

ANALYTICAL METHOD VALIDATION AND DETERMINATION OF PYRIDOXINE, NICOTINAMIDE, AND CAFFEINE IN ENERGY DRINKS USING THIN LAYER CHROMATOGRAPHY-DENSITOMETRY

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ABSTRACT

Food supplement which contains vitamins and stimulants such as caffeine were classified as energy drink. TLC-densitometry method was chosen to determine the pyridoxine, nicotinamide, and caffeine in the energy drink sample. TLC plates of silica gel 60 F₂₅₄ was used as the stationary phase and methanol : ethyl acetate : ammonia 25% (13:77:10) was used as the mobile phase. The correlation coefficient for each pyridoxine, nicotinamide, and caffeine were 0.9982, 0.9997, and 0.9966, respectively. Detection and quantitation limits of from the three analytes were 4.05 and 13.51 µg/mL; 13.15 and 43.83 µg/mL; 5.43 and 18.11 µg/mL, respectively. The recovery of pyridoxine, nicotinamide, and caffeine were within the required limit range of 95-105%. The percent of RSD were below the limit value of 5.7% for caffeine and nicotinamide and 8% for pyridoxine. The content amount of pyridoxine in the sample 1 and 2 were 33.59 ± 0.981 and 30.29 ± 2.061 µg/mL, respectively. The content amount of nicotinamide in the sample 1 and 2 were 106.53 ± 3.521 and 98.20 ± 3.648 µg/mL, respectively. The content amount of caffeine in the sample 1 and 2 were 249.50 ± 5.080 and 252.80 ± 2.640 µg/mL, respectively. Robustness test results showed that the most optimal method conditions should be applied for the analysis.

Keywords: TLC-densitometry; energy drinks; caffeine; vitamins

ABSTRAK

Suplemen makanan yang mengandung vitamin dan stimulan seperti kafein digolongkan sebagai minuman berenergi. Metode KLT-densitometri dipilih untuk menetapkan kandungan senyawa piridoksin, nikotinamid, dan kafein dalam sampel minuman berenergi. Fase diam yang digunakan adalah lempeng KLT silika gel 60 F₂₅₄ sedangkan fase gerak yang digunakan adalah campuran metanol : etil asetat : amonia 25% (13:77:10). Koefisien korelasi yang dihasilkan oleh baku piridoksin, nikotinamid, dan kafein berturut-turut adalah 0,9982, 0,9997, dan 0,9966. Batas deteksi dan batas kuantitasi dari ketiga analit berturut-turut adalah 4,05 dan 13,51 µg/mL; 13,15 dan 43,83 µg/mL; 5,43 dan 18,11 µg/mL. Nilai persen perolehan kembali piridoksin, nikotinamid, dan kafein berada dalam rentang 95-100%. Nilai persen simpangan baku relatif kafein dan nikotinamid berada di bawah 5,7% untuk sedangkan untuk nikotinamid berada di bawah 8,0%. Konsentrasi piridoksin dalam sampel 1 dan 2 berturut-turut adalah 33,59±0,981 dan 30,2 ±2,061 µg/mL. Konsentrasi nikotinamid dalam sampel 1 dan 2 berturut-turut adalah 106,53±3,521 dan 98,20±3,648 µg/mL. Konsentrasi kafein dalam sampel 1 dan 2 berturut-turut adalah 249,50±5,080 dan 252,80±2,640 µg/mL. Uji ketangguhan metode menunjukkan bahwa analisis harus dilakukan pada kondisi paling optimalnya.

Kata Kunci: KLT-densitometri; minuman berenergi; kafein; vitamin

INTRODUCTION

Active people need more energy to perform their activities. The energy can be obtained either from food or supplements consumed everyday [1-2]. Since 2006 the beverages denominating "energy drinks" and "sport drinks" have gained popularity between active persons due to the attribution of energy-giving properties. Most consumers of supplements tend to prefer energy drinks

than to foods because they are easier to consume and obtain the desired result [2]. In previous study, the ingestion of low to moderate doses of caffeinated energy drinks has been associated with adverse side effects such as insomnia or increased nervousness [3]. The pharmacology study of agents included in such drinks, combined with reports of toxicity, raises concern for potentially serious adverse effects in association with energy drink use [3-4]. The importance of control

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over the amount of energy drink consumption is associated with the accurate amount of ingredient information in these supplements. The improvements of toxicity surveillance and regulations of energy drink sales and consumption should be based on appropriate research [4-5]. It is necessary to develop a high-throughput analytical method which should be capable of detecting simultaneously the relevant compounds contained in an energy drink [6].

The previous studies reported the analytical methods to determine compounds contained in energy drink. Determination of caffeine in energy drinks were performed by HPLC/UV [7-9], HPLC/PDA and fluorescence detection [10], microemulsion electrokinetic chromatography [11], mass spectrometry [12], and spectrophotometry UV [13,19]. Determination of vitamin B in energy drinks and multivitamins were performed by HPLC/UV methods [14-17], HPLC with DAD, ELSD, and MS detection [18], and spectrophotometry UV [19]. Determination of caffeine and vitamin B were performed by TLC and HPTLC [6,20-21].

In this study, analytical method validation and determination of pyridoxine, nicotinamide, and caffeine in energy drinks was performed using thin-layer chromatography (TLC)-densitometry method. The purpose of this study was to determine the optimal conditions for TLC-densitometry method; to validate the analytical method for pyridoxine, nicotinamide, and caffeine determination in energy drinks sample simultaneously, quantitatively, and cost effectively.

EXPERIMENTAL SECTION

Materials

Pyridoxine and nicotinamide standard were obtained from PT. Erela, Semarang, Indonesia. Caffeine standard was obtained from PT. Kalbe Farma, Cikarang, Indonesia. Energy drinks of two different brand names were purchased from local market. Methanol, ethyl acetate, and ammonia 25% were purchased from Merck (Germany) and distilled water was purchased from PT. Ikapharmindo Putramas (Indonesia); all solvents were at least of analytical grade or distilled before use. TLC plates of silica gel 60 F₂₅₄ were also purchased from Merck.

Instrumentation

Samples and standard solution were applied with an *CAMAC Automatic TLC Sampler Linomat 5*, with the following settings for 20 tracks per plate: band length, 3.0 mm; track distance, 9 mm; band velocity, 50 nL/s; and first application x axis and y axis were 10.0 and 15.0 mm, respectively. Sample application volumes of 1 μ L

for all standards and samples were used. Chromatography was carried out in a 20 \times 20 cm flat bottom chamber *CAMAG* up to a migration distance of 75 mm using methanol-ethyl acetate-ammonia 25% 13:77:10 (v/v/v) as the mobile phase. The chamber was saturated with mobile phase (without filter paper) for 30 min. After development, the plate was dried in a stream of warm air for 2 min. The plate was scanned with the *CAMAC TLC Scanner 3* CAT. No. 027.6485 SER. No.160602 slit dimension 6.00x0.10 mm and a scanning speed of 20 mm/s. In absorption mode, pyridoxine, nicotinamide, and caffeine were measured at UV 293, 263, and 274 nm, respectively. All instruments were controlled via the software platform *winCats 1.4.4.6337* Planar Chromatography Manager (*CAMAG*).

Procedure

Standard solution

For the pyridoxine standard stock solution: 50.0 mg of pyridoxine were accurately weighed into a 5 mL volumetric flask, dissolved, and diluted to volume with water. The calibration standard solution of pyridoxine was obtained by transferring 70, 100, 130, 160, 190, dan 220 μ L of standard stock solution of pyridoxine into 5 mL volumetric flask for each concentration and dilute to volume with water.

For the nicotinamide standard stock solution: 50.0 mg of nicotinamide were accurately weighed into a 5 mL volumetric flask, dissolved, and diluted to volume with water. The calibration standard solution of nicotinamide was obtained by transferring 75, 125, 175, 225, 275, and 325 μ L of standard stock solution of nicotinamide into 5 mL volumetric flask for each concentration and dilute to volume with water.

For the caffeine standard stock solution: 50.0 mg of caffeine were accurately weighed into a 5 mL volumetric flask, dissolved, and diluted to volume with water. The calibration standard solution of caffeine was obtained by transferring 180, 240, 300, 360, 420, and 480 μ L of standard stock solution of caffeine into 5 mL volumetric flask for each concentration and dilute to volume with water.

Analytical method validation

The method was validated for selectivity, linearity and range, detection limit, quantitation limit, precision, accuracy, and robustness according to the USP guidelines for validation of analytical methods.

Sample preparation

Ten bottles of energy drinks for each brand name were transferred and homogenized in a beaker glass. Sample for the determination was obtained by

transferring 3.75 mL of mixed sample into a 5 mL volumetric flask and dilute to volume with water.

RESULT AND DISCUSSION

Determination of the Maximum Absorption Wavelength

The maximum absorption wavelength of each compound was determined at the beginning of the study by scanning the compounds spots on the TLC plate at 200–400 nm (Fig. 1). The maximum absorption wavelength of pyridoxine, nicotinamide, and caffeine, was 293 nm, 263 nm, and 274 nm, respectively.

Analytical Method Validation

Validation of analytical methods performed in this research includes the following parameters:

Selectivity test

Selectivity test was conducted on both the standard and the sample solution. Selectivity was determined by calculating the resolution value indicates the value of each peak separation of the analytes. R_f values and resolution of caffeine, nicotinamide, and pyridoxine in the raw mixture was 0.72 ($R_s = 2.0$), 0.55 ($R_s = 2.0$), and 0.18 ($R_s = 3.47$), respectively, while the sample was 0.71 ($R_s = 1.80$), 0.53 ($R_s = 1.80$), and 0.19 ($R_s = 1.40$), respectively. Fig. 2 was presented the chromatograms of the energy drinks sample. The peaks of the analytes was separated from the others, thus it can be concluded that the developed method is selective for determining caffeine, nicotinamide, and pyridoxine.

Linearity and range

The absorbance data versus concentration of the three analytes were treated by linear correlation coefficient. In the linearity test, calibration curve equation of caffeine, nicotinamide, and pyridoxine was obtained $y = 12.5434x + 1460.4352$ ($r = 0.9966$), $y = 6.6081x + 120.3904$ ($r = 0.9997$), and $y = 10.4417x + 410.0181$ ($r = 0.9982$), respectively. This method is linear in the range of 180–480 $\mu\text{g/mL}$, 75–325 $\mu\text{g/mL}$, 70–220 $\mu\text{g/mL}$, and 70–220 $\mu\text{g/mL}$ for the caffeine, nicotinamide, and pyridoxine, respectively.

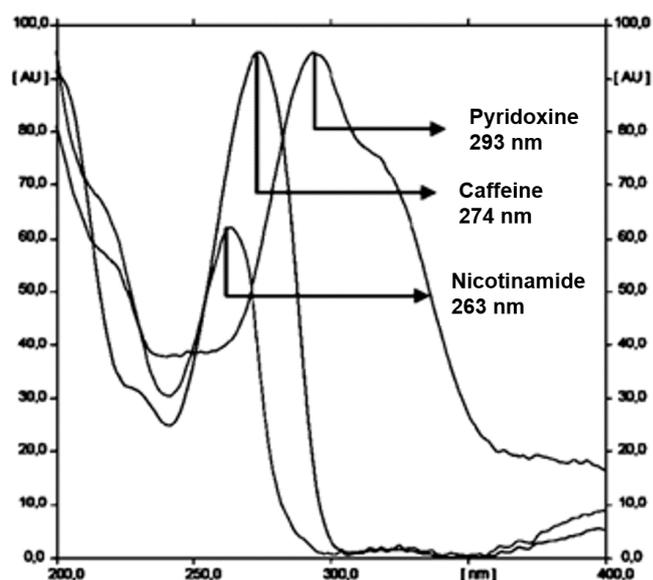


Fig 1. Result of the maximum absorption wavelength determination

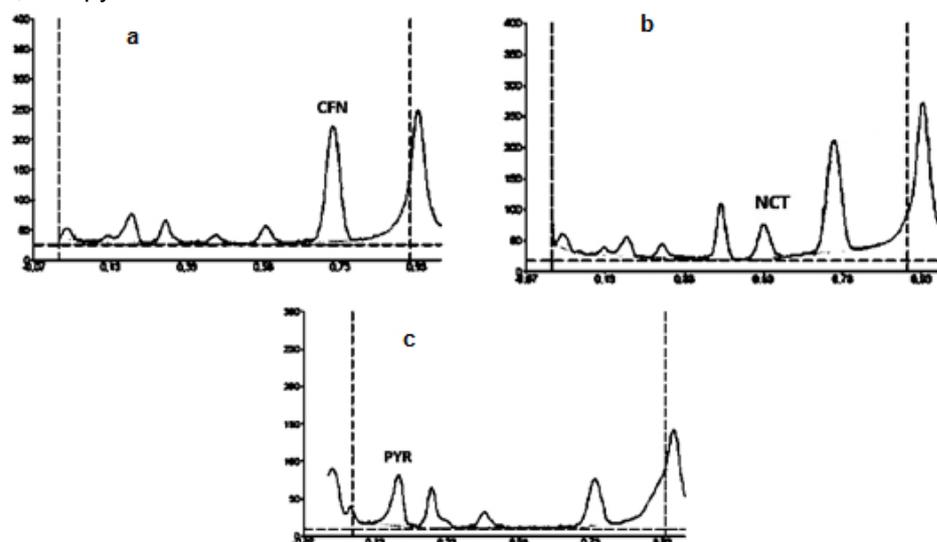


Fig 2. Chromatograms of the energy drinks sample (a) UV detection at 274 nm, (b) UV detection at 263 nm, and (c) UV detection at 293 nm. Chromatographic conditions: mobile phase, methanol-ethyl acetate-ammonia 25% 13:77:10 (v/v/v); CFN: caffeine; NCT: nicotinamide; PYR: pyridoxine

Table 1. Evaluation of intra-day accuracy and precision (n=3)

Concentration levels		Analyte taken ($\mu\text{g/mL}$)	Analyte found ($\mu\text{g/mL}$)	Recovery (%)	SD	RSD (%)	RSD Horwitz (%)
Low	Caffeine	240.0	236.96	98.73	11.03	4.65	7.65
	Nicotinamide	125.0	125.63	100.50	5.81	4.62	7.94
	Pyridoxine	100.0	92.439	92.44	7.74	8.38	8.00
Medium	Caffeine	360.0	375.49	104.30	11.52	3.07	7.34
	Nicotinamide	225.0	227.81	101.25	6.19	2.72	7.69
	Pyridoxine	160.0	162.65	101.66	1.47	0.90	7.85
High	Caffeine	480.0	478.18	99.62	12.04	2.52	7.03
	Nicotinamide	325.0	321.38	98.89	3.45	1.07	7.43
	Pyridoxine	220.0	213.76	97.16	4.00	1.87	7.70

Table 2. Evaluation of inter-day accuracy and precision (n=3)

Concentration levels		Analyte taken ($\mu\text{g/mL}$)	Analyte found ($\mu\text{g/mL}$)	Recovery (%)	SD	RSD (%)	RSD Horwitz (%)
Low	Caffeine	240.0	235.53	98.14	1.89	0.81	7.65
	Nicotinamide	125.0	119.06	95.25	4.19	3.52	7.94
	Pyridoxine	100.0	98.18	98.18	1.39	1.42	8.00
Medium	Caffeine	360.0	363.84	101.07	6.77	1.86	7.34
	Nicotinamide	225.0	218.23	96.99	10.07	4.62	7.69
	Pyridoxine	160.0	154.97	96.86	7.59	4.89	7.85
High	Caffeine	480.0	475.21	99.00	7.30	1.54	7.03
	Nicotinamide	325.0	313.46	96.45	4.88	1.56	7.43
	Pyridoxine	220.0	219.32	99.69	8.03	3.66	7.70

Determination of detection limit and quantitation limit

Detection limit of caffeine, nicotinamide, and pyridoxine was 5.43, 13.15, and 4.05 $\mu\text{g/mL}$, respectively. Quantitation limit of caffeine, nicotinamide, and pyridoxine was 18.11, 43.83, and 13.51 $\mu\text{g/mL}$, respectively. The result was obtained by the calculation using standard deviation approach.

Accuracy and precision of standard solution

Determination of standard solution accuracy and precision was assessed using three concentration levels (low, medium, high) and three replicates of each concentration. Subsequently, the recovery and RSD value were calculated.

Intraday evaluation. Intraday evaluation results of caffeine, nicotinamide, and pyridoxine standards in the three concentration levels have a smaller percent RSD value than the Horwitz's RSD (Table 1) [22]. The required values of percent RSD were accepted except at the low levels of pyridoxine levels (100 $\mu\text{g/mL}$). These results show that this method produced highly precise determination of caffeine and nicotinamide in all concentration levels. The precision of pyridoxine determination was obtained only at the medium and high concentration levels.

Interday evaluation. Interday evaluation results caffeine, nicotinamide, and pyridoxine standards in the three concentration levels have a smaller percent RSD

than the Horwitz's RSD (Table 2) [22]. The required values of percent RSD were accepted. These results show that this method produced highly precise determination of caffeine, nicotinamide, and pyridoxine in all concentration levels.

Precision and accuracy test of the samples

Precision and accuracy of test of samples was performed by the standard addition method. The three levels of addition concentration levels consisted of the low (50 $\mu\text{g/mL}$), medium (100 $\mu\text{g/mL}$), and high (150 $\mu\text{g/mL}$) as shown in Table 3. The mean value of the recovery as the accuracy parameter at three concentration levels of caffeine, nicotinamide, and pyridoxine in both samples are within the required range of 95–105% [22]. Percent RSD as the precision parameter at the three levels of caffeine, nicotinamide, and pyridoxine concentrations in both samples were below the maximum limit of Horwitz's RSD which is 5.7% for high level and 8% for low and medium levels [22]. These results show that this method, produced highly precision and accuracy for determining caffeine, nicotinamide, and pyridoxine in all concentration levels.

Determination of caffeine, nicotinamide, and pyridoxine in the samples

Table 4 was presented the assay of analytes in the samples. Caffeine concentration in the sample 1 was 249.50 $\mu\text{g/mL}$ (RSD = 2.04%), while the sample 2

Tabel 3. Results of recovery study by standard addition method

Caffeine evaluation						
Addition level on the sample		Pure Caffeine added ($\mu\text{g/mL}$)	Caffeine found ($\mu\text{g/mL}$)	Recovery (%)	SD	RSD (%)
Sample 1	Low	50.0	51.20	102.39	2.61	5.11
	Medium	100.0	103.30	103.29	4.09	3.97
	High	150.0	155.83	103.88	2.35	1.51
Sample 2	Low	50.0	48.91	97.82	0.70	1.44
	Medium	100.0	100.66	100.66	2.07	2.05
	High	150.0	151.88	101.26	3.88	2.55
Nicotinamide evaluation						
Addition level on the sample		Pure Nicotinamide added ($\mu\text{g/mL}$)	Nicotinamide found ($\mu\text{g/mL}$)	Recovery (%)	SD	RSD (%)
Sample 1	Low	50.0	49.43	98.86	2.84	5.74
	Medium	100.0	98.27	98.27	4.75	4.84
	High	150.0	145.83	97.21	5.92	4.06
Sample 2	Low	50.0	50.81	101.63	2.36	4.64
	Medium	100.0	97.98	97.98	3.60	3.68
	High	150.0	150.65	100.43	2.48	1.64
Pyridoxine evaluation						
Addition level on the sample		Pure Pyridoxine added ($\mu\text{g/mL}$)	Pyridoxine found ($\mu\text{g/mL}$)	Recovery (%)	SD	RSD (%)
Sample 1	Low	50.0	48.97	97.94	2.82	5.76
	Medium	100.0	103.53	103.53	2.65	2.56
	High	150.0	156.39	104.26	6.24	3.99
Sample 2	Low	50.0	48.91	97.82	1.26	2.58
	Medium	100.0	101.72	101.72	2.70	2.66
	High	150.0	153.93	102.62	2.41	1.57

Table 4. Results of analysis of the samples

	Caffeine		Nicotinamide		Pyridoxine	
	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
Analyte found ($\mu\text{g/mL}$)	249.50	252.80	106.53	98.20	33.59	30.29
SD (%)	5.08	2.64	3.52	3.65	0.98	2.06
RSD (%)	2.04	1.04	3.31	3.71	2.92	6.80

was 252.80 $\mu\text{g/mL}$ (RSD = 1.04%). Percent RSD obtained on two samples of caffeine was accepted (RSD < 5.7%) according to Horwitz [22]. Nicotinamide concentration in the sample 1 was 106.53 $\mu\text{g/mL}$ (RSD = 3.31%), while the sample 2 was 98.20 $\mu\text{g/mL}$ (RSD = 3.71%). Percent RSD obtained on two samples of caffeine was accepted (RSD < 5.7%) according to Horwitz [22]. Pyridoxine concentration in the sample 1 was 33.59 $\mu\text{g/mL}$ (RSD = 2.92%), while sample 2 was 30.29 $\mu\text{g/mL}$ (RSD = 6.80%). Percent RSD obtained on two samples of caffeine was accepted according to Horwitz (RSD < 5.7% for caffeine and nicotinamide; RSD < 11.3% for pyridoxine).

Robustness test

In this study, the robustness test involves of the usage of re-used mobile phase, the wavelength shift, the shortening of the elution distance, and the variation of the proportion of the mobile phase components.

The effect of using re-use mobile phase. The effect of using re-use mobile phase could be tested by comparing analyte concentration eluted by freshly prepared and re-use mobile phase (Table 5). In the determination of caffeine and nicotinamide using re-use mobile there was no statistical difference between freshly prepared and re-use mobile phase elution at the sample 2 but the difference could be found in sample 1. However the determination of pyridoxine has a statistical difference between freshly prepared and re-use mobile phase elution at the sample 1 and sample 2. Statistical tests performed with T test (two-tailed) using 95% confidence level.

The effect of shifting detection wavelength. Wavelength detection shift test of caffeine was performed at three wavelengths: 272, 274, and 276 nm. The results obtained indicates that in sample 1 there was no differences in response of three wavelengths ($F_{\text{calc}} < 3.60$), while in the sample 2 response differences were found at wavelengths 274

Table 5. Results of robustness test of using re-use mobile phase (n=6)

	Sample 1		Sample 2	
	Re-use mobile phase	Freshly prepared mobile phase	Re-use mobile phase	Freshly prepared mobile phase
Caffeine found ($\mu\text{g/mL}$)	319.79	365.94	352.55	353.46
T_{calc}	3.41		0.33	
T_{table}	2.57		2.57	
Statistical difference	Yes		No	
Nicotinamide found ($\mu\text{g/mL}$)	192.80	204.80	192.78	196.18
T_{calc}	2.66		1.49	
T_{table}	2.57		2.57	
Statistical difference	Yes		No	
Pyridoxine found ($\mu\text{g/mL}$)	180.10	138.72	161.09	132.01
T_{calc}	13.87		10.32	
T_{table}	2.57		2.57	
Statistical difference	Yes		Yes	

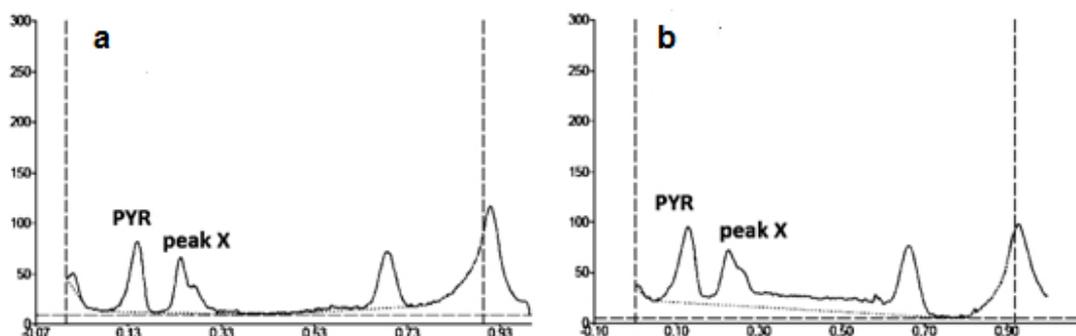


Fig 3. The effect of shortening the elution distance on the pyridoxine selectivity (a) Elution distance 75 mm (Resolution 1.29), (b) Elution distance 50 mm (Resolution 0.95). Chromatographic conditions: mobile phase, methanol-ethyl acetate-ammonia 25% 13:77:10 (v/v/v); PYR: pyridoxine

and 276 nm. Wavelength detection shift test of nicotinamide was performed at three wavelengths: 261, 263, and 265 nm. The results obtained indicates that not only the sample 1 but also sample 2 have differences in response at the three wavelengths ($F_{\text{calc}} > 3.60$). Wavelength detection shift test of pyridoxine was performed at three wavelengths: 291, 263, and 295 nm. The results obtained indicates that in the sample 1 and sample 2 there were differences in response at wavelengths of 291 and 295 nm compared with the maximum wavelength (293 nm). Statistical test was performed by ANOVA test (two-tailed) using confidence level of 95% followed by Post-Hoc test using Least Significant Difference (LSD) method.

The effect of shortening the elution distance. The effect of shortening the elution distance test was performed by changing elution distance from 75 mm to 50 mm and then two densitogram profiles were compared. The resolutions as well as the selectivity

parameter were calculated. Through this comparison, it can be observed that the shortening of the distance elution still provide a good selectivity in the separation of caffeine and nicotinamide. The unknown peak (peak X) produced by the sample matrix near the peak of pyridoxine has resulted in unsatisfying resolution of pyridoxine at 50 mm elution distance (Fig. 3).

The effect of varying the proportion of the mobile phase components. Mobile phase used in this study was a mixture of methanol : ethyl acetate : ammonia 25% (13:77:10), while the test was performed by making two variations of the mobile phase proportion component which are a mixture of methanol: ethyl acetate: ammonia 25% (17:77:6) and a mixture of methanol: ethyl acetate: ammonia 25% (23:67:10). The comparison showed that the composition of the mobile phase consisting of a mixture of methanol : ethyl acetate : ammonia 25% (13:77:10) was the best mobile phase in the determination of analytes in the sample.

CONCLUSION

Optimization of the TLC-densitometry method for caffeine, nicotinamide, and pyridoxine resulted in a good separation with the usage of stationary phase TLC plates silica gel 60 F₂₅₄ and a mobile phase of methanol : ethyl acetate : ammonia 25% (13:77:10). Validation data showed that the selectivity, accuracy, precision, linearity, limit of detection, and limit of quantitation of the assay can be applied to quantitatively determine caffeine, nicotinamide, and pyridoxine in the sample of energy drinks. Optimized and validated method of TLC-densitometry can be applied to determine caffeine, nicotinamide, and pyridoxine in samples of energy drinks.

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