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Simultaneous Analysis of Water-soluble and Fat-soluble Vitamins in Fish by Reversed-Phase LC-MS/MS Method

ASMS 2017 WP 187

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Introduction

Vitamins are a group of compounds that are essential for maintaining good health. As most of them cannot be produced by human body, vitamins are obtained mainly from external sources such as fruits and fish. Analysis methods of HPLC-UV and LC-MS were reported for different types of vitamins. Fat-soluble vitamins are analysed normally using normal-phase chromatography, while water-soluble ones by reversed-phase chromatography [1,2]. Fish is an important source for both fat-soluble vitamins (D and E) and water-soluble vitamins (B) [3]. It is desired to develop methods enabling determination of both fat-soluble and water-soluble vitamins. We describe a novel LC/MS/MS method for simultaneous analysis of 17 fat-soluble and water-soluble vitamins, aiming at quantitation of total vitamins in fish samples using a single method.

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. The pH of WSV mix standard was adjusted to 7 by adding 1% NH_4OH solution. A Shimadzu LCMS-8060 triple quadrupole LC/MS/MS system was used to develop a MRM method for quantitative analysis of the vitamins. A Kinetex PFP (150 mm x 2.1mm I.D., 2.6µm) column was used and a gradient elution program was set up for separation of the seventeen compounds. The detailed conditions are compiled into Table 1. Fish samples were obtained from local market and homogenised using a Resch GM200 grinder at 10,000 rpm for 3 mins. The homogenised samples were then kept at -20°C and thawed for 1 hr before using for extraction. Extraction of WSV and FSV from fish sample are separated into two different procedures.

Water soluble vitamins: add 10 mL of methanol to 100 mg of the homogenised fish sample. The sample was shaken for 10 min at room temperature for extraction, followed by centrifugation at 4°C, 11,000 rpm for 10 min. The pellet was removed and the supernatant was added with 10 mL of hexane, shaking for 10 min and centrifuging at 4°C, 11,000 rpm for 10 min. The methanol layer was transferred and diluted by 10x with methanol. The sample was filtered with 0.22µm nylon filter before injection to LC/MS/MS.

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Table 1. Analytical conditions for water-soluble and fat-soluble vitamins on LCMS-8060.

Column	: Kinetex PFP 100Å (150 x 2.1 mm, 2.6 µm)
Flow rate	: 0.4 mL/min
Mobile phase	: A: Water with 0.1% formic acid
	B: Methanol with 0.1% formic acid
Elution mode	: Gradient elution, LC program 15min
	3% B (0.00 mins to 1.50 mins) → 90% B (4.90 mins to 9.00 mins)
	→ 95% B (9.00 mins to 11.50 mins) → 3% B (11.60 mins to 15.00 mins)
Oven temp.	: 40 °C
Injection vol.	: 5.0 µL for fat soluble vitamin; 2.0 µL for water soluble vitamin
Interface	: ESI
MS Mode	: MRM, Positive
Block Temp.	: 400 °C
DL Temp.	: 250 °C
Interface Temp.	: 350 °C
CID gas	: Ar (270 kPa)
Nebulizing Gas Flow	: Nitrogen, 1.5 L/min
Drying Gas Flow	: Nitrogen, 5.0 L/min
Heating Gas Flow	: Zero air, 15 L/min

Fat soluble vitamins: to 1 gram of the homogenised fish sample, add 5 mL of 5% ascorbic acid in methanol/water and 10mL of hexane/ethyl acetate (8:2). The mixture was vortexed for 30 mins at room temperature. The sample was then centrifuged at 4°C, 11,000 rpm for 10 min. The supernatant was separated, which was added with 10 mL of methanol/water, shaking for another 10 min. The hexane layer was transferred to a glass tube and it was blown to dryness under a gentle stream of N_2 gas. The sample was reconstituted with 10 mL of methanol and filtered with 0.22µm nylon filter before injection to LC/MS/MS.

Results and Discussion

Development of LC/MS/MS method

A detection and quantitation method for 8 FSV and 9 WSV in fish extract was established on LC/MS/MS with ESI interface. Three optimized MRM transitions were selected for each vitamin, one as quantifier ion and the others as confirmation ions.

A gradient elution method was set up for separation of both WSV and FSV. The initial part of the time programme aims to retain and separate the more polar WSV. This is crucial as WSV are easily eluted out even at low percentage of organic solvents under the reversed phase conditions. The latter part of the time programme aims to elute and separate the FSV using reversed phase condition instead of the commonly used normal phase condition.

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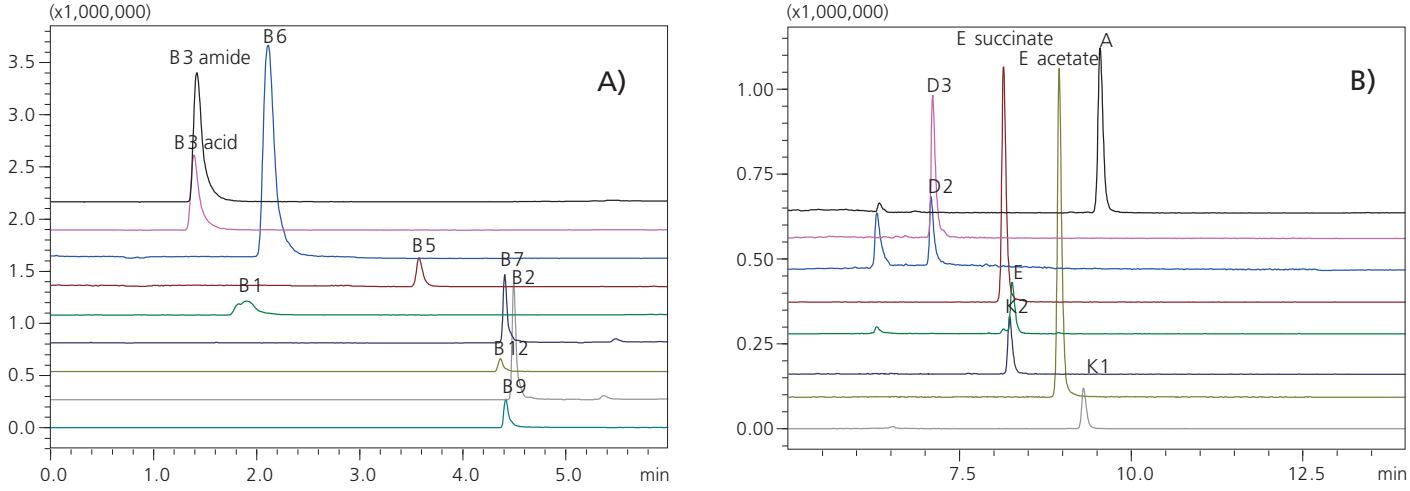

Figure 1. Total MRM chromatograms of (A) 9 water-soluble vitamins and (B) 8 fat-soluble vitamins standards at 10 ng/mL.

Establishment of MRM quantitation method

Red snapper fish (*Lutjanus campechanus*) was used as the blank matrix for calibration curves. Each post-spiked calibrant was injected thrice to obtain an average peak area for calibration curve construction. Calibration curves with good linearity (R^2>0.995) were obtained for all 17 vitamins for concentration up to 100 ng/mL (Figure 2). Water soluble vitamins yielded a low quantitation limit of 0.02~0.45 ng/mL and detection limit of 0.01~ 0.15 ng/mL. On the other hand, fat soluble vitamins displayed a higher quantitation limit of 0.11~4.72 ng/mL and higher detection limit of 0.04~1.56 ng/mL. Good repeatability was obtained for the spiked samples with %RSD results (*n*=3) ranging from 0.7~ 9.9%. The linearity, LOD, LOQ and %RSD results are summarised in Table 2.

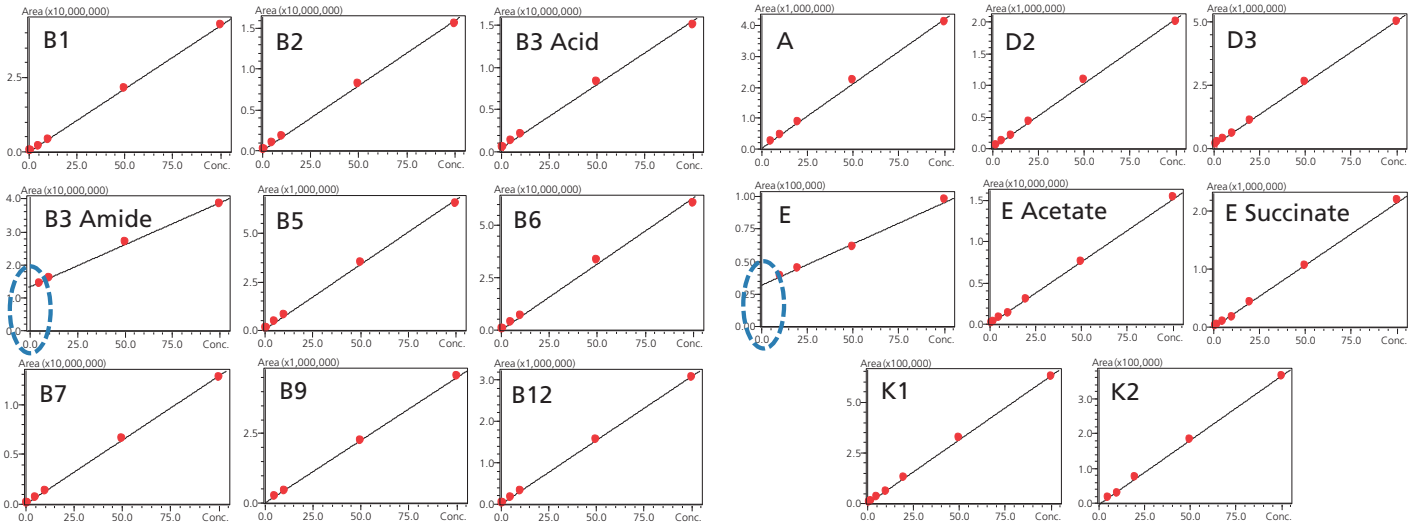

Figure 2. Calibration curves of 9 water-soluble vitamins and 8 fat-soluble vitamins in fish matrix.
(Note: Vitamin B3 Amide and Vitamin E present in the blank are not detected. In addition, very low contents of Vitamin B1, B5, B3 acid, E acetate and B2 are also present.)

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

No	Vitamin	Abbreviation	MRM transition	RT (min)	Range (ng/mL)	R^2	LOQ (ng/mL)	LOD (ng/mL)	RSD% (*n*=3)
1	Thiamine	B1	265.00>122.10	1.97	0.5 – 100	0.9994	0.13	0.04	1.7
2	Riboflavin	B2	377.20>243.20	4.53	0.5 – 100	0.9987	0.06	0.02	2.6
3	Nicontiamide	B3 Amide	123.10>80.10	1.46	5 – 100	0.9978	0.02	0.01	1.7
4	Nicontinic acid	B3 acid	124.10>80.10	1.39	5 – 100	0.9974	0.11	0.04	2.9
5	D- Pantothenic acid hemicalcium salt	B5	220.00>90.15	3.65	0.5 – 100	0.9981	0.06	0.02	1.4
6	Pyridoxine	B6	170.00>134.15	2.19	0.5 – 100	0.9969	0.30	0.09	0.8
7	Biotin	B7	244.90>227.15	4.47	0.5 – 100	0.9995	0.45	0.15	5.4
8	Folic acid	B9	442.20>295.15	4.45	5 – 100	0.9997	0.20	0.07	3.6
9	Cyanocobalamin	B12	678.40>147.10	4.40	0.5 – 100	0.9997	0.04	0.01	0.7
10	Retinol	A	269.30>91.15	9.55	5 – 100	0.9984	4.72	1.56	2.1
11	Ergocalciferol	D2	397.30>105.05	7.09	2 – 100	0.9986	2.02	0.67	5.8
12	Cholecalciferol	D3	385.35>367.30	7.11	1 – 100	0.9991	0.40	0.13	5.5
13	α-Tocopherol	E	431.45>136.85	8.27	10 – 100	0.9959	1.32	0.43	3.3
14	D-α-Tocopherol succinate	E Succinate	531.50>265.10	8.14	1 - 100	0.9979	0.28	0.10	2.3
15	DL-α-Tocopheryl acetate	E Acetate	473.40>207.15	8.96	1 – 100	0.9991	0.11	0.04	2.8
16	Phylloquinone	K1	451.40>185.15	9.31	1 – 100	0.9990	1.01	0.33	1.1
17	Menaquinone 4	K2	445.00>81.15	8.23	5 – 100	0.9983	3.55	1.17	9.9

Matrix effect and recovery studies on spiked fish sample

Extraction of vitamins from complex matrix is generally carried out via saponification and in combination with liquid-liquid extraction [4]. In this study, we developed a faster extraction method by using a modified liquid-liquid extraction. Two spiking concentrations were used to examine the recovery and matrix effect, 10 and 100 ng/mL for WSV and 1 and 10 ng/mL for FSV. The method exhibited decent recovery for WSV (44.9 to 66%, except for B9 and B12) and good values for FSV in the range of 61.3 ~ 100.5% (Table 3). On the contrary, strong matrix suppression was observed for FSV while the fish matrix had no negative effect for WSV in exception of vitamin B5. Further optimization of sample pre-treatment will be investigated in the future.

LC/MS/MS analysis of vitamins in fish sample

The established method was applied to simultaneous analysis of WSV and FSV of a different type of fish, seabass (*Dicentrarchus labrax*). The seabass sample was extracted in a similar manner to blank fish prior to LC/MS/MS analysis.

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Table 3. Extraction recovery and matrix effect studies

No	WSV	Recovery (%)		Matrix effect (%)	
		10 ng/mL	100 ng/mL	10 ng/mL	100 ng/mL
1	B1	52.6	48.4	44.9	86.8
2	B2	62.7	48.7	85.1	100.6
3	B3 Amide	66.0	50.7	91.9	106.7
4	B3 Acid	62.6	48.9	91.7	109.4
5	B5	59.3	45.8	472.0	161.5
6	B6	63.2	46.8	94.0	111.4
7	B7	61.1	44.9	90.7	107.9
8	B9	53.7	39.7	92.5	109.9
9	B12	20.8	26.8	86.9	100.8

No	FSV	Recovery (%)		Matrix effect (%)	
		1 ng/mL	10 ng/mL	1 ng/mL	10 ng/mL
10	A	ND	66.9	ND	61.8
11	D2	83.5	75.1	82.3	24.0
12	D3	71.0	84.4	88.7	10.4
13	E	ND	73.6	ND	10.8
14	E Succinate	88.5	100.5	14.1	13.6
15	E Acetate	71.0	77.5	41.6	50.7
16	K1	61.3	74.8	41.0	45.4
17	K2	ND	99.7	ND	7.8

ND; not detected

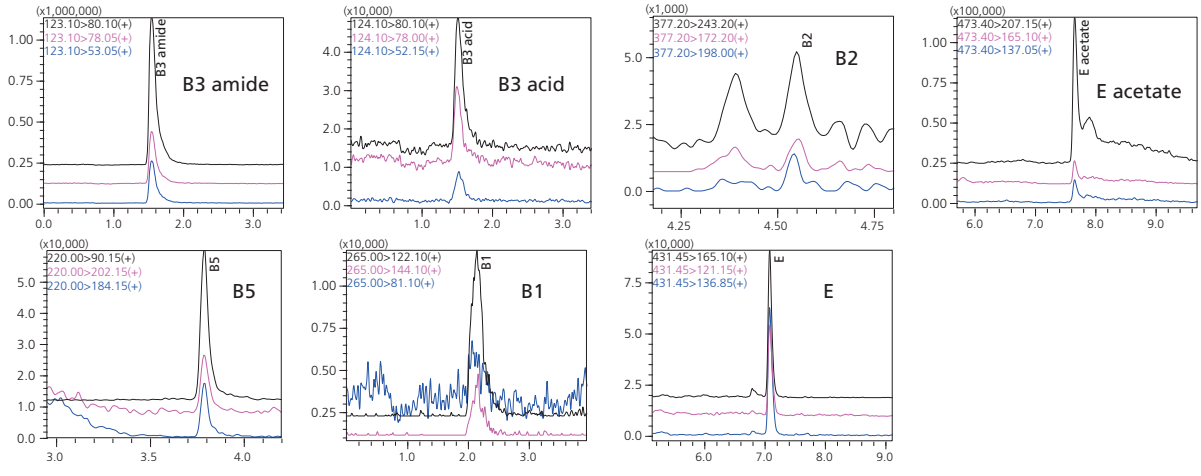

Figure 3. Individual MRM chromatograms of WSV and FSV in non-spiked seabass sample

Seven vitamins were detected in seabass and their content are: vitamin B1 (0.3 ug/g), B2 (0.05 ug/g), B3 acid (1.3 ug/g), B3 amide (12.9 ug/g), B5 (2.5 ug/g), E (6.8 ug/g) and E acetate (0.04 ug/g) after confirmation with post-spiked samples. This results confirm the robustness and sensitivity of newly developed method for simultaneous analysis of diverse vitamins using a single method.

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Conclusions

A quantitation method for simultaneous analysis of nine water-soluble vitamins (B1, B2, B5, B6, B7, B3 acid, B3 amide, B9 and B12) and eight fat-soluble vitamins (D2, D3, E, E acetate, E succinate, K1, K2 and A and) was developed by employing reversed-phase LC/MS/MS platform. This LC/MS/MS method demonstrated high sensitivity for quantitative profiling of vitamins in fish sample.

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First Edition: June, 2017

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