Takadiastase (Aspergillus oryzae carboxyl proteinase) has been used for hydrolyses of thiamin phosphate ester and riboflavin phosphate ester to analyse thiamin and riboflavin, respectively by HPLC.

However, it is difficult to get commercial Takadiastase stable nowadays.

So in this technical note, we introduce a new sample preparation method for thiamin and riboflavin analyses using acid phosphatase hydrolysis instead of Takadiastase hydrolysis.

**Standard Solution (Vitamin B1)**

Separated thiamine by ODS column was derivatized with potassium ferricyanide under alkaline condition. Fluorescent compound thiochrome was produced and detected by fluorescence detector.

**HPLC Conditions (Vitamin B1)**

Column: Inertsil ODS-3 5 mm, 150 µ 4.6 mm I.D.

Eluent: A) CH₃OH

B) 10 mM NaH₂PO₄, 150 mM NaClO₄ (pH 2.2)

A/B = 1/9, v/v (Mixed by a gradient mixer)

Flow Rate: 1.0 mL/min

Col. Temp.: 40 ˆ

Detection: FL Ex. 375 nm, Em. 440 nm

Inj. Vol.: 20 µ L

Reaction Reagent: 0.05 w/v % C₆FeK₃N₆, 15 w/v % NaOH

Flow Rate of Reaction Reagent: 0.4 mL/min

Reaction Coil: 5000 µ 0.33 mm I.D. (PTFE tube)

**Standard Solution (Vitamin B2)**

Riboflavin (100 mg/L)

**HPLC Conditions (Vitamin B2)**

Column: Inertsil ODS-3 5 mm, 150 µ 4.6 mm I.D.

Eluent: A) CH₃OH

B) 40 mL of 4 M CH₃COONa, 20 mL of 50 % CH₃COOH,

1940 mL of H₂O (pH 4.5)

A/B = 35/65, v/v (Mixed by a gradient mixer)

Flow Rate: 1.0 mL/min

Col. Temp.: 40 ˆ

Detection: FL Ex. 445 nm, Em. 530 nm

Inj. Vol.: 20 µ L

More technical information is here ··· http://www.glsciences.com/applications.html
Sample preparation method example

**Sample**
- 2 g

**Extraction**
- 50 mL of 0.1 M HCl
- Heating and Mixing (30 min)
- Cooling (below 50 °C)

**Hydrolysis (Enzyme-treated)**
- Adjust pH 4.0 – 4.5 with 4 M CH₃COONa
- 1 mL of 1 % Acid phosphatase
- Heating (37 °C, 12 hours)
- Cooling

**Dilute to Volume**
- 100 mL with Eluent B for V.B2 Analysis

**Filtration**
- Centrifugation (3000 rpm, 10 min)
- 0.45 µm Chromato Disk

**Extract**
HPLC-FL
Vitamin B2 Analysis

**Solid Phase Extraction**
InertSep SCX-2
500 mg/6 mL

**Conditioning**
- 5 mL of CH₃OH
- 5 mL of H₂O
- 5 mL of Eluent B for V.B Analysis

**Applying Extract**
- 10 mL of Extract

**Wash**
- 5 mL of H₂O
- 5 mL of CH₃OH/H₂O = 70/30, v/v

**Elution**
- 10 mL of CH₃OH/25 % KCl, 0.1 M HCl = 2/8, v/v
- 8 mL of 25 % KCl, 0.1 M HCl

**Dilute to Volume**
- 20 mL with Eluent A/B = 1/9, v/v for V.B1 Analysis
- Dilution with Eluent A/B = 1/9, v/v for V.B1 Analysis

**Post column derivatization HPLC-FL**
Vitamin B1 Analysis

1. Please do these steps as necessary.

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**An effect of Solid Phase Extraction (a case of commercial instant noodle)**

**No Sample preparation**

**With Sample preparation**

1. Thiamine
Analyses of foods example

Vitamin B1 in foods
An extract of beef

1. Thiamine
2. Hydroxyethylthiamine (HET)

A commercial instant noodle

Vitamin B2 in foods
An extract of beef

3. Riboflavin

A commercial instant noodle
Post column derivatization HPLC system was used for Vitamin B1 analysis. On the other hand, Vitamin B2 is a natural fluorescence compound and was analyzed by normal HPLC system. In this time, HPLC system in which switching valve was mounted like below.

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