

SIMULTANEOUS ANALYSIS METHOD OF TEN WATER-SOLUBLE VITAMINS WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN FISH FEED ADDITIVES

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ABSTRACT

Water-soluble vitamins (WSVs) as feed additives are useful for increasing growth and immunity against diseases. Analysis of WSVs has been carried out using HPLC with fluorescence or diode arrays detector. Currently, there is no simultaneous analysis method for 10 WSVs with an HPLC UV detector at two wavelengths detection. This study aimed to develop a simultaneous analysis method for 10 WSVs (vitamin B₁, B₂, B₅, B₆, B₇, B₉, B₁₂, ascorbic acid, niacin, and nicotinamide) with an HPLC-UV at 210 and 254 nm. The results showed that this method was able to analyze 10 WSVs in 25 min, with an isocratic mobile phase (24:76) consisting of methanol (A) and 0.05 M phosphate buffer pH 3.0 containing 0.01 M sodium 1-hexane sulfonate and 0.1% TEA (B), Shimpack C₁₈ (250 mm x 4.6 mm, 5µm) column, at flow rate 1 ml/min. The method has been validated with parameters based on the Association of Official Analytical Chemists (AOAC). The method has good specificity with resolution >1.15. Linearity was expressed as a correlation coefficient of $r > 0.99$. The LOD ranged from 0.71 mg/L and 16.32 mg/L; whereas the LOQ ranged from 2.23 mg/L and 54.40 mg/L. Intra-day and inter-day precision were expressed as relative standard deviations in the range of 0.16% to 4.18% and 0.40% to 5.59%, respectively. While the accuracies were in the range of 81.6% to 109.71%. The method can be used to quantify 10 WSVs in fish feed additives for routine analysis in the laboratory.

Keywords: HPLC, UV Detector, Simultaneous, Water-Soluble Vitamins, Validation, AOAC.

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INTRODUCTION

Currently, water-soluble vitamins are not only used as a dietary supplement in humans but also as a feed additive in animal husbandry and fisheries. Specifically, in the fisheries sector, registered fish medicines in 2018 showed that 21.2% is premix multivitamin.¹ It shows that multivitamins are widely used as fish feed additives. Therefore, it is necessary to control the quality of the WSVs composition in various preparations. Quality control is intended to determine the concentration of each WSV according to the label claims made by both the company's Quality Control (QC) and the government as the competent authority before a fish medicine is registered. Determination of the concentration of several WSVs in various preparations has been carried out using High-Performance Liquid Chromatography (HPLC).² Most determination of WSVs uses HPLC with UV, fluorescence (FLD), diode array (DAD), and electrochemical detectors.² Reverse phase HPLC (RP-HPLC) is the most popular technique for WSVs separation because it is easier, faster in equilibrium, better in shape, and produces satisfactory final separation when compared to normal phase HPLC.² Analysis of 12 WSVs was performed using column C₁₈ with FLD for vitamin B₂ and B₆ and DAD for niacin, nicotinamide, vitamin B₅, B₁, B₉, B₁₂, and ascorbic acid.³ Vitamin B₇ was not analyzed because it prioritized the separation of B₆ vitamers (pyridoxal, pyridoxamine, and pyridoxine) and B₂ vitamers (flavin mononucleotide and riboflavin) and detection at various wavelengths between 202-450 nm. HPLC with UV detector was reported to be able to separate six WSVs (ascorbic acid, vitamin, B₁, B₂, niacin, B₆, and B₉), fat-soluble vitamins, lycopene, and beta-carotene.⁴ In addition, Hydrophilic Interaction Liquid Chromatography (HILIC) is an alternative use of RP-HPLC for polar analytes, such as WSVs.² Seven

WSVs (vitamin B₁, B₂, B₆, B₉, niacin, nicotinamide, and ascorbic acid) were analyzed by HILIC comparing the use of DAD, FLD, and coulometric detector.⁵ In that study, ascorbic acid could not be identified with a coulometric detector. UPLC-MS is very sensitive and specific for the determination of vitamins, especially in complex matrices, and allows identification at very low concentrations. However, the application of LC-MS is very limited, due to the high costs of the instrument.² Most of these methods use DAD, FLS, and MS detectors. The analysis is only carried out on a few components of WSVs. Analysis of 10 WSVs (vitamin B₁, B₂, B₆, B₇, B₅, B₉, B₁₂, ascorbic acid, niacin, and nicotinamide) in multivitamins has not been simultaneous using RP-HPLC with a UV detector. The analysis of 10 WSVs is currently carried out with different test methods, so it requires a long time and more chemicals. This condition reduces the service effectiveness to customers, due to the longer testing time. Therefore, it is necessary to develop a simultaneous analysis method of 10 WSVs using RP-HPLC with a UV detector. The simultaneous analysis is one of the most sensitive separation techniques; the process is fast, simple, selective, reduces chemical consumption, and easily and accurately results.^{6,7} In addition, a simultaneous multicomponent analysis will reduce the separation steps, such as extraction and cleanup.^{6,7} In this study, WSVs analysis uses RP-HPLC with UV detector, due to the first choice in routine analysis² and both governments and private institutions have this instrument, rather than HPLC with DAD, FLD, or MS detectors, because the price of these instruments is more expensive. According to the advantages of the simultaneous analysis and the limitations of the institution's instruments, the development of analysis methods for 10 WSVs with HPLC UV detectors needs to be done to reduce time and chemical consumption, speed up the service and shorten customer queues. It is expected that this method can be applied in WSVs routine analysis in government and private institutions for analyzing the quality of multivitamins for registration.

EXPERIMENTAL

Materials

All reagents and chemicals were analytical grades, except methanol. Standards of WSVs (vitamin B₁, B₂, B₅, B₆, B₇, B₉, B₁₂, ascorbic acid, niacin, and nicotinamide) and metaphosphoric acid were purchased from Sigma Aldrich. HPLC grade of methanol, sodium 1-hexane sulfonate, potassium dihydrogen phosphate, phosphoric acid, triethylamine, and sodium hydroxide were purchased from Merck. Double distilled water was purchased from Ika Pharmindo.

Preparation of Standard Solution

The stock standard solutions of vitamin B₁, B₅, B₆, B₁₂, ascorbic acid, niacin, and nicotinamide were dissolved in 1% metaphosphoric acid,^{4,8} while the stock standard solutions of vitamin B₂, B₇, and B₉ were dissolved with distilled water containing 0.1 M NaOH.⁹ The working standard solutions (mixed standard) were obtained by diluting a certain volume of the stock standard solution according to the desired concentration with 1% metaphosphoric acid. The stock solutions of vitamin B₅, B₇, and ascorbic acid were prepared at 5000 mg/L; stock solutions of vitamins B₁, B₂, niacin, nicotinamide, and B₆ at 1000 mg/L; and stock solutions of vitamin B₉ and B₁₂ at 500 mg/L. The mixed standard solution was prepared at 100 mg/L of vitamin B₁, B₂, niacin, nicotinamide, and B₆; 200 mg/L of vitamin B₅, B₇, and ascorbic acid; and 8 mg/L of vitamin B₉ and B₁₂.¹⁰

Method Optimization

Method optimization aimed to obtain maximum results with a high level of sensitivity to obtain the optimum process conditions that produce the best criteria values.¹¹ The HPLC condition was optimized to separate 10 WSVs, that met the requirements of the system suitability test. Optimization was carried out on the ratio and composition of the mobile phases, the gradient and isocratic mobile phase elution systems, the mobile phase flow rate, the column temperature, and the detector wavelength.

Detection Method

Separation of 10 WSVs was achieved at 30 °C on Shimpack C₁₈ column, 250 x 4.6 mm id, 5 µm particle size with isocratic elution using a mobile phase of 24% methanol (A) and 76% 0.05 M KH₂PO₄ phosphate buffer containing 0.01 M sodium 1-hexane sulfonate and 0.1% triethylamine (B), adjusted pH 3.0 with H₃PO₄, at a flow rate of 1 ml/min in 25 min. The sample injection volume was 10 µL, and simultaneous UV detection was 210 and 254 nm. The elution order of the 10 WSVs and their retention times (min) were

ascorbic acid (3.14), niacin (3.75), nicotinamide (4.25), B₅ (5.37), B₆ (6.32), B₁ (9.40), B₁₂ (10.45), B₉ (11.48), B₇ (15.65), and B₂ (22.31).

Sample Preparation

Three samples of fish feed additives from three different brands and each of the three packages were used. About 100-1000 mg of samples were extracted using an extracting solution, i.e. a mixture of 4 ml of distilled water; 0.4 ml of 2 M NaOH, and 5 ml of 1 M phosphate buffer, then filled to the mark with distilled water in 10 ml of volumetric flask.¹² This solvent was used because not all WSVs are dissolved in water, so it needed adjustment to the WSVs properties to be analyzed. Furthermore, the sample was sonicated at 40 °C for 15 min. After the sample was kept at room temperature, then it was centrifuged at 6500 rpm for 5 min. The supernatant was filtered with 0.2 µm of nylon syringe filter, then put into the vial, and ready to be injected into the HPLC system.

RESULTS AND DISCUSSION

Method Development and Optimization of the LC Condition

During the method development, various parameters, such as gradient and isocratic elution systems with various mobile phase ratios, the composition of mobile phase, mobile phase additives, column temperature, and flow rate were investigated. Initially, the method was carried out at 0.005 M sodium 1-hexane sulfonate as an ion pair reagent. However, it resulted in the good separation of vitamin B₅ and B₆ ($R_s < 1.0$). Therefore, the ion pair concentration was increased to 0.01 M and resulted in a good separation of vitamins B₅ and B₆ (Fig.-1). A 0.1% triethylamine (TEA)¹² was added in the mobile phase to improve the shape of the peak and reduce retention. Mobile phase without TEA resulted in peak asymmetry of vitamin B₆ and 32 min of separating time. Significant improvement in the peak shape, symmetry, and retention time occurred when 0.1% TEA was added to the mobile phase with 29 min of separating time (Fig.-2). TEA is known to minimize the interaction between silanol in the silica-based column with ionic samples, especially basic compounds.¹¹ Gradient elution systems have been investigated with mobile phase methanol (A) and phosphate buffer pH 3.0 (B), including the gradient elution system 0-5 min (10% A), 5-20 min (30% A), and 20-30 min (10% A) at 35°C and flow rates of 0.8 and 1.0 ml/min and a gradient system of 0-5 min (15% A), 5-15 min (30% A), and 15-30 min (15% A) at 30°C and flow rates of 0.8 and 1.0 ml/min. However, it resulted in poor separation and the peaks tended to be irregular, due to interference from the absorption of methanol. Methanol with UV-cutoff at 205 nm still provides absorption at 205 nm and 210 nm.¹¹ Hence, the increased percentage of methanol in the mobile phase causes changes or fluctuations in the baseline which disrupt the analysis. Therefore, this study was further carried out by elution of the isocratic system at 210 nm, i.e. the detection wavelength of vitamin B₅ and B₇. The isocratic elution system carried out several investigations on the ratio of the mobile phase of methanol (A) with a phosphate buffer pH 3.0 containing 0.01 M sodium 1-hexane sulfonate (B), from 20-30% of methanol. In the mobile phase with 20, 22, and 24% of methanol, 10 WSVs could be separated (Fig.-3), whereas, in the comparison of other methanol ratios, there were several vitamins poorly separated. Therefore, the system suitability test was carried out at 30°C, the flow rate of 1 ml/min using the mobile phase ratio of 20:80, 22:78, and 24:76 of methanol (A): 0.05 M phosphate buffer pH 3.0 containing 0.01 M sodium 1-hexane sulfonate and 0.1% TEA (B).

System Suitability Test

System suitability was assessed to ensure that the resolution, capacity factor, tailing factor, theoretical plate number, and RSD of the AUC of the method was adequate to the requirements for developing analytical methods.^{11,13} The system suitability was evaluated by making six replicate injections of the mixed standard solution of WSVs.¹⁴ System suitability was applied with mobile phase 24% of methanol and 76% of phosphate buffer generated acceptable results meeting the requirements of the system suitability criteria according to the Center for Drug Evaluation and Research (CDER) Validation of Chromatographic Methods as part of the USP, covering the resolution > 1.0 -1.5; tailing factor ≤ 2 , capacity factor $0.5 < k < 20$; theoretical plate number > 2000 , and RSD of AUC $\leq 1\%$.¹³ Summary of the system suitability parameters and results are detailed in Table-1. Data were presented as mean $\pm$ standard deviation.

Table-1: Summary of System Suitability (n = 6)

Parameters	Resolution, Rs	Capacity factor, k	Theoretical plate number, N	Tailing factor, Tf	RSD AUC, %
Requirement*	> 1.0 – 1.5	$0.5 < k < 20$	> 2000	≤ 2	≤ 1
Vit C	5.42 ± 0.03	0.52 ± 0.00	9372 ± 128.63	1.19 ± 0.01	0.10
B ₁	8.80 ± 0.05	3.60 ± 0.00	6863 ± 42.50	0.67 ± 0.00	0.02
B ₂	17.08 ± 0.04	9.61 ± 0.01	12174 ± 37.90	1.13 ± 0.00	0.08
Niacin	4.16 ± 0.02	0.83 ± 0.00	7839 ± 30.56	1.29 ± 0.00	0.10
Nicotinamide	2.81 ± 0.01	1.07 ± 0.00	8532 ± 20.62	1.22 ± 0.00	0.21
B ₅	2.61 ± 0.01	1.62 ± 0.00	9325 ± 15.81	1.16 ± 0.00	0.22
B ₆	4.00 ± 0.02	2.07 ± 0.00	10754 ± 31.79	1.18 ± 0.00	0.07
B ₇	9.11 ± 0.01	6.54 ± 0.01	13959 ± 25.72	1.11 ± 0.00	0.06
B ₉	1.64 ± 0.02	4.36 ± 0.01	9146 ± 17.84	1.12 ± 0.00	1.00
B ₁₂	1.15 ± 0.02	3.90 ± 0.02	3303 ± 10.56	1.08 ± 0.00	0.21

*Centre for Drug Evaluation and Research (CDER) Validation of Chromatographic Methods

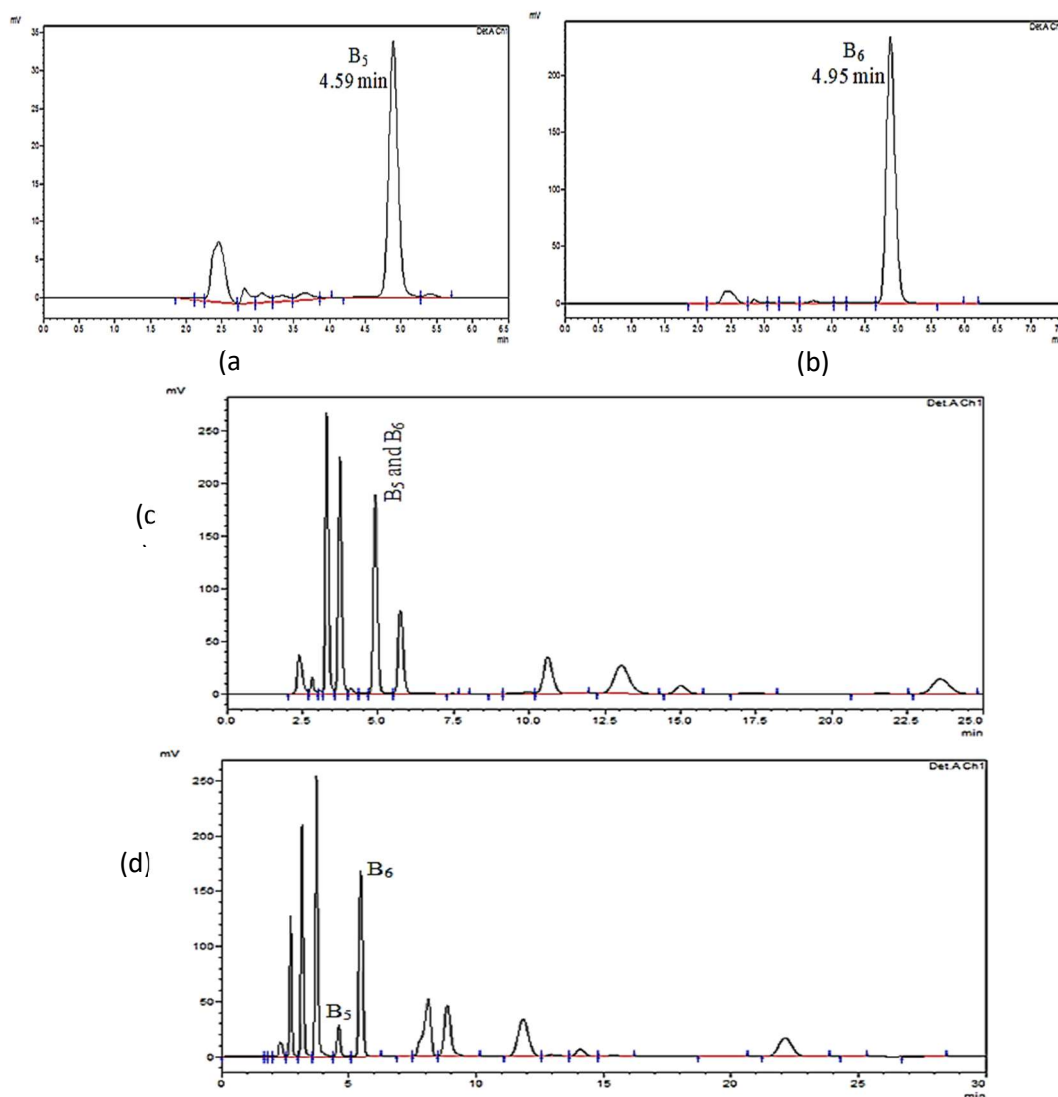


Fig.-1: Chromatograms of the Effect Concentration of Ion Pair Reagent. (a) The peak of Vitamin B₅, (b) Peak of Vitamin B₆, (c) Peak of Vitamin B₅ and B₆ with 0.005 M Ion Pair, (d) Peak of Vitamin B₅ and B₆ with 0.01 M Ion Pair

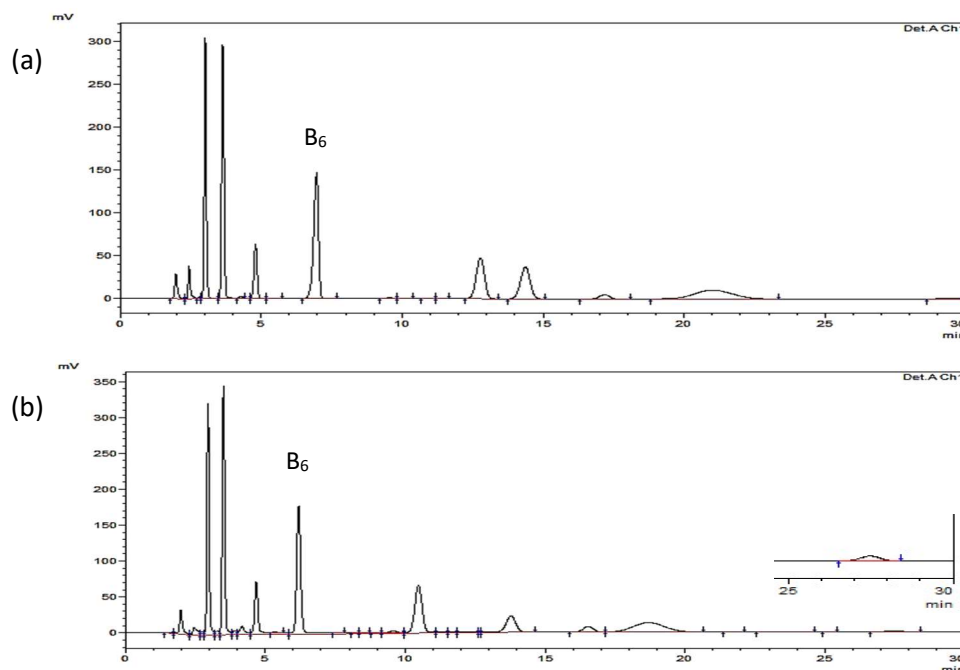


Fig.-2: Chromatogram of the Effect of Application TEA. (a) without TEA, (b) with 0.1% TEA

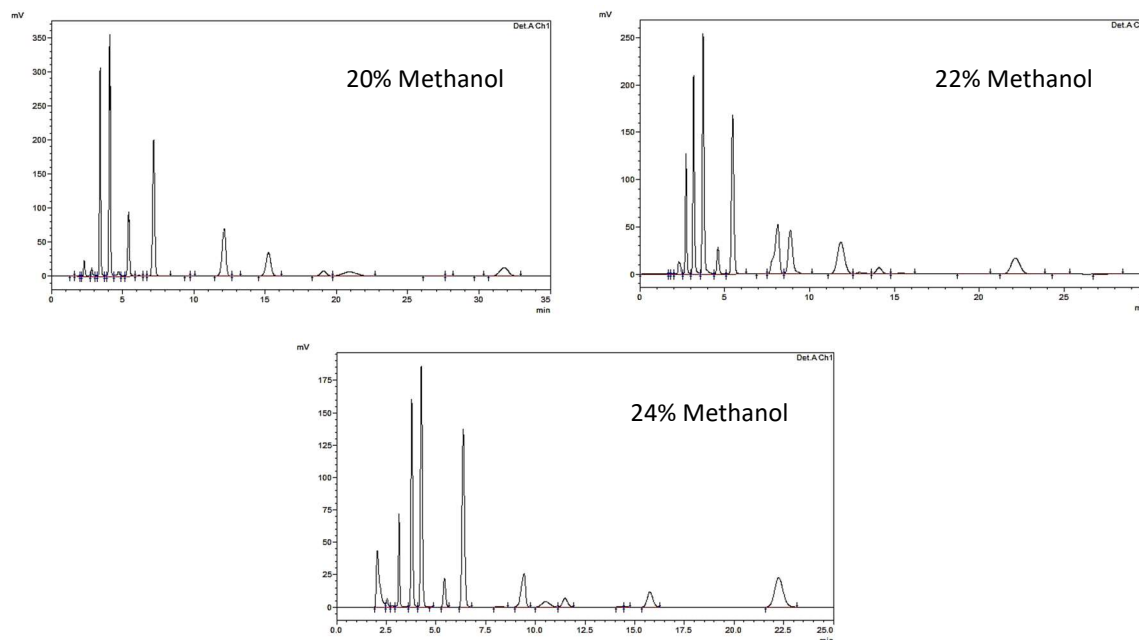


Fig.-3: Chromatograms of Isocratic Elution at ratios 20%, 22%, and 24% of Methanol

Method Validation

Specificity

The method specificity was determined from the resolution of the mixed standard solution of WSVs. The results of the system suitability test showed that the resolution met the requirements, $R_s > 1.0$ – 1.5 because the separation consists of 10 analytes.¹¹ Fig.-4 was a chromatogram for the separation of WSVs at 210 nm and 254 nm.

Linearity and Range

Linearity was performed by analyzing the standard response of WSVs in six concentrations in the range of 10–140 mg/L for vitamin B₁, B₂, B₆, niacin, and nicotinamide, 10–400 mg/L for vitamin B₅, B₇, and ascorbic

acid; and five concentration of vitamin B₉ and B₁₂ in the range 1-16 mg/L. The concentration range was adjusted to the concentration of vitamins in the sample. The individual regression curve was established by plotting the AUC versus the standard concentration. The results showed that the standard concentration of 10 WSVs and AUC had a linear relationship with $r > 0.990$.¹⁴ A summary of the range, linearity equation, and correlation coefficient (r) was presented in Table-2.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were calculated from the standard deviation of the intercept divided by the slope of the linear regression equation. LOD is the smallest level of analyte that gives a measurable response, while LOQ is the smallest concentration of analyte which gives a response that can be accurately and precision quantified.¹⁴ The obtained values of LOD and LOQ of 10 WSVs are summarized in Table-2.

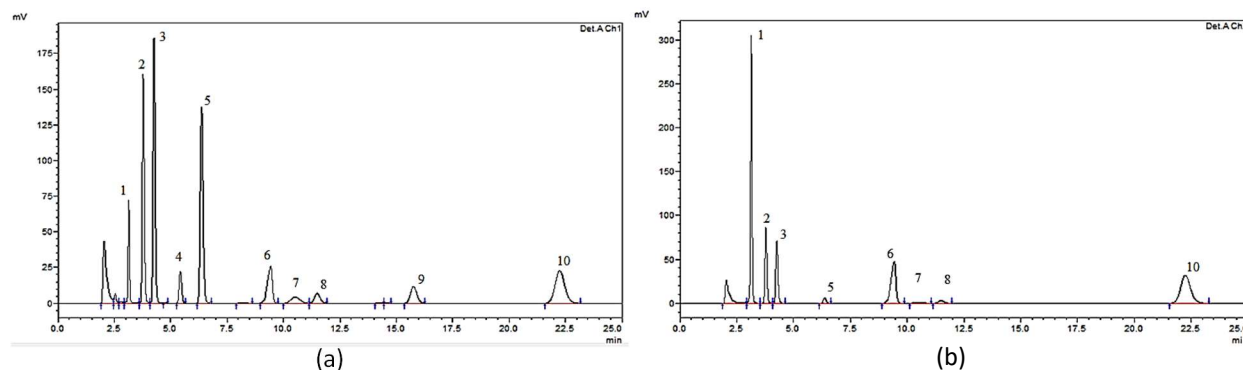


Fig.-4: Chromatograms of Separation of 10 WSVs. (a) at 210 nm and (b) at 254 nm. Peak no. 1. Ascorbic Acid, 2. Niacin, 3. Nicotinamide, 4. Vitamin B₅, 5. B₆, 6. B₁, 7. B₁₂, 8. B₉, 9. B₇, and 10. B₂

Table-2: Linearity Equation, Correlation Coefficient, Range, LOD, and LOQ

Vitamin	Range of Linearity (mg/L)	Linearity Equation	Correlation coefficient (r)	LOD (mg/L)	LOQ (mg/L)
B ₁	10 - 140	$y = 23251x - 6068.8$	0.9999	1.242	4.138
B ₂	10 - 140	$y = 31164x + 21424$	0.9998	3.006	10.019
B ₅	10 - 400	$y = 3327.4x + 7486.2$	0.9999	7.443	24.809
B ₆	10 - 140	$y = 39573x + 33263$	0.9999	1.735	5.784
B ₇	10 - 400	$y = 4616.2x + 5172.7$	0.9999	6.006	20.022
B ₉	1 - 16	$y = 27320x - 355.2$	0.9961	1.894	6.292
B ₁₂	1 - 16	$y = 31049x - 7355.2$	0.9996	0.668	2.226
Niacin	10 - 140	$y = 31590x - 91.437$	0.9999	0.705	2.350
Nicotinamide	10 - 140	$y = 38982x + 10889$	0.9999	0.940	3.134
Ascorbic acid	10 - 400	$y = 24737x + 126235$	0.9995	16.321	54.403

Accuracy

The method accuracy was evaluated by the simulation method (spiked-placebo recovery). Analysis was performed by adding the WSVs standard with three different concentrations (80, 100, and 120%) to the placebo of the fish feed additive with three replications for each concentration. The accuracy values were calculated as recovery. The results showed that the analysis method had the smallest recovery value of 81.60% in 6.6 mg/L vitamin B₁₂ and the largest of 109.71% in 162 mg/L vitamin B₅. These results were acceptable according to AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals.¹⁴ Results of the accuracy for the 10 WSVs were summarized in Table-3.

Precision

In this study, intra-day and inter-day precision were determined. Determination of precision was performed by preparing three concentrations of standard vitamin solutions (80, 100, and 120%) with three replications. Precision is expressed as the relative standard deviation (RSD) of the AUC response. Inter-day precision

was evaluated by analyzing three replicates on three consecutive days. The method has good precision with RSD of intra-day and inter-day precision in the range 0.16 – 4.18% and 0.40 – 5.59%, respectively. The values of intra-day and inter-day precision were presented in Table-3.

Table-3: Accuracy, Intra-Day, and Inter-Day Precision

Vitamin	Concentration (mg/L)	Intra-day (n=3)			Inter-day (n=3)		
		Mean C $\pm$ SD	% Accuracy	% RSD	Mean C $\pm$ SD	% Accuracy	% RSD
Requirement (AOAC)			*85 – 110 / 80 - 115	* $< 4 /$ < 6		*85 – 110 / 80 - 115	** $< 8 /$ < 11
B ₁	80	80.89 $\pm$ 0.13	101.13 $\pm$ 0.17	0.16	81.48 $\pm$ 1.65	101.84 $\pm$ 2.06	2.03
	100	99.11 $\pm$ 1.46	99.12 $\pm$ 1.46	1.47	104.78 $\pm$ 3.35	104.78 $\pm$ 3.35	3.20
	120	119.92 $\pm$ 1.42	99.95 $\pm$ 1.18	1.35	123.57 $\pm$ 3.94	102.98 $\pm$ 3.28	3.18
B ₂	81.5	79.22 $\pm$ 0.53	97.15 $\pm$ 0.65	0.67	79.96 $\pm$ 2.54	98.11 $\pm$ 3.12	3.18
	102	99.90 $\pm$ 0.67	98.02 $\pm$ 2.01	2.05	99.45 $\pm$ 2.36	97.50 $\pm$ 2.31	2.37
	122	115.35 $\pm$ 1.60	94.32 $\pm$ 1.31	1.39	116.32 $\pm$ 0.47	95.35 $\pm$ 0.39	0.40
Niacin	83	84.93 $\pm$ 0.69	102.59 $\pm$ 0.83	0.81	81.89 $\pm$ 2.85	98.93 $\pm$ 3.44	3.48
	103	105.39 $\pm$ 1.63	101.85 $\pm$ 1.57	1.54	98.99 $\pm$ 3.80	97.72 $\pm$ 3.68	3.84
	124	123.45 $\pm$ 1.31	99.42 $\pm$ 1.05	1.06	119.28 $\pm$ 0.56	96.06 $\pm$ 0.45	0.47
Nicotinamide	82	86.48 $\pm$ 0.41	104.95 $\pm$ 0.50	0.48	82.99 $\pm$ 3.03	100.71 $\pm$ 3.68	3.65
	103	107.98 $\pm$ 1.95	104.84 $\pm$ 1.89	1.80	105.31 $\pm$ 2.56	102.16 $\pm$ 2.48	2.43
	123.6	126.00 $\pm$ 1.45	101.95 $\pm$ 1.17	1.14	122.28 $\pm$ 0.76	98.93 $\pm$ 0.62	0.62
B ₅	162	178.00 $\pm$ 1.60	109.71 $\pm$ 0.98	0.89	167.39 $\pm$ 3.13	103.53 $\pm$ 5.86	1.87
	203	217.99 $\pm$ 1.93	107.49 $\pm$ 0.95	0.88	208.22 $\pm$ 5.95	102.57 $\pm$ 2.93	2.86
	243	253.52 $\pm$ 1.10	107.02 $\pm$ 0.45	0.42	249.98 $\pm$ 3.39	102.87 $\pm$ 1.39	1.35
B ₆	87	90.24 $\pm$ 0.49	105.43 $\pm$ 0.56	0.52	89.11 $\pm$ 2.18	102.43 $\pm$ 2.51	2.45
	109	111.89 $\pm$ 2.24	100.75 $\pm$ 2.06	1.94	108.22 $\pm$ 3.18	99.28 $\pm$ 2.92	2.94
	130.5	130.26 $\pm$ 1.69	103.45 $\pm$ 1.29	1.24	128.96 $\pm$ 2.12	98.82 $\pm$ 1.63	1.65
B ₇	162.5	166.02 $\pm$ 2.77	102.13 $\pm$ 1.70	1.65	160.21 $\pm$ 5.09	98.59 $\pm$ 3.13	3.18
	203	206.95 $\pm$ 3.33	101.85 $\pm$ 1.64	3.05	202.86 $\pm$ 6.55	99.93 $\pm$ 3.23	3.23
	244	244.07 $\pm$ 0.67	100.09 $\pm$ 0.27	0.27	238.35 $\pm$ 2.84	97.68 $\pm$ 1.16	1.19
Ascorbic acid	169	176.82 $\pm$ 2.93	104.72 $\pm$ 1.74	1.66	171.91 $\pm$ 6.88	101.72 $\pm$ 4.07	4.00
	211	220.00 $\pm$ 0.10	104.23 $\pm$ 0.05	0.05	213.78 $\pm$ 6.77	101.32 $\pm$ 3.21	3.17
	253	274.72 $\pm$ 3.73	108.46 $\pm$ 1.47	1.35	260.95 $\pm$ 10.69	103.50 $\pm$ 3.78	3.65
B ₉	6.6	6.15 $\pm$ 0.05	93.46 $\pm$ 0.81	0.87	6.52 $\pm$ 0.33	99.14 $\pm$ 5.02	5.07
	8.2	8.27 $\pm$ 0.08	100.57 $\pm$ 0.92	0.91	8.17 $\pm$ 0.22	98.60 $\pm$ 2.69	2.71
	9.9	9.35 $\pm$ 0.14	94.53 $\pm$ 1.46	1.55	9.90 $\pm$ 0.55	100.26 $\pm$ 5.61	5.59
B ₁₂	6.6	5.42 $\pm$ 0.12	81.60 $\pm$ 1.80	2.28	6.25 $\pm$ 0.33	94.01 $\pm$ 4.96	5.27
	8.3	8.04 $\pm$ 0.20	96.69 $\pm$ 2.45	2.59	8.41 $\pm$ 0.47	100.31 $\pm$ 5.60	5.53
	10	10.07 $\pm$ 0.41	100.97 $\pm$ 4.15	4.18	9.83 $\pm$ 0.32	98.56 $\pm$ 3.21	3.26

*85-110% accuracy and RSD < 4% for 100 mg/L concentration; 80-115% accuracy and RSD < 6 for 10 mg/L concentration

**RSD < 8% for 100 mg/L concentration and < 11% for 10 mg/L concentration.

Application to the Fish Feed Additives

The validated method was applied to quantify 10 WSVs in three fish feed additives. Initially, the extracting solution to extract the WSVs was a mixture of 5 ml of distilled water, 200 μ l of 0.1 M NaOH, and 1%

metaphosphoric acid,^{4,15} and also a mixture of methanol and water (5:95) with 1% acetic acid.¹⁶ However, both extracting solvents could not completely extract WSVs in the sample, which showed low recovery (less than 50%) in vitamins B₂, B₇, and B₉. Furthermore, the WSVs extraction from the sample was carried out using a mixture of distilled water, 2 M NaOH, and 1 M phosphate buffer pH 5.5 as the extracting solution.¹² The results of the determination of WSVs concentration in the sample were presented in Table-4. The recovery of vitamin B₁ in sample 1 was only 45.04 ± 5.23%, while in samples 2 and 3 was 102.78 ± 2.89% and 97.25 ± 2.35%, respectively. Meanwhile, the recovery of niacin in sample 3 was only 50.49 ± 2.55%, while in sample 2 it was 95.50 ± 2.30%. Spiking 50 mg/L standard vitamin B₁ into sample 1 and 25 mg/L standard niacin into sample 3 were performed to ensure the extraction accuracy. The results showed that the addition of standard vitamin B₁ and niacin caused an increased AUC corresponding to the concentration theoretical addition of both vitamins. These indicated that the developed method and the WSVs extracting solvent were correct. Therefore, it can be concluded that the concentration of vitamin B₁ in sample 1 and niacin in sample 3 mismatch the label claims. Meanwhile, the concentration of vitamins B₇ and B₁₂ in samples 1 and 2 were too small and lower than LOD. The standard vitamin was added to the sample solution, and the obtained recovery values matched the label claims.

Table-4: Results of Determination 10 WSVs in Samples

Vitamin	Sample 1 (n = 3)			Sample 2 (n = 3)			Sample 3 (n = 3)		
	Label claim (mg/g)	Result (mg/g)	% Recovery	Label claim (mg/g)	Result (mg/g)	% Recovery	Label claim (mg/g)	Result (mg/g)	% Recovery
Ascorbic acid	55.86	54.31±0.69	97.21±1.23	100	100.26±2.38	100.26±2.38	150	153.57±0.36	102.38±0.24
B ₁	6.21	2.80±0.32	45.04±5.23	20	21.23±0.50	102.78±2.90	3.0	2.92±0.07	97.25±2.35
B ₂	6.21	5.71±0.10	91.96±1.55	35	35.85±1.32	100.49±0.44	3.0	2.94±0.04	97.89±1.25
Niacin	-	-	-	75	71.62±1.72	95.50±2.30	2.5	1.26±0.06	50.49±2.55
Nicotinamide	12.41	13.09±0.12	105.46±0.96	-	-	-	-	-	-
B ₅	6.21	6.17±0.12	99.36±1.89	50	49.79±3.13	96.34±1.24	1.5	1.38±0.05	91.92±3.31
B ₆	6.21	6.28±0.05	101.15±0.79	25	25.02±0.44	100.06±1.77	1.5	1.57±0.03	104.74±2.14
B ₇	0.028*	0.0281±0.0007	100.18±2.48	-	-	-	-	-	-
B ₉	0.25	0.25±0.02	100.74±6.51	4	4.09±0.07	102.27±1.63	0.5	0.46±0.03	92.93±5.82
B ₁₂	0.02*	0.0174±0.0001	91.79±0.37	0.025*	0.0232±0.0005	94.29±2.64	-	-	-

*: level < LOD, performed by spiking standard of vitamin

:- sample does not contain the substance

CONCLUSION

Simultaneous analysis method of 10 WSVs (vitamin B₁, B₂, B₅, B₆, B₇, B₉, B₁₂, ascorbic acid, niacin, and nicotinamide) was achieved at 30°C, the flow rate of 1 ml/min with HPLC-UV at two wavelengths detection, 210 and 254 nm. This method uses the isocratic mobile phase (24:76) of methanol (A) and 0.05 M phosphate buffer pH 3.0 containing 0.01 M sodium 1-hexane sulfonate and 0.1% TEA (B). The method can properly separate 10 WSVs in 25 min. The method met the validation requirements according to AOAC, including specificity, linearity, range, accuracy, precision, LOD, and LOQ. The method can be used as a routine analysis in the laboratory to determine the concentration of 10 WSVs in fish feed additives.

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