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# Simultaneous Analysis of Water-soluble and Fat-soluble Vitamins in Fish by Novel SFC-MS/MS Method

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Udi Jumhawan, Jun Xiang Lee, Liw Wan Tung, Jie Xing, and Zhaoqi Zhan
Application Development & Support Centre,
Shimadzu (Asia Pacific) Pte Ltd, 79 Science Park Drive, Singapore

# Simultaneous Analysis of Water-soluble and Fat-soluble Vitamins in Fish by Novel SFC-MS/MS Method

## Introduction

Vitamins, essential dietary nutrients, are principally classified into water-soluble and fat-soluble groups based upon the solubility. Multiple chromatographic systems are commonly employed to analyse water-soluble and fat-soluble vitamins separately as both groups exhibit different chemical properties [1]. Supercritical fluid carbon dioxide (SF-$CO_2$) has been extensively utilized as a common solvent due to its advantages including non-toxic, low viscosity, high diffusivity, and easily removed from the reservoir [2]. Despite its nonpolar characteristic, the polarity of SF-$CO_2$ can be easily attuned by adding polar solvents such as methanol and ethanol. With the use of SF-CO2 as the mobile phase, supercritical fluid chromatography - mass spectrometry (SFC-MS) has attracted much attention for separation and analysis of diverse compounds. By changing the composition of mobile phase from supercritical to near liquid phase with addition of polar modifier (e.g., MeOH) up to 90%, we develop a novel SFC-MS/MS method for simultaneous analysis of 17 water soluble and fat soluble vitamins in fish samples.

## Experimental

A total of 17 water-soluble (WSV) and fat-soluble vitamins (FSV) were acquired from Sigma Aldrich and AccuStandard. Individual stock solutions of vitamin standards were diluted in either water, methanol, methanol:chloroform (1:1), diethyl ether or 0.01N NaOH. Working solutions were made by mixing and diluting individual stocks of the vitamins in methanol. Extraction of WSV and FSV from fish sample is performed separately as shown in Figure 1 and the SFC/MS/MS method is described in Table 1.

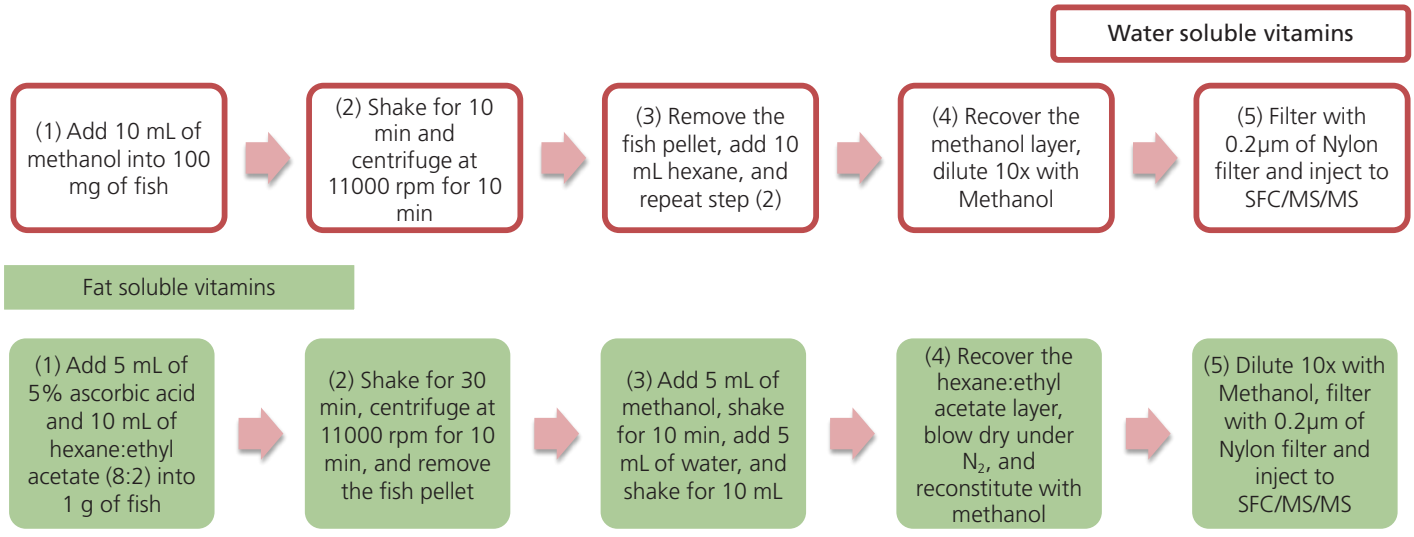


Figure 1. Flowcharts of sample pretreatment for water-soluble and fat-soluble vitamins

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Table 1. Analytical conditions of water-soluble and fat-soluble vitamins on Nexera UC with LCMS-8050

| | |
|---|---|
| Column | : Raptor Biphenyl (100 x 2.1 mm, 2.7 µm) |
| Flow rate | : 0.8 mL/min<br>0.4 mL/min (make up pump of MS) |
| Mobile phase | : A: Supercritical fluid carbon dioxide (SF-$CO_2$)<br>B: Methanol with 0.1% ammonium formate (w/v)<br>C: Methanol with 0.1% formic acid (make-up) |
| Elution mode (A/B) | : Gradient elution 6.5 min, 0min (3% B) → 0.5-1.5mins (90% B) →<br>1.51-3.50mins (90% B) → 3.51-3.60mins (3% B) → 3.61-6.50 mins (3% B) |
| Temp. | : Column oven: 45°C, SFC unit: 50°C |
| Injection vol. | : 5.0 µL |
| Interface & temp. | : ESI, 300°C |
| MS mode | : Positive, MRM |
| Block temp. | : 450°C |
| DL temp. | : 250°C |
| CID gas | : Ar (270 kPa) |
| Nebulizing gas flow | : $N_2$, 3 L/min |
| Drying gas flow | : $N_2$, 10 L/min |
| Heating gas flow | : Zero air, 10L/min |

# Results and Discussion

## Development of SFC/MS/MS method

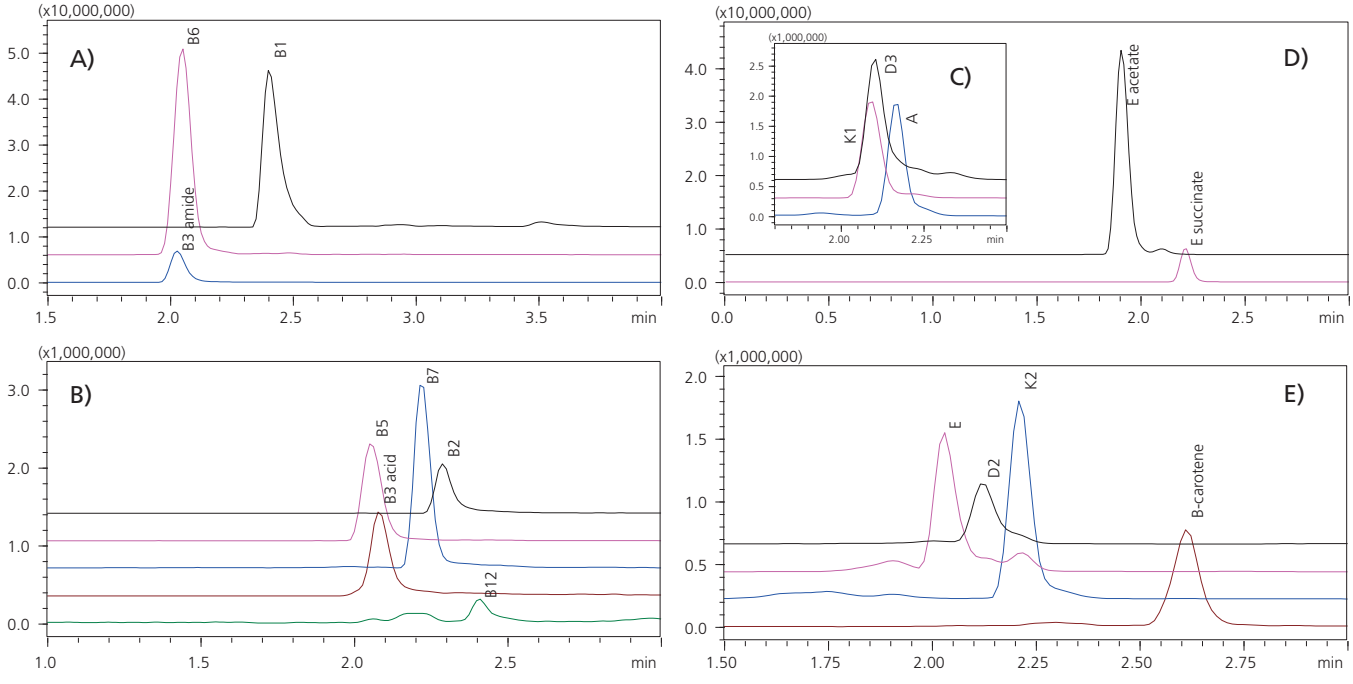


Figure 2. Total MRM chromatograms of 8 WSV (A, B) and 9 FSV standards (C, D, and E) at 250 ng/mL.

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A simultaneous analysis method of WSV and FSV was established on SFC-MS/MS platform using a Raptor Biphenyl column with a 6.5 min gradient elution program (Figure 2). Three MRM transitions were employed for identification of each vitamin, one for quantitation and the others for confirmation. It was reported [3] that the stationary phase in SFC plays more critical role to determine retentions of analytes. Therefore, screening for best column was conducted among BEH, C18, C8, PFP, phenyl-hexyl and biphenyl columns with methanol supplemented with 0.1% ammonium formate and 0.1% formic acid as the polar modifier. Based upon the peak shapes of the analytes, PFP and biphenyl columns were selected in the first round of evaluation.

Table 2. Extraction recovery and matrix effect of WSV and FSV in fish sample

| No | Vitamin | MRM transition for quantitation | Average extraction recovery (%) (*n*=3) | | Average matrix effect (%) (*n*=3) | |
|---|---|---|---|---|---|---|
| | | | 10ng/mL | 100ng/mL | 10ng/mL | 100ng/mL |
| 1 | Thiamine (B1) | 265.0>122.1 (+) | 131.5 | 62.8 | 316.7 | 191.7 |
| 2 | Riboflavin (B2) | 377.2>243.2 (+) | 55.1 | 55.6 | 41.6 | 70.2 |
| 3 | D-panthothenic acid hemicalcium salt (B5) | 220.0>90.2 (+) | 58.1 | 74.3 | 74.2 | 64.3 |
| 4 | Pyridoxine (B6) | 170.0>134.2 (+) | 54.6 | 74.7 | 106.6 | 122.7 |
| 5 | Biotin (B7) | 244.9>227.2 (+) | 52.9 | 65.4 | 63.9 | 89.4 |
| 6 | Nicotinic acid (B3 acid) | 124.1>80.1 (+) | 59.4 | 66.0 | 78.3 | 72.5 |
| 7 | Nicotinamide (B3 amide) | 123.1>80.1 (+) | 97.3 | 73.6 | 121.5 | 55.7 |
| 8 | Cyanocobalamin (B12) | 678.5>147.1 (+) | 66.1 | 72.3 | 103.6 | 106.9 |
| 9 | Ergocalciferol (D2) | 397.1>379.3 (+) | 95.0 | 100.2 | 90.0 | 57.2 |
| 10 | Cholecalciferol (D3) | 385.4>367.4 (+) | 102.2 | 91.1 | 72.4 | 65.9 |
| 11 | α-Tocopherol (E) | 431.5>165.1 (+) | 134.3 | 91.4 | 32.1 | 36.6 |
| 12 | DL-α-Tocopherol acetate (E acetate) | 473.4>207.2 (+) | 102.9 | 80.0 | 47.1 | 85.6 |
| 13 | DL-α-Tocopherol succinate (E succinate) | 531.5>265.1 (+) | 88.1 | 97.6 | 42.3 | 49.5 |
| 14 | Phylloquinone (K1) | 451.4>187.2 (+) | 81.4 | 83.9 | 51.3 | 65.0 |
| 15 | Menaquinone 4 (K2) | 445.0>187.2 (+) | 94.2 | 86.9 | 34.3 | 45.4 |
| 16 | Retinol (A) | 269.3>91.2 (+) | 68.8 | 87.5 | 37.4 | 28.5 |
| 17 | β-carotene | 536.5>444.4 (+) | 32.3 | 36.4 | 14.9 | 13.4 |

Further testing revealed that the PFP column had weaker retention for FSV and thus produced poorer peak shape in comparison with biphenyl column. Ultimately, a biphenyl column was selected for this work. An optimized gradient elution program was set up, which rammed up to 90% methanol to provide separation of both FSV and WSV (Table 1).

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## Optimization of extraction method and evaluation of method performance

A faster extraction method was optimized to shave off the conventional yet laborious saponification step (Figure 1). Extraction method for WSV and FSV was performed separately but employing almost the same set of solvents in different chronological order. Red snapper fish (*Lutjanus campechanus*) was utilized as the blank matrix. In general, fish species contain higher abundance of WSV than FSV in the flesh [4]. Consequently, different initial sample amounts and post-extraction dilutions were applied to minimize the interference from fish matrix. In exception to β-carotene, good extraction recovery was obtained based on the analysis results of thrice injections at 10 and 100 ng/mL ranged from 52.9 to 131.5% (Table 2). However, strong matrix enhancement and suppression were observed for B1 (WSV) and most FSV, respectively.

Table 3. Calibration results and performance evaluation of WSV and FSV in fish matrix (n=3).

| No | Vitamin | MRM transition for quantitation | RT (min) | LOQ (ng/mL) | LOD (ng/mL) | Range (ng/mL) | Linearity ($R^2$) | Repeatability at 50 ng/mL ($n$=3) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Area RSD (%) | Conc. RSD (%) |
| 1 | B1 | 265.0>122.1 | 2.51 | 0.05 | 0.01 | 0.5-100 | 0.999 | 4.9 | 5.0 |
| 2 | B2 | 377.2>243.2 | 2.34 | 0.55 | 0.18 | 5-250 | 0.998 | 3.2 | 3.3 |
| 3 | B5 | 220.0>90.2 | 2.16 | 0.18 | 0.06 | 5-250 | 0.998 | 3.5 | 3.8 |
| 4 | B6 | 170.0>134.2 | 2.10 | 0.06 | 0.02 | 5-100 | 0.996 | 16 | 16.6 |
| 5 | B7 | 244.9>227.2 | 2.28 | 4.5 | 1.5 | 5-250 | 0.999 | 11.9 | 11.8 |
| 6 | B3 acid | 124.1>80.1 | 2.21 | 0.97 | 0.32 | 5-250 | 0.999 | 4.8 | 5.4 |
| 7 | B3 amide | 123.1>80.1 | 2.09 | 0.06 | 0.02 | 5-250 | 0.999 | 4.2 | 7.9 |
| 8 | B12 | 678.5>147.1 | 2.52 | 13.5 | 4.5 | 10-250 | 0.999 | 7.8 | 9.8 |
| 9 | D2 | 397.1>379.3 | 2.16 | 7.7 | 2.5 | 10-250 | 0.999 | 10.5 | 18.2 |
| 10 | D3 | 385.4>367.4 | 2.14 | 1.8 | 0.6 | 10-250 | 0.998 | 3.9 | 7.0 |
| 11 | E | 431.5>165.1 | 2.07 | 0.13 | 0.04 | 10-250 | 0.998 | 6.8 | 16.6 |
| 12 | E acetate | 473.4>207.2 | 1.94 | 0.03 | 0.01 | 10-250 | 0.998 | 2.6 | 3.3 |
| 13 | E succinate | 531.5>265.1 | 2.28 | 0.23 | 0.08 | 0.5-250 | 0.999 | 1.8 | 1.8 |
| 14 | K1 | 451.4>187.2 | 2.12 | 0.89 | 0.29 | 5-250 | 0.998 | 6.7 | 6.5 |
| 15 | K2 | 445.0>187.2 | 2.23 | 1.8 | 0.6 | 1-100 | 0.999 | 21.6 | 22.4 |
| 16 | A | 269.3>91.2 | 2.19 | 2.9 | 1.0 | 5-250 | 0.998 | 6.3 | 7.5 |
| 17 | β-carotene | 536.5>444.4 | 2.63 | 1.5 | 0.5 | 1-100 | 0.998 | 2.8 | 2.8 |

The calibration curves were constructed in red snapper matrix spiked with vitamin standards from 0.5 to 250 ng/mL. All vitamins showed excellent linearity with $R^2 \geq 0.996$ across four calibration points or more (Table 3). The calibration range is varied for each vitamin due to the natural abundance in fish sample.

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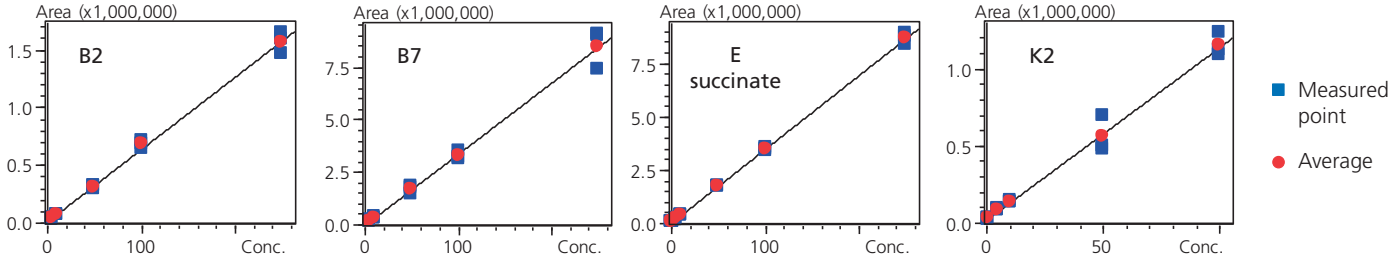


Figure 3. Representative calibration curves of B2, B7, E succinate, and, K2 in fish matrix

Using post-spiking standards, quantitation limit (LOQ) of most vitamins studied are estimated under 7.7 ng/mL, except for B12 (13.5 ng/mL), based upon signal-to-noise ratio (S/N) > 10. Vitamin B1, B6, B3 amide and E acetate exhibited the lowest LOQ values in the red snapper matrix. Repeatability of the method was evaluated from relative standard deviation of each vitamin at 50 ng/mL. The RSD are within the acceptance level (20%), except for vitamin K2 (22.4%).

## SFC/MS/MS analysis of vitamins in fish sample

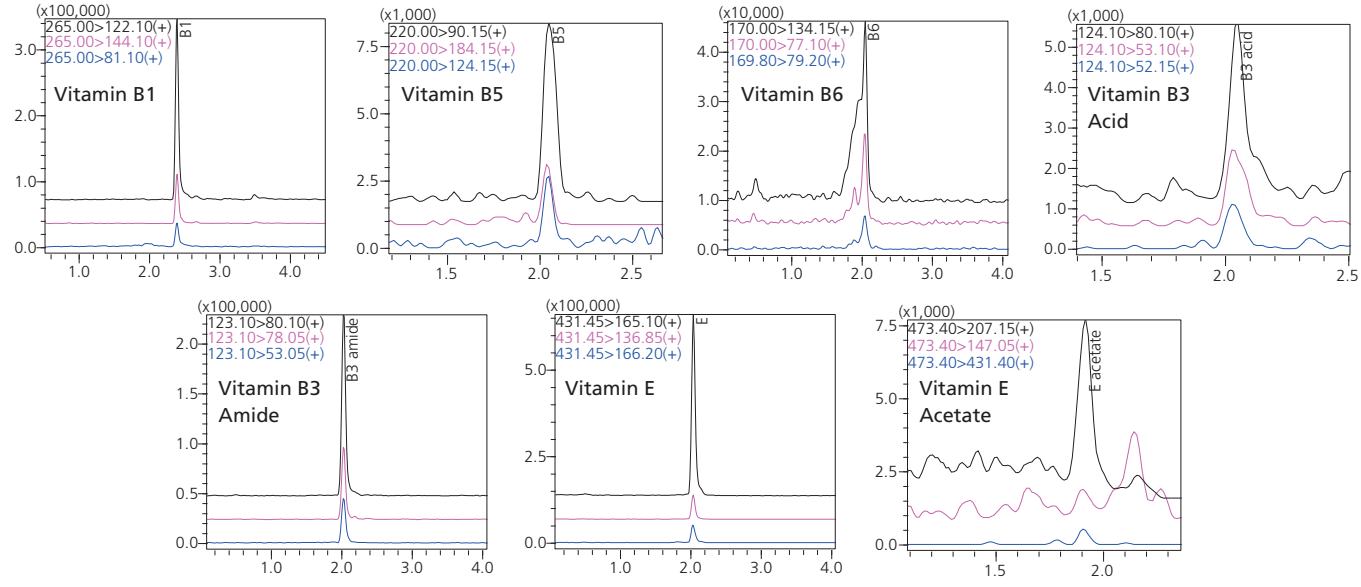


Figure 4. Individual MRM chromatograms of WSV and FSV in non-spiked seabass sample.

Seabass (*Dicentrarchus labrax*) sample was analysed using the established SFC-MS/MS method. Both WSV and FSV are detected in the seabass sample (Figure 4). The quantitative results are: B1 (1.2 ug/g), B5 (1.5 ug/g), B6 (0.6 ug/g), B3 acid (1.6 ug/g), B3 amide (12.7 ug/g), E (3.2 ug/g) and E acetate (0.004 ug/g).

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## Conclusions

A novel SFC-MS/MS method for quantitative analysis of vitamins in fish sample was developed using a Raptor Biphenyl SFC column. Simultaneous analysis of eight water-soluble vitamins (B1, B2, B5, B6, B7, B3 acid, B3 amide and B12) and nine fat-soluble vitamins (D2, D3, E, E acetate, E succinate, K1, K2, A and β-carotene) was achieved in 6.5 minute using a gradient supercritical fluid chromatographic program with MeOH as the modifier.

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