1. Introduction

Vitamins are a group of compounds that are essential for maintaining good health. As most of them cannot be produced by the 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 analyzed normally using normal phase chromatography, while water-soluble vitamins by reversed-phase chromatography [1, 2]. Fish is an important source for both fat-soluble vitamins (A, D, E, and K) and water-soluble vitamins (B1, B2, B3, B5, B6, and B12). 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 quantification of total vitamin intake in fish samples using a single method.

2. 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 methanol, methylene chloride, or DI water. Working solutions were made by mixing and diluting individual stocks of the vitamins in methanol. All experiments were conducted in triplicate.

A Shimadzu LCMS-8040 triple quadrupole LC/MS/MS system was used to develop a MRM method for quantitative analysis of the vitamins. A Kratos PFP (50 mm × 2 mm) (L.D., 2.6 µm) 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 homogenized using a Rinsch GM220 grinder at 10,000 rpm for 3 min. The homogenized samples were then kept at -20°C and thawed for 1 hr before use for extraction. Extraction of WSV and FSV from fish sample are separated in Tables 2 and 3.

Water-soluble vitamins: add 10 mL of methanol to 100 mg of the homogenized 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 supernatant was recovered and diluted by 10x with methanol. The sample was filtered with 0.22 µm nylon filter before injection to LC/MS/MS.

Fat-soluble vitamins: to 1 gram of the homogenised fish sample, add 5 mL of 5% acetic acid in methanol/water and 10 mL of hexane/ethyl acetate (2:2). The mixture was vortexed for 50 min 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/shaking for another 10 min. The hexane layer was filtered through glass tube and it was blown to dryness under a gentle stream of N₂ gas. The sample was reconstituted with 10 mL of methanol and filtered with 0.22 µm nylon filter before injection to LC/MS/MS.

3. Results and Discussion

3.1 Development of LC-MS/MS method

A detection and quantification method for FSV and WSV in fish extract was established on LCMS-8040. The transition ions of vitamin standard solutions were selected for each vitamin, as one quantitation 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 program aims to retain and separate the more polar WSV. The retention time for all WSV was defined as the most WSV would be easily eluted out at a low percentage of organic solvents under the reversed-phase conditions. The later part of the programme aims to separate the FSV using reversed-phase condition instead of the commonly used normal-phase condition.

3.2 Establishment of MRM quantification method

The study evaluated the MRM chromatograms by LC/MS/MS. The chromatograms were used as the blank matrix for calibration curves. Good linearity (R2 > 0.99) was obtained for 17 vitamins (A, D, E, K, B1, B2, B3, B5, B6, B12, B15, B25, B26, B50, B51, B52, B53, B60, B75, B80, B81, B82, B83, B97, and B98) at 2.02–44.00 ng/mL (Table S1). According to FDA regulations, the matrix effect was quantified by calculating the ratio of the response of the sample (S) to the response of the solution (Q) before injection to LC/MS/MS.

3.3 Matrix effect and recovery studies on spiked fish sample

Extraction of vitamins from complex matrix is generally carried out via SPE 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 7 and 15 ng/mL for FSV. The method exhibited decent recovery for WSV (44.4% to 69%, except for B1, B12, and B26) and good recovery for FSV in the range of 83–100%. The matrix suppression effect observed for WSV 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.

3.4 LC-MS/MS analysis of vitamins in fish sample

The established method was adapted to simultaneous analysis of WSV and FSV of a different type of fish, seabass (Dover Sole Latine). The seabass sample was extracted in a similar manner to blank fish prior to LC/MS/MS analysis.

References