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ORIGINAL

Determination of 13 Vitamin B and the Related Compounds Using HPLC with UV Detection and Application to Food Supplements

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Abstract

A high-performance liquid chromatographic method was developed for the separation and determination of 13 vitamin B compounds, including thiamin, riboflavin, riboflavin 5′-monophosphate, pyridoxine, hydroxocobalamin, cyanocobalamin, adenosylcobalamin, methylcobalamin, niacin, niacinamide, pantothenic acid, folic acid, and biotin. The method consisted of an ODS column, a gradient elution using pH 3.0 phosphate buffer–acetonitrile, ion-pairing reagent as mobile phase at 1.0 mL min^{-1}, and UV detection at 210 nm. As a result, the 13 vitamin B compounds were separated in 60 min, and the specificity, linearity, range, limit of detection, limit of quantitation, intra- and inter-day precision, and recovery were satisfactory. The limit of detections ranged from 0.03 to 0.41 μg mL^{-1}. Intra- and inter-day precision of peak area, expressed as RSD, were 0.542–5.144% and 0.216–6.367%, respectively. The method was used to evaluate vitamin B compounds in energy drinks and multivitamin pills. In case of energy drink analysis, the method identified and evaluated not only vitamin B compounds but also major ingredients, such as caffeine, vanillin, and benzoic acid. The vitamin B compounds in energy drinks and multivitamin pills were also quantitated and compared with values listed on each product label. Each quantitative value was close to the label values, suggesting high quantitative ability of the developed method.

Keywords Vitamin B · HPLC–UV · Gradient · Ion pairing · Energy drinks · Multivitamins

Introduction

Vitamin B is a coenzyme that catalyzes the conversion of carbohydrate to energy and biosynthesis of DNA and fatty acids in an organism [1–6]. Lack of vitamin B intake can cause a deficiency disease; therefore, intake of vitamin B is essential for health maintenance. The vitamin B complex is water soluble and comprises eight vitamins, which include thiamin (B_1), riboflavin (B_2), pyridoxine (B_6), cobalamin (B_{12}), niacin (B_3, PP, and nicotinic acid), pantothenic acid (B_5), folic acid (B_9), and biotin (B_7). The vitamin B complex is present as various structures in organisms and foods [7]; thus, it suggests that the group has a wide variety of compounds.

Presently, the official analytical methods used for the estimation of vitamin B include microbiological, high-performance liquid chromatography (HPLC), and spectrophotometric methods [8, 9]. The microbiological method that is mainly used to quantitate vitamin B is time consuming and has a poor reproducibility in terms of treating microorganisms. Furthermore, since the method treats different microbial strains depending on the vitamin B to be analyzed, they can be analyzed only individually. Thus, this method has a poor specificity. The spectrophotometric method also has poor specificity for analysis of limited vitamins B, such as thiamin and riboflavin. The HPLC method can quantitate the vitamin B complex with high specificity and sensitivity. Some HPLC-based methods for the simultaneous estimation of vitamins in the vitamin B complex have been reported [10–18]. However, to the best of the authors knowledge, there is no chromatographic method that can determine all eight vitamin B complex containing four vitamin B_{12}-related substances in dietary supplements, food, and biological samples with single application using a UV detector. The four vitamin B_{12}-related substances are cyanocobalamin, adenosylcobalamin, methylcobalamin, and hydroxocobalamin. Analysis of the cobalamin-related substances indicated only cyanocobalamin as the substance artificially added to

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food and dietary supplements; however, there is no method for the simultaneous analysis of all the eight vitamins in the vitamin B complex, including adenosylcobalamin and methylcobalamin that play an important role in metabolism and hydroxocobalamin that is a natural form of vitamin B_{12}. Furthermore, the vitamin B_2-related substances contain mainly riboflavin and riboflavin 5′-monophosphate. They are contained not only in organisms but also in dietary supplements containing vitamin B_2; thus, selective analysis of these is necessary.

From the above, it is important that vitamin B complex contained in the various structures in organisms and food can be easily analyzed. This study is aimed to develop an analytical method for the simultaneous detection of 13 vitamin B compounds, including thiamin: B_1, riboflavin and riboflavin 5′-monophosphate (RMP): B_2, pyridoxine: B_6, hydroxocobalamin, cyanocobalamin, adenosylcobalamin and methylcobalamin: B_{12}, niacin and niacinamide: B_3, pantothenic acid: B_5, folic acid: B_9, and biotin: B_7, in dietary supplements and biological samples using simple equipment and a UV detector. The developed method was used for the analysis of vitamin B compounds in commercially available supplements.

Materials and Methods

Materials and Instruments

Thiamin hydrochloride, riboflavin, pyridoxine hydrochloride, cyanocobalamin, sodium (+)-pantothenic acid, and folic acid were purchased from FUJIFILM Wako Pure Chemical (Tokyo, Japan); hydroxocobalamin, adenosylcobalamin, and methylcobalamin from Sigma-Aldrich (Tokyo, Japan); riboflavin 5′-monophosphate sodium salt (RMP) from TCI (Tokyo, Japan); niacin from AccuStandard (New Haven, CT, USA); niacinamide from ChromaDex (LA, CA, USA), and biotin from TRC (Toronto, Canada).

Acetonitrile (HPLC grade), phosphoric acid (special grade), and 1-hexanesulfonic acid sodium salt were purchased from FUJIFILM Wako Pure Chemical (Tokyo, Japan). Triethylamine (special grade) was purchased from TCI, PTFE membrane filter (0.45 μm) from Merck Millipore (Burlington, MA, USA), Sep-Pak Plus C18 Short Cartridge (360 mg, 55–105 μm particle size) from Waters (Milford, MA, USA), filter paper (No.2, 110 mm) from ADVANTEC (Tokyo, Japan), and Ultrasonic bath from ASONE (Osaka, Japan).

Deionized water was obtained from TAKASUGI SEIYAKU (Fukuoka, Japan) and filtered by Millipore system. Energy drinks and multivitamin pills were purchased from local supermarkets and pharmacies.

Preparation of Standard Solutions

Stock solutions of thiamin, RMP, pyridoxine, hydroxocobalamin, cyanocobalamin, adenosylcobalamin, methylcobalamin, niacin, niacinamide, and pantothenic acid were prepared by dissolving 10 mg of the respective compound in 10 mL of deionized water (1 mg mL^{-1}), and stock solutions of riboflavin, folic acid, and biotin were prepared by dissolving 10 mg of the respective compound in 10 mL of 0.1 mol L^{-1} NaOH (1 mg mL^{-1}).

Sample Preparation and Quantification

For energy drinks, 0.25 mL of sample was placed in a plastic tube with 0.75 mL of deionized water. For multivitamin pills, 25 mg of sample was weighed into a 25-mL volumetric flask and mixed with deionized water. The mixture was sonicated using supersonic waves for 2 min, and the volume was made up to 25 mL with deionized water. The solution was subsequently transferred into 50-mL plastic centrifuge tubes and centrifuged at 3000 rpm for 5 min. The energy drink or the supernatant of multivitamin pill was filtered through a 0.45-μm nylon membrane filter after vortexing, and 20 μL of the filtrate was analyzed using HPLC.

The samples were concentrated to quantitate biotin and cobalamins, modifying the method that Morii et al. performed [19]. For energy drinks, 200 mL of sample was passed through a Sep-Pak Plus C18 Short Cartridge connected to a 0.45-μm nylon membrane filter, which had been prewashed with 10 mL of acetonitrile and deionized water. The sample on the cartridge was washed with 10 mL of deionized water and 3 mL of 10% acetonitrile solution, and the analytes ware eluted with 2 mL of 30% acetonitrile solution. 20 μL of the eluted solution was analyzed using HPLC. For multivitamin pills, 1.25 g of sample was weighed into a 50-mL volumetric flask and mixed with deionized water. The mixture was sonicated with ultrasonic bath for 2 min, and the volume was made up to 50 mL with deionized water. The solution was subsequently transferred into 50-mL plastic centrifuge tubes and centrifuged at 3000 rpm for 5 min. The supernatant was filtered through a filter paper, and the subsequent procedures were the same as those used for energy drinks.

HPLC System

Vitamin B mixtures were analyzed using an HPLC system that included a PU-1580 pump, DG-980-50 degasser, CO-965 column oven (40 °C), UV-970 UV–VIS detector (wavelength, 210 nm) (JASCO Corporation, Tokyo, Japan), DMC675 Mixer (GLscience, Tokyo, Japan), and a 7725i

Rheodyne sampling injector connected to a 20-µL sample loop (Rheodyne, Rohnert Park, CA, USA). This system was equipped with a CAPCELL PAK C18 SG120 column (250 nm × 4.6 mm, 5-µm particle size, OSAKA SODA, Tokyo, Japan). The mobile phase was consisted of solutions A (5 mmol L^{-1} sodium hexanesulfonic acid, 20 mmol L^{-1} phosphoric acid, 16 mmol L^{-1} triethylamine; pH 3.0) and B [mobile phase A: acetonitrile, 75:25 (v/v)]. The gradient employed was 0–5 min, isocratic at 20% B; 5–50 min, 20% B to 80% B; and 50–60 min, isocratic at 80% B. The flow rate was 1.0 mL min^{-1}. HPLC was conducted at room temperature. The HPLC system was also connected to a diode array detector (DAD) (5430 DAD: Hitachi High-Tech Science Corporation, Tokyo, Japan) to identify 13 vitamin B compounds and other ingredients in samples. Identification of 13 vitamin B compounds and other ingredients was accomplished on the basis of the retention times of the analytes and by comparison between the UV–VIS absorption spectra (scan range: 200–700 nm) of the reference compounds and those of the detected peaks in the samples.

Method Validation

Validation of the HPLC method included specificity, linearity, range, limit of detection (LOD), limit of quantitation (LOQ), and intra- and inter-day precision. For the specificity, the analytical solution was prepared by mixing 100 µL of each stock solutions in 10 mL of mobile phase A. The retention time (tR), peak area, retention factor (k), resolution factor (Rs), separation factor (α), and tailing factor (TF) were estimated using the results of these replicate analyses. The parameters were defined using the standard formulae: $k = (tR_n - tR_0)/tR_0$, $Rs = 2(tR_{n+1} - tR_n)/(W_{n+1} + W_n)$, $\alpha = k_{n+1}/k_n$, and $TF = (W_A + W_B)/2W_A$, where tR_n is the retention time of each peak, tR_0 is the retention time of the first solvent peak ($tR_0 = 2.54$ min), W_n is the width of the peak at baseline, W_A is the front half width of the peak measured at 5% of peak height, and W_B is the back half width of the peak measured at 5% of peak height. The subscript "n" refers to the order of vitamin B compounds in the elution. For the intra- and inter-day precision tests, two concentrations of analytical solution were prepared by mixing 100 and 10 µL of each stock solutions in 10 mL of mobile phase A, respectively (10 µg mL^{-1} and 1 µg mL^{-1} of each vitamin B compound). The intra-day precision test was performed five times in a day, the inter-day precision test was for 3 days, and the precision was evaluated by calculating the relative standard deviation (RSD). For testing the linearity, range, LOD, and LOQ, the analytical solutions were prepared by mixing the stock solutions in the appropriate proportions and diluting them with mobile phase A. Calibration curves were created by plotting peak areas against six corresponding concentrations (µg mL^{-1}) of each vitamin B compound. The linearity, range, LOD, and LOQ were estimated using the results of these analyses. LOD was calculated as the concentration at which the peaks of vitamin B compounds could be detected without any baseline noise disturbances (> 3 times the baseline noise). LOQ was calculated as the concentration at which the analyte responses were > 10 times the baseline noise.

Recovery Assay

Recoveries were determined according to the standard addition procedure at three different concentrations. Standard solution for recovery tests of energy drinks was prepared by mixing 1 mL of stock solutions of thiamin, RMP, pantothenic acid, and biotin and 100 µL stock solutions of other vitamin B compounds in 10 mL of deionized water. Standard solution for recovery tests of multivitamin pills was prepared by mixing 0.5, 1.0, or 1.5 mL of each stock solution in 25 mL of deionized water.

For energy drinks, 0.25, 0.5, or 0.75 mL of the standard solution was transferred to a plastic tube with 0.25 mL of energy drink and 0.5, 0.25, or 0 mL of deionized water, respectively. For multivitamin pills, 25 mg of multivitamin pill was weighed in a 25-mL flask with 0.5, 1.0, or 1.5 mL of each stock solution and deionized water and centrifuged at 3000 rpm for 5 min. The energy drink or the supernatant of multivitamin pill was filtered through a 0.45-µm nylon membrane filter after vortexing, and 20 µL of filtrate was analyzed. All other sample preparation was carried out as described previously in "Sample Preparation and Quantification" section.

Recovery was evaluated using standard formulae; Recovery (%) = $100(A - B)/C$, where A is the peak area of sample spiked with standard, B is the peak area of sample alone (not spiked), and C is the peak area of standard alone (same concentration used to spike the sample).

Result and Discussion

Method Validation

The chromatogram of mixed vitamin B compounds is shown in Fig. 1 and the spectra of each vitamin B compound are shown in Fig. 2. To investigate the optimal separation of mixed vitamin B compounds by UV-HPLC analysis, the optimal column, pH of eluent, and isocratic or gradient elution program were determined. After examining the resolution of vitamin B compounds by the isocratic elution program with reference to various column catalogs, CAPCELL PAK C18 SG120 column was selected. However, when the mixed vitamin B compounds were analyzed by the method in the catalog of the column [20], it was difficult to separate

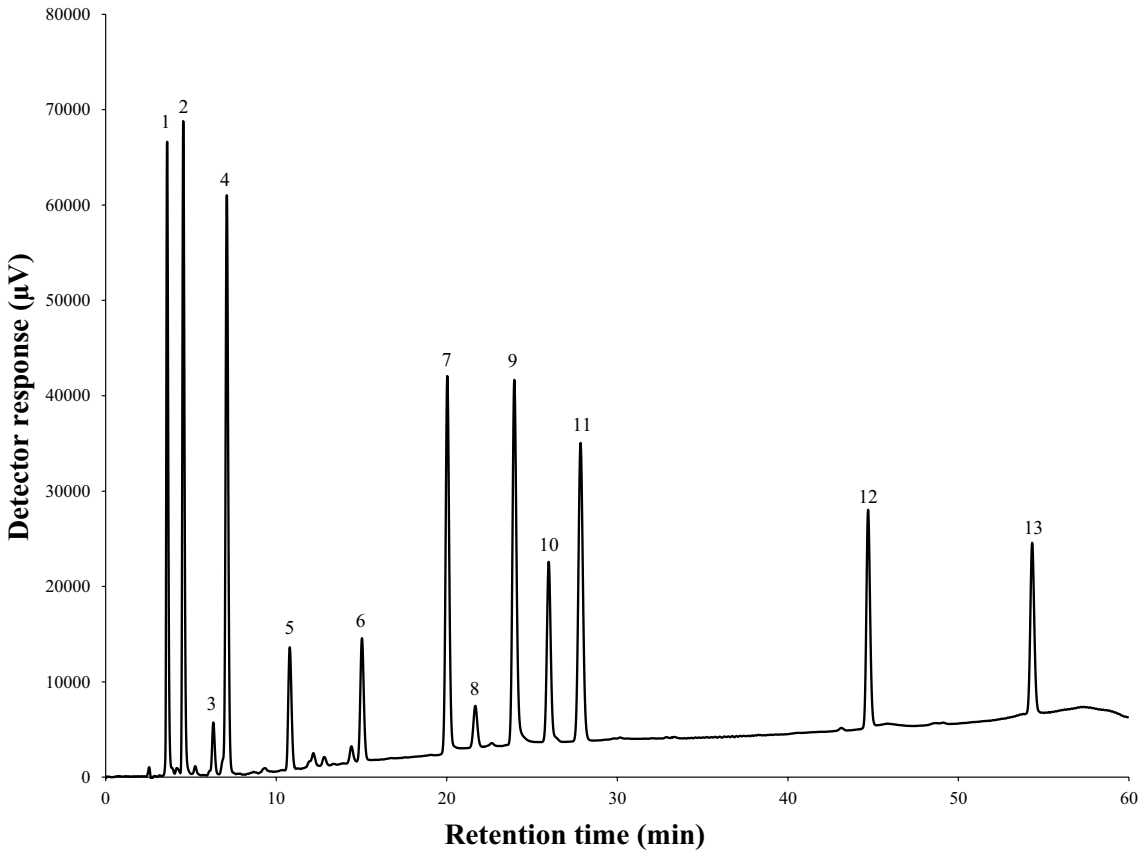

Fig. 1 HPLC chromatogram of 13 vitamin B compounds monitored at 210 nm. The number on the chromatogram represents the following compounds: (1) Niacin; (2) Niacinamide; (3) Pantothenic acid; (4) Pyridoxine; (5) Thiamin; (6) RMP; (7) Folic acid; (8) Biotin; (9) Hydroxocobalamin; (10) Riboflavin; (11) Cyanocobalamin; (12) Adenosylcobalamin; and (13) Methylcobalamin

adenosylcobalamin and methylcobalamin from hydroxocobalamin. To improve the separation of these compounds, the pH of mobile phase was adjusted using triethylamine and the gradient elution program was applied. The resulting appropriate pH of mobile phase was 3.0; the gradient elution program has been explained in "Materials and Methods" section.

To confirm the specificity of the method developed here, the tR, k, RS, α, and TF were investigated; the results are presented in Table 1. All the RSs between each vitamin B compounds were > 1.5 and all the TFs of each vitamin B compound were around 1.1. The separation of each vitamin B compound exhibited good specificity. The linearity, range, LOD, and LOQ were investigated, and the results are shown in Table 2. The correlation factor for each vitamin B compound was > 0.9914, suggesting that the developed method exhibits good linearity. The linear range varied depending on the individual vitamin B compound. The LODs and LOQs for the various vitamin B compounds were found to be in the range of 0.03–0.41 μg mL^{-1} and 0.09–1.38 μg mL^{-1}, respectively. To confirm the intra- and inter-day precision of the developed method, the tR and peak area were investigated; the results are displayed in Table 3. In the test using the 10 μg mL^{-1} of each vitamin B compound mixed solution, the RSDs of the intra- and inter-day on tRs for all vitamin B compounds were < 0.911% and 0.425%, respectively. The RSDs of the intra-day peak areas for all vitamin B compounds were 0.542–2.302%. The RSDs of the inter-day peak areas for all vitamin B compounds were 0.216–3.674%. In the test using the 1 μg mL^{-1} of each vitamin B compound mixed solution, the RSDs of the intra- and inter-day on tRs for all vitamin B compounds were < 0.097% and 0.304%, respectively. The RSDs of the intra-day peak areas for all vitamin B compounds were 1.043–5.144%. The RSDs of the inter-day peak areas for all vitamin B compounds were 0.814–6.367%. These results suggest that the developed method exhibits high reproducibility.

Contents of Vitamin B Compounds in Energy Drinks and Multivitamin Pills

The vitamin B contents of energy drinks and multivitamin pills were investigated using the method developed in this paper. Typical chromatograms of energy drinks and

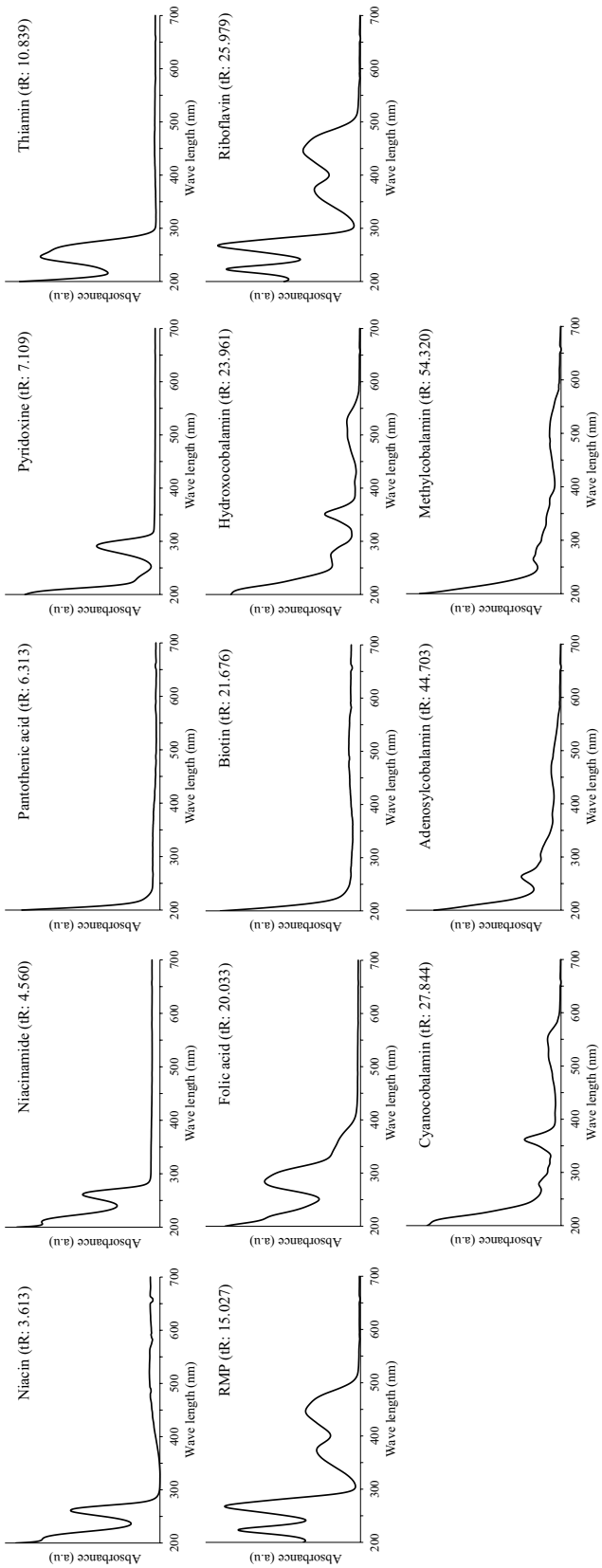

Fig. 2 Retention time (tR) and UV–vis absorbance spectrum obtained by a HPLC–DAD of 13 vitamin B compounds

Table 1 Results from the evaluation of the specificity of the LC method

Compound	tR (min)	*k*	RS	*α*	TF
Niacin	3.613	0.417	–	–	1.213
Niacinamide	4.560	0.788	4.368	1.891	1.153
Pantothenic acid	6.313	1.476	6.812	1.872	1.165
Pyridoxine	7.109	1.788	2.795	1.211	1.111
Thiamin	10.839	3.251	11.424	1.818	1.134
RMP	15.027	4.893	11.207	1.505	1.118
Folic acid	20.033	6.856	13.143	1.401	1.063
Biotin	21.676	7.500	4.045	1.094	1.167
Hydroxocobalamin	23.961	8.396	5.332	1.119	1.108
Riboflavin	25.979	9.188	4.802	1.094	1.065
Cyanocobalamin	27.844	9.919	4.145	1.080	1.083
Adenosylcobalamin	44.703	16.531	37.938	1.667	1.109
Methylcobalamin	54.320	20.302	23.200	1.228	1.102

Chromatographic parameters of 13 vitamin B compounds with UV–VIS HPLC (detection, wavelength 210 nm), mixed 13 vitamin B compounds (each 10 μg mL^{-1}) per 20 μL injection ($n=5$)

tR retention time, *k* retention factor, *Rs* resolution factor, *α* separation factor, *TF* tailing factor

multivitamin pills are shown in Figs. 3 and 4, respectively. Identification of vitamin B compounds in samples was performed by comparing the retention times and spectra of their standard (Fig. 2). To quantitate cobalamins in energy drink 3 and biotin and cobalamins in multivitamin pills 1 and 2, the samples were concentrated 100 times and 10 times, respectively. These chromatograms of concentrated samples are shown in Fig. 5. These chromatograms indicate that the method could be used to analyze many kinds of drinks and pills. The mean recovery for each of the vitamin B compounds was investigated; the results are shown in Tables 4 and 5. The mean recoveries for all the vitamin B compounds for energy drink 1 were 80.2–112%, energy drink 2 were 66.8–121%, energy drink 3 were 90.3–114%, multivitamin pill 1 were 70.2–106%, and for multivitamin pill 2 were 77.5–106%, indicating that the developed method was sufficiently quantitative. The analysis values of vitamin B compounds obtained from five samples, and the values described on product labels are shown in Table 6, where "niacin" indicates the sum of niacin and niacinamide contents, "riboflavin" indicates the sum of RMP and riboflavin contents, and "cobalamin" indicates the sum of hydroxo-, cyano-, adenosyl-, and methylcobalamin contents. The energy drink 1 contained 228.65 ± 0.69 μg mL^{-1} of niacin, 58.07 ± 0.58 μg mL^{-1} of pyridoxine, 61.51 ± 0.33 μg mL^{-1} of thiamin, and 59.71 ± 0.18 μg mL^{-1} of riboflavin, and no pantothenic acid, folic acid, biotin, and cobalamin were detected. The energy drink 2 contained 573.15 ± 0.94 μg mL^{-1} of niacin, 213.66 ± 0.36 μg mL^{-1} of pyridoxine, and 46.26 ± 0.16 μg mL^{-1} of riboflavin, and no pantothenic acid, thiamin, folic acid, biotin, and cobalamin were detected. The energy drink 3 contained 41.29 ± 0.70 μg mL^{-1} of niacin, 42.47 ± 0.71 μg mL^{-1} of pantothenic acid, 29.56 ± 0.34 μg mL^{-1} of pyridoxine, 1.09 ± 0.01 μg mL^{-1} of riboflavin, and 0.03 ± 0.01 μg mL^{-1} of cobalamin, and no thiamin, folic acid, and biotin were detected. The multivitamin pill 1 contained 53.36 ± 0.53 mg g^{-1} of niacin, 28.04 ± 0.37 mg g^{-1} of pantothenic acid, 57.98 ± 0.66 mg g^{-1} of pyridoxine, 130.06 ± 1.52 mg g^{-1} of thiamin, 55.00 ± 0.60 mg g^{-1} of riboflavin, 0.52 ± 0.02 mg g^{-1} of folic acid, 0.09 ± 0.02 mg g^{-1} of biotin, and 0.07 ± 0.03 mg g^{-1}

Table 2 Results from the evaluation of the linearity and range of the LC method

Compound	Regression equation	Correlation factor	Linear range (μg mL^{-1})	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Niacin	$y^a = 5.19 \times 10^4 x^b + 3.51 \times 10^4$	0.9997	0.10–100	0.03	0.10
Niacinamide	$y = 5.99 \times 10^4 x + 3.88 \times 10^4$	0.9997	0.09–100	0.03	0.09
Pantothenic acid	$y = 5.51 \times 10^3 x + 1.17 \times 10^5$	0.9990	1.20–1000	0.36	1.20
Pyridoxine	$y = 6.29 \times 10^4 x + 8.43 \times 10^4$	0.9993	0.11–100	0.03	0.11
Thiamin	$y = 2.04 \times 10^4 x - 2.21 \times 10^4$	0.9998	0.98–1000	0.29	0.98
RMP	$y = 2.12 \times 10^4 x + 8.00 \times 10^5$	0.9914	0.39–600	0.12	0.39
Folic acid	$y = 5.93 \times 10^4 x + 3.15 \times 10^4$	0.9998	0.15–100	0.05	0.15
Biotin	$y = 7.83 \times 10^3 x - 1.81 \times 10^4$	0.9994	1.38–600	0.41	1.38
Hydroxocobalamin	$y = 5.53 \times 10^4 x + 1.33 \times 10^5$	0.9988	0.18–100	0.05	0.18
Riboflavin	$y = 4.05 \times 10^4 x - 7.94 \times 10^4$	0.9985	0.26–100	0.08	0.26
Cyanocobalamin	$y = 5.77 \times 10^4 x + 1.84 \times 10^4$	0.9998	0.21–100	0.06	0.21
Adenosylcobalamin	$y = 3.91 \times 10^4 x - 1.73 \times 10^4$	0.9998	0.25–100	0.08	0.25
Methylcobalamin	$y = 4.06 \times 10^4 x - 9.36 \times 10^4$	0.9991	0.31–100	0.09	0.31

[a] y = mass concentration (μg mL^{-1})

[b] x = HPLC peak area

Table 3 Results from the evaluation of the intra- and inter-day precision of the LC method

Compound	10 μg mL^{-1} of each vitamin B compound				1 μg mL^{-1} of each vitamin B compound			
	tR-RSD (%)		AREA-RSD (%)		tR-RSD (%)		AREA-RSD (%)	
	Intra-day[a]	Inter-day[b]	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Niacin	0.057	0.074	1.030	0.688	0.097	0.171	2.072	4.913
Niacinamide	0.254	0.425	0.582	1.403	0.026	0.148	2.263	4.634
Pantothenic acid	0.536	0.250	0.816	0.714	0.030	0.150	5.144	0.814
Pyridoxine	0.636	0.113	0.690	0.755	0.053	0.183	1.718	3.764
Thiamin	0.911	0.077	0.980	0.216	0.075	0.304	1.043	4.759
RMP	0.674	0.346	0.742	0.923	0.058	0.129	1.826	3.694
Folic acid	0.609	0.103	2.302	3.674	0.048	0.086	4.694	5.961
Biotin	0.460	0.232	0.859	0.978	0.027	0.074	1.751	4.206
Hydroxocobalamin	0.611	0.205	1.789	0.985	0.026	0.063	3.953	4.385
Riboflavin	0.598	0.248	0.542	0.781	0.024	0.066	1.881	4.108
Cyanocobalamin	0.553	0.039	0.561	0.759	0.021	0.072	2.147	4.854
Adenosylcobalamin	0.047	0.081	0.822	1.044	0.017	0.081	2.391	6.367
Methylcobalamin	0.278	0.058	1.928	1.640	0.015	0.078	2.946	4.596

tR retention time, *AREA* peak area

[a]Intra-day at five times in 1 day ($n=5$)

[b]Inter-day on 3 different days ($n=3$)

of cobalamin. The multivitamin pill 2 contained 87.67 ± 1.18 mg g^{-1} of niacin, 124.79 ± 1.71 mg g^{-1} of pantothenic acid, 76.74 ± 1.15 mg g^{-1} of pyridoxine, 117.43 ± 1.78 mg g^{-1} of thiamin, 50.58 ± 0.78 mg g^{-1} of riboflavin, 0.28 ± 0.01 mg g^{-1} of folic acid, 0.08 ± 0.01 mg g^{-1} of biotin, and 0.10 ± 0.01 mg g^{-1} of cobalamin. The results obtained by the presently developed method and the label values were generally comparable in energy drinks and multivitamin pills.

In the analysis of energy drinks, some ingredients other than vitamin B compounds were detected (Fig. 3). As the ingredients were labeled on the sample product, their standards were also analyzed. As a result, the ingredients in the energy drinks were identified as caffeine (tR; 16.664 min), vanillin (tR; 22.693 min), and benzoic acid (tR; 31.195 min), and their spectra are also shown in Fig. 3. These ingredients did not interfere with the determination of the vitamin B compounds. Therefore, the method could not only analyze vitamin B compounds but also other main ingredients in energy drinks.

Conclusion

A simple HPLC–UV method was developed that successfully and simultaneously analyzed 13 vitamin B compounds (eight vitamin B complex) with high sensitivity. This sample was analyzed within 60 min and showed sufficient reproducible and quantitative ability. Using this method, vitamin B compounds in energy drinks and multivitamin pills can be determined and quantitated simultaneously. Moreover, the method can identify and evaluate not only vitamin B compounds but also major ingredients, such as caffeine, vanillin, and benzoic acid.

This study is aimed to evaluate vitamin B compounds in biological samples that may contain other vitamin B compounds using this method in the future.

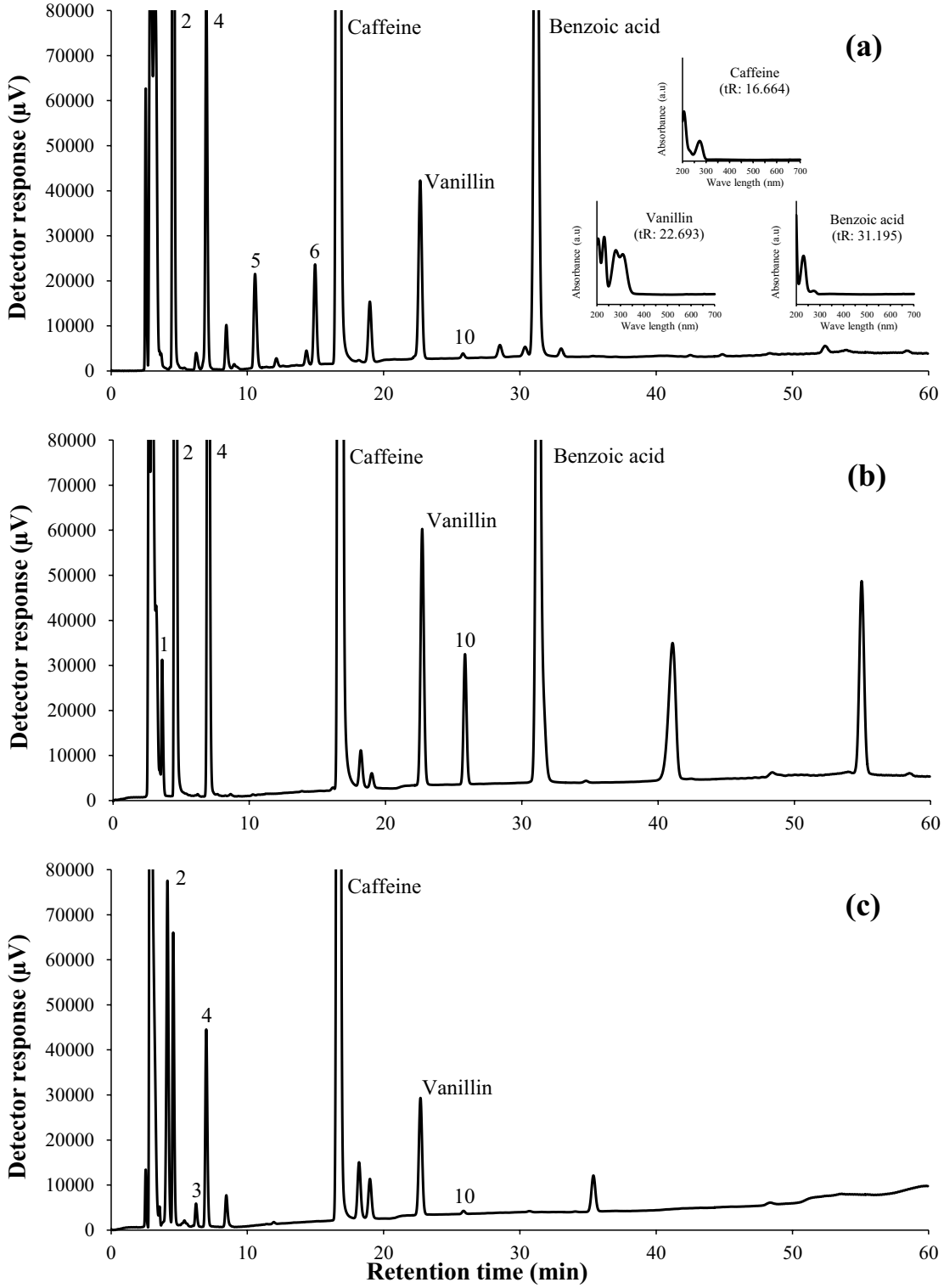

Fig. 3 HPLC chromatograms of vitamin B compounds in energy drinks monitored at 210 nm and absorbance spectrum of caffeine, vanillin, and benzoic acid. **a** Energy drink 1, **b** Energy drink 2, and **c** Energy drink 3. Energy drink 1 contains vitamin B compounds (2, Niacinamide; 4, Pyridoxine; 5, Thiamin; 6, RMP; and 10, Riboflavin) and other ingredients (caffeine, vanillin, and benzoic acid). Energy drink 2 contains vitamin B compounds (1, Niacin; 2, Niacinamide; 4, Pyridoxine; and 10, Riboflavin) and other ingredients (caffeine, vanillin, and benzoic acid). Energy drink 3 contains vitamin B compounds (2, Niacinamide; 3, Pantothenic acid; 4, Pyridoxine; and 10, Riboflavin) and other ingredients (caffeine and vanillin)

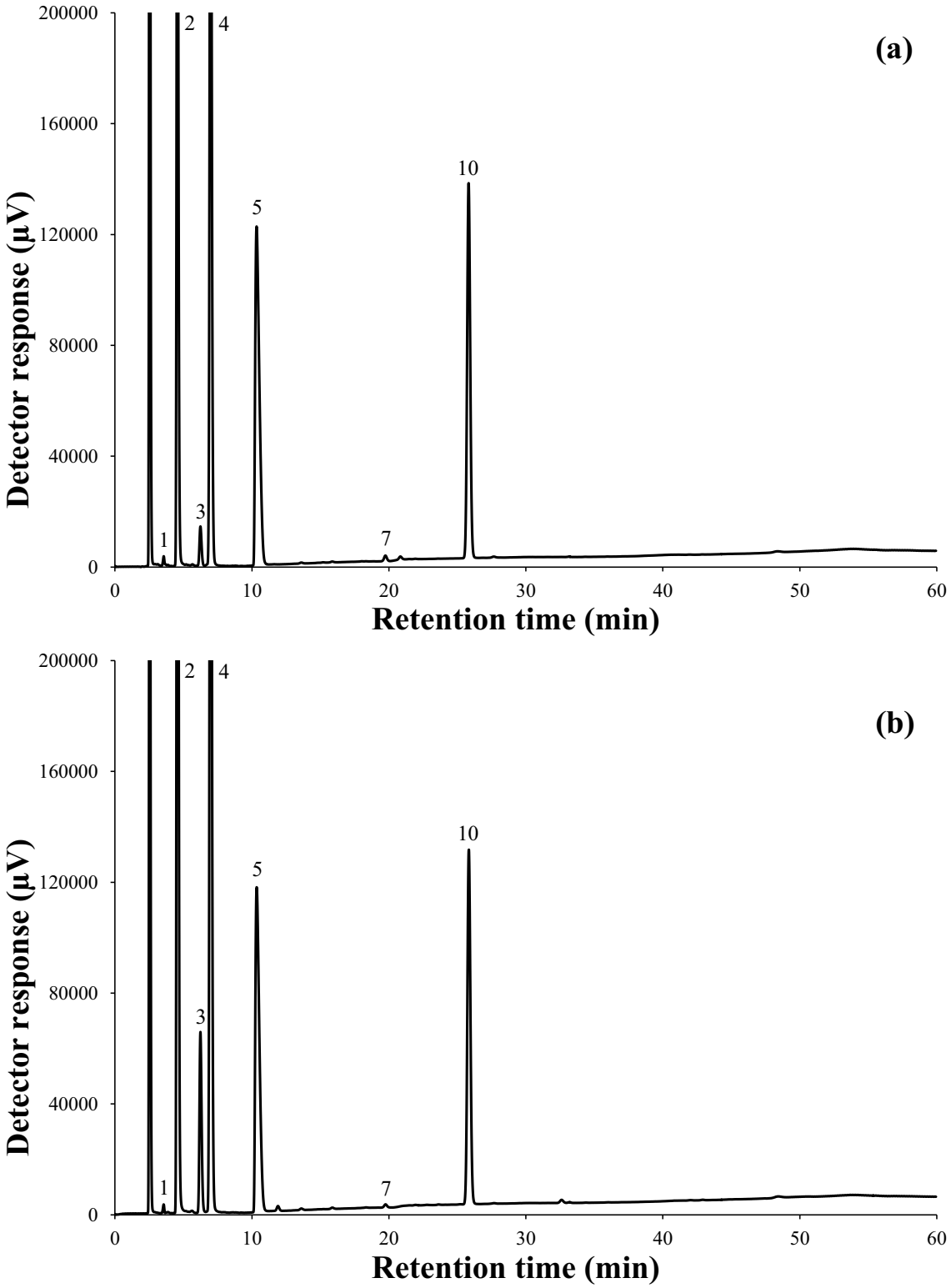

Fig. 4 HPLC chromatograms of vitamin B compounds in multivitamin pills monitored at 210 nm. **a** Multivitamin pill 1 and **b** multivitamin pill 2. The number on the chromatogram represents the following compounds: (1) Niacin; (2) Niacinamide; (3) Pantothenic acid; (4) Pyridoxine; (5) Thiamin; (7) Folic acid; (10) Riboflavin

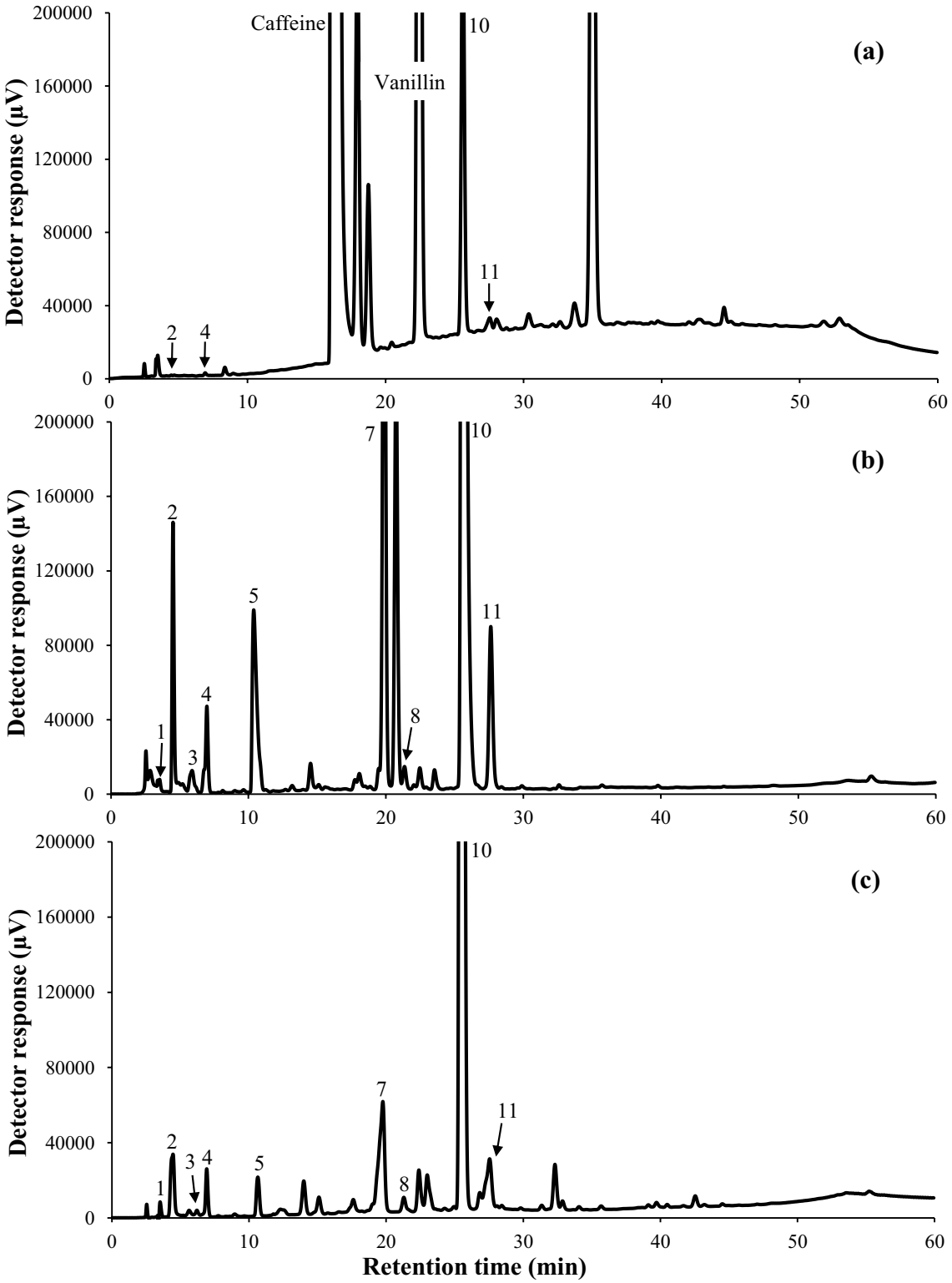

Fig. 5 HPLC chromatograms of vitamin B compounds concentrated 100 times in energy drink 3 and 10 times in multivitamin pills monitored at 210 nm. **a** Energy drink 3, **b** multivitamin pill 1, and **c** multivitamin pill 2. The number on the chromatogram represents the following compounds: (1) Niacin; (2) Niacinamide; (3) Pantothenic acid; (4) Pyridoxine; (5) Thiamin; (7) Folic acid; (8) Biotin; (10) Riboflavin; (11) Cyanocobalamin

Table 4 Results from the evaluation of the recovery in energy drinks of the LC method

Sample	Energy drink 1					Energy drink 2					Energy drink 3				
Spiked standard Niacin	Orig.[a] ND	0.75[b] 108	0.5[b] 107	0.25[b] 122	Ave.[c] 112	Orig 21.16	0.75 106	0.5 108	0.25 108	Ave 107	Orig 2.55	0.75 104	0.5 102	0.25 99.4	Ave 102
Niacinamide	228.65	99.4	96.2	96.7	97.5	551.99	55.6	66.6	78.2	66.8	38.74	100	100	95.0	98.5
Pantothenic acid	ND	96.5	96.7	96.3	96.5	ND	103	97.0	103	101	42.47	100	98.2	99.4	99.3
Pyridoxine	58.07	107	101	99.3	102	213.66	110	128	125	121	29.56	99.4	101	107	102
Thiamin	61.51	104	93.0	92.7	96.5	ND	97.3	94.2	93.3	95.0	ND	103	102	108	104
RMP	57.96	105	97.0	93.2	98.3	ND	100	98.4	100	99.5	ND	100	97.7	100	99.2
Folic acid	ND	100	85.8	82.9	89.4	ND	95.8	90.7	88.8	91.8	ND	95.2	88.4	87.2	90.3
Biotin	ND	96.7	92.0	96.5	95.1	ND	97.4	96.8	111	102	ND	100	97.5	99.3	98.9
Hydroxocobalamin	ND	82.6	90.0	128	100	ND	93.0	89.2	108	96.8	ND	97.9	112	83.2	97.6
Riboflavin	1.75	90.5	94.6	94.5	93.2	46.26	95.2	105	109	103	1.09	101	99.2	101	100
Cyanocobalamin	ND	98.4	93.9	94.2	95.5	ND	99.0	97.4	101	99.3	0.03	100	98.1	98.1	98.8
Adenosylcobalamin	ND	138	110	77.0	108	ND	98.7	97.8	81.1	92.5	ND	97.5	93.7	135	109
Methylcobalamin	ND	124	80.7	77.6	94.0	ND	91.1	107	85.7	94.6	ND	96.0	82.5	163	114

[a]The average levels of original analytes in each sample (μg mL^{-1}), *ND* not detected

[b]Spiked standard 0.75; this solution contained 0.75 mL of the standard solution (10 μg mL^{-1} of each vitamin B compound), 0.25 mL energy drink, and 0 mL deionized water, 0.5; this solution contained 0.5 mL of the standard solution, 0.25 mL energy drink, and 0.25 mL deionized water, 0.25; this solution contained 0.25 mL of the standard solution, 0.25 mL energy drink, and 0.5 mL deionized water. Each data; Recoveries of each vitamin B compound at three concentrations (%)

[c]The average recoveries for the analyses of three concentrations of spiked standard solutions (%)

Table 5 Results from the evaluation of the recovery in multivitamin pills of the LC method

Sample	Multi-vitamin pill 1					Multi-vitamin pill 2				
Spiked standard Niacin	Orig.[a] 0.68	60[b] 91.4[d]	40[b] 98.6[d]	20[b] 100[d]	Ave.[c] 96.7	Orig 0.43	60 103	40 99.6	20 97.0	Ave 99.7
Niacinamide	52.67	94.1	87.5	77.1	86.2	87.24	101	94.1	86.0	93.9
Pantothenic acid	28.04	104	95.7	112	104	124.79	104	86.2	78.5	89.7
Pyridoxine	57.98	94.3	90.0	87.6	90.6	76.74	93.3	93.1	84.3	90.2
Thiamin	130.06	77.4	73.2	59.8	70.2	117.43	80.5	73.8	78.1	77.5
RMP	ND	94.1	101	101	98.8	ND	100	99.9	97.5	99.1
Folic acid	0.52	101	101	102	101	0.28	102	104	99.9	102
Biotin	0.09	100	91.3	111	101	0.08	104	98.1	99.0	100
Hydroxocobalamin	ND	98.9	89.4	108	98.8	0.04	99.0	91.3	88.1	92.8
Riboflavin	55.00	108	97.2	114	106	50.58	124	119	70.9	105
Cyanocobalamin	0.07	102	97.1	102	100	0.06	107	108	105	106
Adenosylcobalamin	ND	91.2	108	94.1	97.7	ND	95.0	98.7	103	98.8
Methylcobalamin	ND	92.2	109	86.3	95.8	ND	93.6	96.9	104	98.0

[a]The average levels of original analytes in each sample (mg g^{-1}), *ND* not detected

[b]Spiked standard 60; this solution contained 1.5 mL of the each stock solution (1 mg mL^{-1} of the vitamin B compound), 25 mg multivitamin pill, and deionized water to fill up 25 mL (60 μg mL^{-1} of each vitamin B compound), 40; this solution contained 1.0 mL of the each stock solution, 25 mg multivitamin pill, and deionized water to fill up 25 mL (40 μg mL^{-1} of each vitamin B compound), 20; this solution contained 0.5 mL of the each stock solution, 25 mg multivitamin pill, and deionized water to fill up 25 mL (20 μg mL^{-1} of each vitamin B compound). Each data; Recoveries of each vitamin B compound at three concentrations (%)

[c]The average recoveries for the analyses of three concentrations of spiked standard solutions (%)

Table 6 Analytical results of the vitamin content in energy drinks and multivitamin pills

Sample	Vitamin found Drinks: (μg mL^{-1}) Pills: (mg g^{-1})							
	Niacin[a]	Pantothenic acid	Pyridoxine	Thiamin	Riboflavin[b]	Folic acid	Biotin	Cobalamin[c]
Energy drink 1	228.65 ± 0.69 (200)[d]	ND (nd)	58.07 ± 0.58 (50)	61.51 ± 0.33 (50)	59.71 ± 0.18 (50)	ND (nd)	ND (nd)	ND (nd)
Energy drink 2	573.15 ± 0.94 (500)	ND (nd)	213.66 ± 0.36 (200)	ND (nd)	46.26 ± 0.16 (40)	ND (nd)	ND (nd)	ND (nd)
Energy drink 3	41.29 ± 0.70 (30)	42.47 ± 0.71 (20)	29.56 ± 0.34 (20)	ND (nd)	1.09 ± 0.01 (0.9)	ND (nd)	ND (nd)	0.03 ± 0.01 (0.02)
Multivitamin pill 1	53.36 ± 0.53 (44)	28.04 ± 0.37 (22)	57.98 ± 0.66 (40)	130.06 ± 1.52 (100)	55.00 ± 0.60 (48)	0.52 ± 0.02 (0.8)	0.09 ± 0.02 (0.18)	0.07 ± 0.03 (0.08)
Multivitamin pill 2	87.67 ± 1.18 (100)	124.79 ± 1.71 (100)	76.74 ± 1.15 (75)	117.43 ± 1.78 (100)	50.58 ± 0.78 (75)	0.28 ± 0.01 (0.5)	0.08 ± 0.01 (0.125)	0.10 ± 0.01 (0.05)

Mean ± standard deviation ($n = 3$), *ND* not detected

[a]Sum of niacin and niacinamide contents

[b]Sum of RMP and riboflavin contents

[c]Sum of hydroxocobalamin, cyanocobalamin, adenosylcobalamin, and methylcobalamin contents

[d]Values in the brackets indicate the values in the labels of the products, *nd* not declared (not labeled)

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Compliance with Ethical Standards

Conflict of interest All the authors declared that they have no conflict of interest.

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