applied this optimized process to the determination of thiamin in three different legumes (chick peas, beans and lentils) and lyophilized samples of green beans, pork muscle and full-cream milk powder. The method discribed here does not require the conversion of thiamin to thiochrome nor fluorescence detection. The clean-up procedure established allows for the removal of the major interfering substances present in legumes and makes the method sufficiently sensitive for these food samples; the detection limit achieved (0.5 ng/injection) is reasonably low. A knowledge of the factors affecting the retention time of thiamin has helped to select the appropiate mobile phases and column temperatures that place the thiamin peak in a zone free of interfering substances.

This method can be applied to the thiamin analysis of unfortified or unenriched food products using standard columns and ultraviolet detector facilities. The purification method, coupled to the specific chromatographic conditions proposed here, allows for a clean separation of thiamin from interfering matrix peaks.

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