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A Rapid and Simple Gas Chromatographic Method for Direct Determination of Nicotinamide in Commercial Vitamins and Tonic Drinks

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ABSTRACT

A simple and rapid method was developed to determine the presence of nicotinamide in tonic drinks using the megapore semi-polar column (CP-Sil 8 CB, 30 m × 0.53 mm) with direct injection gas chromatography. Direct quantitative analysis of nicotinamide in amino acid drinks, vitamin drinks, essence of chicken and tonic drinks was carried out without any sample pretreatment procedure. Water soluble compound 1,9-nonanediol was used as an internal standard. The detection limit for nicotinamide was 2-5 μg/mL. Recovery studies were performed using 1 mL of vitamin drink and tonic drink, each spiked with nicotinamide at 105.5, and 211.0 μg, respectively. The recoveries were found in the range of 94~99% and 93-108%, respectively. The coefficients of variation being less than 9.8%. Forty-two commercial amino acid drinks, vitamin drinks, essences of chicken and tonic drinks were analyzed with this method. The nicotinamide contents were found as: 131-246, 24-112, 0-134 and 0-263 μg/mL, respectively. These results indicated that, one bottle each of the amino acid drink, vitamin drink, essences of chicken and tonic drink contain nicotinamide levels of : 39-74, 5-22, 0-10 and 0-21 mg/bottle. The results also showed that in 12 out of 4 of commercial amino acid and vitamin drinks, the nicotinamide exceeded the ROC's acceptable daily intake (RNDA, 14.4 mg) level; and 3 out of 10 commercial tonic drinks exceeded the ROC's RNDA level for nicotinamide.

Key words: **tonic drink, nicotinamide, direct injection, gas chromatography, quantitative analyses.**

INTRODUCTION

Nicotinic acid and nicotinamide are members of the vitamin B family. In living creatures, nicotinic acid can be converted to nicotinamide, which bonds with phosphoric acid, nucleosides and purines to form coenzymes, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP)[1]. Both NAD and NADP are involved in oxidation-reduction reac-

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tions and are distributed widely in animal tissues[2-3]. Free NAD and NADP are commonly used as color fixatives to develop and maintain fresh color in beef and fish products[4]. In addition, free NAD and NADP are usually added in tonic and vitamin drinks. However, an overdose of nicotinic acid due to consumption of processed meat products can cause hyperemia symptoms, such as flushed and itchy skin, headaches, peptic ulcers and liver damage[6]. Therefore, NAD and NADP were banned in meat products by the Japanese government in 1982[4]. Because of the recent popularity of tonic and vitamin drinks in Taiwan, it is important and necessary to establish rapid and precise analysis methods for nicotinamide and/or nicotinic acid in these drinks[5].

The current standard methods (AOAC methods) for total nicotinic acid and nicotinamide analysis are: (1) the microbiological turbidimetric method[2,7]; (2) the colorimetric method[2,8-11] and (3) the continuous-flow analysis method[2,8-9]. The disadvantages of the microbiological turbidimetric method include high cost, low accuracy, poor repeatability and time consuming, thus, this method is not appropriate for a rapid analysis or handling a large quantity of samples. Cyanogen bromide, a reagent used in colorimetric method, is unstable and especially toxic, and is difficult to analyze precisely. Likewise, the use of cyanogen bromide in the continuous-flow analysis method generates several concerns. For instance, the high toxicity and volatility of cyanide bromide make it hazardous to deal with and the waste produced may create a handling problem. In addition, cyanide bromide is under a strict regulation in Taiwan; therefore, it requires tedious procedures to obtain this chemical. Other methods, such as thin-layer chromatography (TLC)[12], high performance liquid chromatography (HPLC)[7,13-17], and gas chromatography (GC)[18-19], are also available for total nicotinic acid and nicotinamide analysis. The TLC method always results in low accuracy. The complexity of purification steps of the HPLC method may cause deviation in data. Tanaka *et al*.[18] used GC methods to analyze total nicotinic acid and nicotinamide, however, the tediousness of the sample derivatization and other analytical steps may reduce the precision of quantitation.

The GC method is one of the most important modern analytical techniques because of its advantages of high resolution and sensitivity. Owing to years of research using GC, we found that insertion of a ball of glass wool into a liner of the GC injector or application of a guard column (ca. 1-2 cm) could effectively prevent the nonvolatile compounds from getting into the analytical column and reduce the interference of the contaminants. We also found that the commercially available megapore capillary GC column was superior in water resistance. Even though the samples were in an aqueous solution, direct injection of the samples into GC with this column resulted in a comparable repeatability of resolution and retention time to a new column. It was not necessary to frequently clean the retained salts and other impurities in the liner. Under normal circumstances, the liner could be used for more than 100 samples before cleaning. The cleaning procedure for the liner is quite simple. The liner should be removed from the GC and soaked in hydrochloric acid solution for 10 minutes. The old glass wool was removed from the liner, which was then washed in water. A ball of new glass wool was inserted into the dried liner, which was then placed into the injector for use.

Due to the above advantages for the GC method, we chose to analyze samples of tonic and vitamin drinks by direct injection into GC without further treatments, but with the addition of an internal standard before analysis. The purpose of this study was to establish a simple, fast, and accurate method through the use of an adequate GC column and analytical conditions to quantify nicotinic acid and nicotinamide in vitamin and tonic drinks.

MATERIALS AND METHODS

I. *Materials*

All samples, including 12 vitamin drinks, 10 tonic drinks and 20 chicken essences, were purchased from supermarkets in the Pingtung and

Tainan areas. Nicotinamine, 1,5-pentanediol, 1,6-hexanediol, 1,4-dihydroxybenzene, and 1,9-nonanediol (purity > 98% for all chemicals) were purchased from TCI Co. (Tokyo, Japan).

II. *Method*

(I) *Preparation of Standard Compounds and Internal Standard Solutions*

Nicotinamide (0.2 g) and 1,9-nonanediol (0.2 g) were dissolved in methanol in separate volumetric flasks and the volume was brought to 100 mL. 1,9-Nonanediol was used as the internal standard in this study.

(II) *Determination of Relative Response Factor (RRF) of Nicotinamide to 1,9-Nonanediol*

Nicotinamide (0.2%, w/v) and 1,9-nonanediol (0.2%, w/v) in methanol were mixed together in different ratios (nicotinamide/1,9-nonanediol = 2/1, 1/1, and 1/2). RRF was calculated as the following formula:

$$RRF = (A_S/W_S)/(A_{IS}/W_{IS}) \quad (1)$$

A_S: GC peak area of nicotinamide
A_{IS}: GC peak area of 1,9-nonanediol
W_S: weight (μg) of nicotinamide
W_{IS}: weight (μg) of 1,9-nonanediol

III. *Quantitation of Nicotinamide*

(I) *Direct Injection GC Method*

Each sample of vitamin drink, tonic drink and chicken essence (1 mL) was placed in a 7-mL sample vial, in which 0.1 mL of 0.2% internal standard was added. The sample was properly mixed and a volume of 0.1 μL was injected into GC for analysis. The amount of nicotinamide was calculated as the following formula:

$$\text{Nicotinamide } (\mu g) = (A_S/A_{IS})(W_{IS}/RRF)/V \quad (2)$$

V: volume (mL) of samples

(II) AOAC Method(2,8)

The amount of nicotinamide was quantified by the spectrophotometric method.

IV. *Determination of the Lowest Quantitatively Determinable Concentration of GC-FID*

Nicotinamide standard solution (2.0 mg/mL) was diluted with water to concentrations of 100, 50, 25, 10, 5, and 2 μg/mL. One mL of each mixed separately with 0.1 mL of internal standard (1,9-nonanediol). The mixture was injected into GC in triplicate for analysis. The coefficient of variation (CV%) for nicotinamide recovery was set at 15%; the lowest concentration of nicotinamide obtained was the lowest quantitatively determinable concentration of GC-FID.

V. *Fortification Recovery Test*

A volume of 0.1 mL of nicotinamide standard solutions (2.11, 1.055, and 0.264 mg/mL) were added separately to 1 mL of vitamin drink (in a 7-mL vial). The blank sample was prepared without addition of the nicotinamide solution. The mixtures were spiked with 0.1 mL of internal standard solution (0.2%, w/v), after mixing, 0.1 μL of each mixture was injected into GC for analysis. The recovery of each level was measured in triplicate.

VI. *GC Conditions*

For this study, a GC (GL Science 390B, Tokyo, Japan) equipped with a flame ionization detector (FID) was used with the H_2 flow rate at 30 mL/min and air flow rate at 300 mL/min for this study. The flow rate of carrier gas (N_2) was set at 5 mL/min. The temperatures of injection port and detector were set at 280ºC and 300ºC, respectively. The oven temperature was programmed to initiate at 110ºC and held for 1.5 min. The temperature was raised to 190ºC at a rate of 8ºC/min and held for 1 min, and finally increased to 290ºC at a rate of 40ºC/min. The injection volume was 0.1 μL with direct injection mode.

RESULTS AND DISCUSSION

I. Study of GC Conditions

Without a preparation procedure, the samples spiked with an internal standard were directly injected into a GC for analysis. The selection of a

suitable GC column and analytical conditions were the only two considerations for the method development.

When the solutions of nicotinic acid and nicotinamide were analyzed using a high-polar column (CP-Wax, 30 m × 0.53 mm) and a mid-polar column (CP-SIL 8CB, 30 m × 0.53 mm), respectively, only nicotinamide was observed. Nicotinic acid was not detected due to its low volatility. The results of the current study suggest that the GC method could only analyze nicotinamide but not nicotinic acid. This GC method with direct injection was easier than that reported by Tanaka[18] with derivatization steps for nicotinamide prior to GC analysis. In addition, nicotinamide is a more common ingredient than nicotinic acid in vitamin and tonic drinks, thus, development of a nicotinamide analytical method using a GC with direct injection is necessary.

A CP-SIL 8CB column was chosen for nicotinamide analysis since it was more stable and durable than a CP-Wax column. Without a preparation procedure, the samples were analyzed by direct injection and with GC conditions as described in MATERIALS AND METHODS. The retention time for the nicotinamide standard was 11.18 min. As shown in Figure 1, it took only 15 min to complete an analysis.

Selection of the internal standard was conducted by adding a small amount of 1,5-pentanediol, 1,6-hexandiol, 1,4-dihydroxybenzne, and 1,9-nonanediol standard solutions into vitamin, amino acid, and tonic drinks, respectively, and analyzed under the above GC conditions. As shown in Table 1, the retention times of these four internal standards were 3.82, 5.17, 8.14, and 9.45 min, respectively. The 1,9-nonanediol peak was the only one that did not overlap with other ingredient peaks of each drink, thus, 1,9-nonanediol was chosen as the internal standard for this study.

II. *Determination of Relative Response Factor (RRF) of Nicotinamide to 1,9-Nonanediol*

Since 1,9-nonanediol was selected as the internal standard to quantify nicotinamide in vitamin, amino acid, and tonic drinks, it was necessary to determine the RRF of nicotinamide to 1,9-nonanediol in order to quantify nicotinamide accurately. With RRF, the content of nicotinamide in different samples could be calculated using formula (2). The coefficient of linearity of the standard curve was obtained by plotting the peak area ratios (Y axis) of nicotinamide to the internal standard versus the concentration ratios (X axis) of nicotinamide to the internal standard. As shown

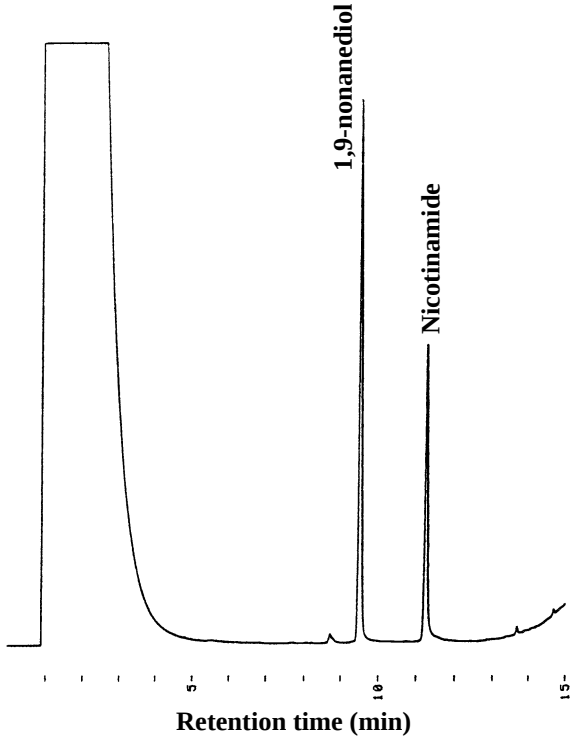

Figure 1. Gas chromatogram of nicotinamide and 1,9-nonanediol internal standard by direct injection method.

Table 1. Gas chromatographic retention time of nicotinamide and the internal standards

Compound	Retention time (min)[a]
1,5-Pentanediol	3.82
1,6-Hexanediol	5.17
1,4-Dihydroxybenzene	8.14
1,9-Nonanediol	9.45
Nicotinamide	11.18

[a] CP-Sil 8CB column (30 m × 0.53 mm, 1.5 μm) was used.
Oven condition = 110ºC(1.5 min)→8ºC/min→190ºC(1 min)→40ºC/min→290ºC.

in Figure 2, R^2 was better than 0.99 and RRF was 0.64.

III. *The Lowest Quantitatively Determinable Concentration of Nicotinamide on GC-FID*

The standard solution of nicotinamide (0.2%, w/v) was diluted to a series of concentrations and a proper amount of internal standard was added to each dilution. The mixtures were directly injected into the GC in which an FID detector with the setting of range: 1 and attenuation: 2 was used. The lowest quantitatively determinable concentration of nicotinamide in the GC condition described above and CV% for nicotinamide recovery at 15% was 2-5 µg/mL (Table 2).

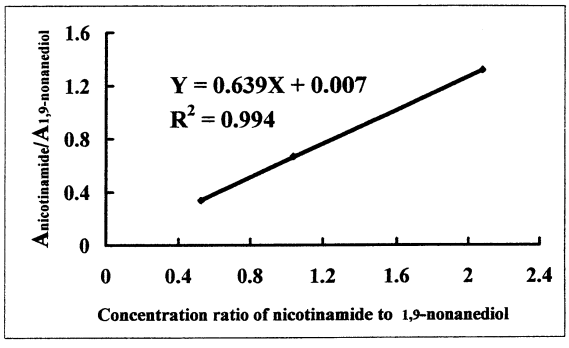

Figure 2. Calibration curve of nicotinamide to 1,9-nonanediol internal standard by direct injection GC method.

IV. *Fortification Recovery Test*

The recoveries of nicotinamide in vitamin and tonic drinks are shown in Table 3. Addition of 211.0 and 105.5 µg of nicotinamide to 1 mL of vitamin and tonic drinks produced recoveries of 93-108% and 94-99%, respectively, with CV%

Table 2. Lowest quantitatively determination level of nicotinamide using gas chromatography with FID detector by direct injection method

Nicotinamide content (µg/mL)[a]	Recovery (%)[b]	CV (%)[c]
100	103.5	3.9
50	101.7	6.9
25	94.8	5.4
10	108.4	8.1
5	107.4	6.8
2	141.3	23.4

[a] FID range=1, Attenuation=2.
[b] Average of tripicate analyses.
[c] Coefficient of variation (cv %).

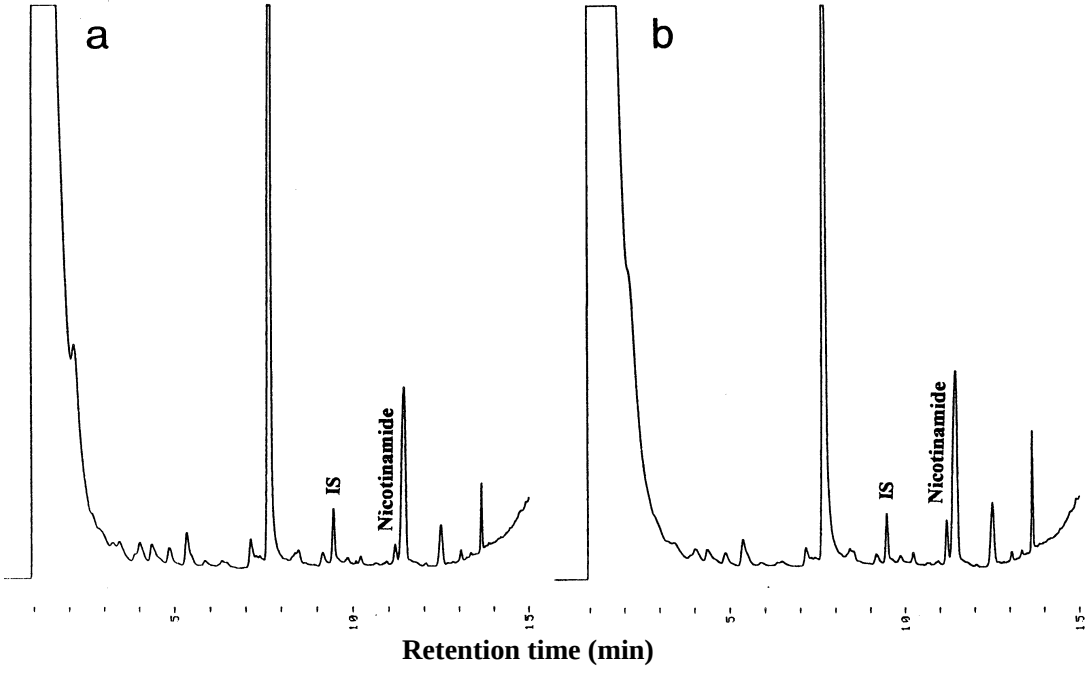

Figure 3. Gas chromatograms of the nicotinamide in (a) vitamin B drink, and (b) spiked vitamin B drink by direct injection method.

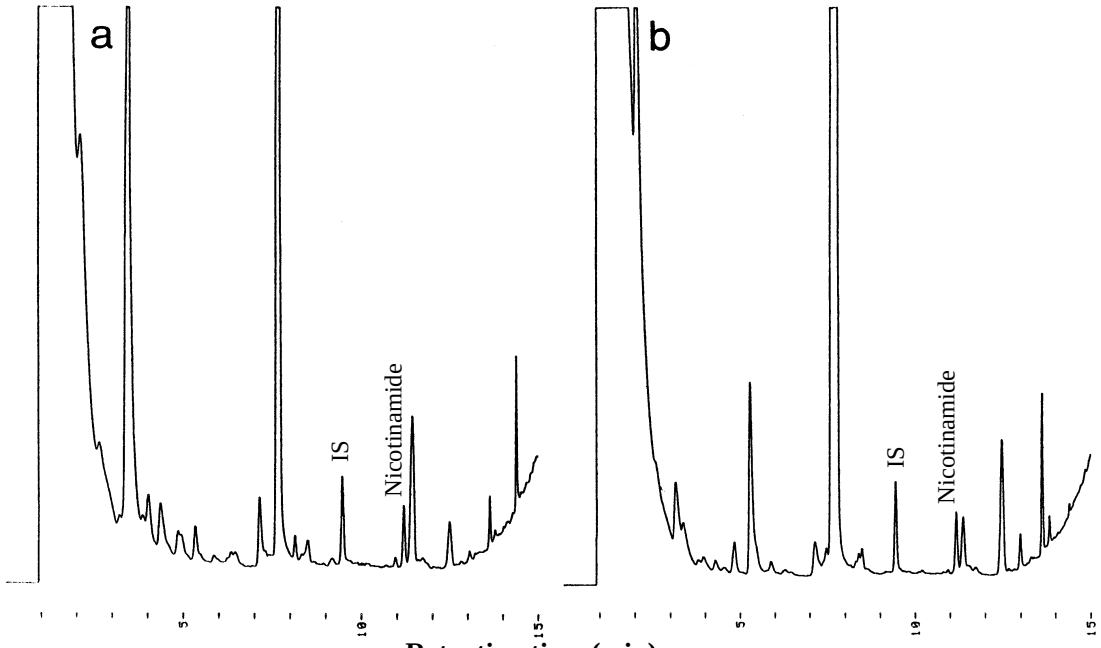

Figure 4. Gas chromatograms of the nicotinamide in (a) tonic drink, and (b) amino acid drink by direct injection method.

Table 3. Recoveries of the nicotinamide in spiked commercial vitamin and tonic drinks by direct injection gas chromatographic method

Sample	Blank [a] (μg)(A)	Amount added (μg)(B)	Amount found [b] (μg)(C)	Recovery (%)[c]	CV (%)[d]
TD-3	32.5	211.0	228.3	92.8	7.4
	32.5	105.5	136.9	107.6	9.8
	32.5	26.4	60.2	102.2	9.2
VD-8	111.7	211.0	310.7	94.3	8.3
	111.7	105.5	225.8	98.5	7.9
	111.7	26.4	144.3	104.5	9.6

[a] Nicotinamide in 1 mL vitamin drink (VD-8) and tonic drink (TD-3).
[b] Average of triplicate analyses.
[c] Recovery(%)=(C–A)/B × 100%.
[d] Coefficient of variation (cv %).

Table 4. Comparison of AOAC method and proposed method for determinning of nicotinamide in commercial vitamin and tonic drink

Sample	Proposed method[a]		AOAC method[b]	
	Nicotinamide (μg/mL)	CV (%)	Nicotinamide (μg/mL)	CV (%)
TD-3	32.5	8.4	34.7	6.7
VD-8	121.7	5.7	127.5	5.9

[a] Direct injection GC method in this study.
[b] Spectrophotometric method[2,8].
[c] Average of triplicate analyses, coefficient of variation (cv%).

Table 5. Nicotinamide contents of various commercial amino acid, vitamin and tonic drinks

Drink[a]	Nicotinamide Content (μg/mL)[b]	Volume per bottle (mL)	Total nicotinamide per bottle (mg)
Amino acid drink			
AD -1	130.55	300	39.17
AD -2	246.11	300	73.83
Vitamin drink			
VD -3	71.81	200	14.36
VD -4	54.13	160	8.66
VD -5	106.9	160	17.12
VD -6	86.91	160	13.92
VD -7	64.56	200	12.91
VD -8	111.91	200	22.38
VD -9	49.02	200	9.81
VD -10	23.88	200	4.78
VD -11	70.78	200	14.16
VD -12	25.48	200	5.10
Tonic drink			
TD -1	87.71	100	8.77
TD -2	119.12	100	11.91
TD -3	32.50	100	3.25
TD -4	160.67	100	16.07
TD -5	212.55	100	21.26
TD -J91-1	trace[c]	30	trace[c]
TD -J91-2	trace[c]	60	trace[c]
TD -J91-3	81.34	60	4.88
TD -J91-4	262.89	60	15.77
TD -J91-5	27.18	60	1.63

[a] From different food or drug factories. [b] Average of duplicate analyses. [c] trace < 2 μg/mL.

under 9.8%. This method skipped the sample preparation step and analyzed directly; furthermore, it took only 15 min (Figure 3 and 4) to examine one sample. When this method is compared to the GC method with derivatization steps (need 1 hr for analysis) reported by Tanaka[18], the former is faster and simpler than the latter. This method is considered the fastest and simplest method reported to date, and is, therefore, recommended for use in routine nicotinamide analysis.

V. *Comparison of the Direct Injection GC Method and AOAC Method*

The results of using the direct injection GC method and the AOAC method (spectrophotometric method) to analyze nicotinamide in vitamin and tonic drinks are compared in Table 4. There is no significant difference between these two methods. The direct injection GC method was not only reliable but also simple and fast as compared to the AOAC method. Therefore, the direct injection GC method would be a good alternative for nicotinamide analysis.

VI. *Contents of Nicotinamide in Commercially Available Amino Acid, Vitamin, and Tonic Drinks*

A total of 22 samples was collected from several supermarkets including 2 amino acid, 10 vita-

Table 6. Nicotinamide contents of commercial chicken essences

Tonic drink[a]	Nicotinamide content (μg/mL)[b]	Volumn per bottle (mL)	Total nicotinamide per bottle (mg)
EC -1	ND	70	-
EC -2	33.94	70	2.38
EC -3	Trace[c]	70	Trace
EC -4	ND	70	-
EC -5	Trace	70	Trace
EC -6	Trace	100	Trace
EC -7	ND	100	-
EC -8	ND	100	-
EC -9	ND	70	-
EC-10	ND	70	-
EC-11	62.32	70	4.36
EC-12	47.65	100	4.77
EC-13	Trace	70	Trace
EC-14	133.86	70	9.37
EC-15	ND	100	-
EC-16	20.59	100	2.06
EC-17	ND	100	-
EC-18	103.61	100	10.36
EC-19	Trace	100	Trace
EC-20	52.58	100	5.26

[a] From different food or drug factories. [b] Average of duplicate analyses. [c] Trace < 2 μg/mL.
ND = not detected.

min, and 10 tonic drinks. Nicotinamide in these drinks was analyzed by the direct injection GC method. As reported in Table 5, the contents of nicotinamide were at 131-246, 24-112, and 0-263 μg/mL for amino acid, vitamin, tonic drinks, respectively. The volume of each bottle of amino acid or vitamin drinks ranged from 160 to 300 mL and for tonic drinks from 30 to100 mL. Therefore, the total amount of nicotinamide in each bottle was calculated at 39-74, 5-22, and 0-21 mg/bottle in amino acid, vitamin, and tonic drinks, respectively. In addition, the content of nicotinamide in twenty chicken essence samples was also investigated, which were at 0-134 μg/mL. The volume of each bottle of tonic drink varied from 70 to 100 mL and the total amount of nicotinamide was calculated at 0-10 mg/bottle.

The above results indicated that consumption of a bottle (300 mL) of amino acid drink could reach 3-5 times of the Recommended Daily Nutrient Allowances (RDNA) for nicotinamide (14.4 mg)[6]. Consumption of a bottle of vitamin drink (160-200 mL) resulting in exceeding the RNDA occurred in two samples, and in 3 samples of tonic drink (30-100 mL). Drinking a bottle of chicken essence did not surpass the average ADI of nicotinamide.

It was reported that, to adults, no hyperemia symptoms occurred by consuming of 20 mg/day, however, ingestion of 100 mg/day could cause hyperemia symptoms such as flushed skin, itchy skin, an upsurge of blood sugar levels, peptic ulcers, and even lever damage[6]. The amounts of nicotinamide in commercially available amino acid and vitamin drinks (belong to food category) were higher than in tonic drinks; thus, an additional awareness is needed in consuming these drinks.

CONCLUSIONS

The samples of amino acid, vitamin, and tonic drinks were spiked with an internal standard (1,9-nonanediol) and 0.1 μL of which was injected into GC directly for analysis. The direct injection GC method developed in the current study was simple, quick and precise in quantifying nicotinamide in various drinks. The analysis of each sample could be completed within 15 min. Nicotinamide was examined in 42 samples, including amino acid, vitamin, tonic drinks, and chicken essences, using the direct injection GC method. The results found concentrations of nicotinamide were between 0 and 263 μg/mL. Based on the volume of a bottle of the above drinks (30-300 mL), the total amounts of nicotinamide can be 0-74 mg/bottle. The total amounts of nicotinamide in 7 out of 42 samples exceeded the RNDA of Taiwan.

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市售維生素飲料及口服液中菸鹼醯胺之直接注入氣相層析快速定量分析法

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摘　要

本研究建立了直接注入氣相層析分析市售胺基酸飲料、維生素飲料、雞精及口服液等液體樣品中菸鹼醯胺之快速、簡便之測定方法。採用直接注入（direct injection）之方式，以中間極性之CP-SIL 8CB管柱（30 m × 0.53 mm）分析定量上述液體樣品之菸鹼醯胺，選擇水溶性之1,9-壬二醇（1,9-nonanediol）為內標準，最低檢出量約為2-5 μg/mL左右。添加菸鹼醯胺105.5及211.0 μg 於1 mL維生素飲料及口服液檢體中，直接注入GC分析，其回收率分別為94-99%及93-108%，變異係數均在9.8%以下。以本方法分析市售不同廠牌之胺基酸飲料、維生素飲料、雞精及口服液等液體樣品共42件之菸鹼醯胺含量分別為131-246、24-112、0-134及0-263 μg/mL。相當於每瓶上述檢體中分別含菸鹼醯胺39-74、5-22、0-10及0-21 mg/bottle。此結果顯示，12件胺基酸及維生素飲料中，每瓶菸鹼醯胺含量有4件超過國人每日平均建議攝取量（RNDA＝14.4 mg）許多；而10件口服液中每瓶菸鹼醯胺亦有3件超過國人每日平均建議攝取量。

關鍵詞：維生素飲料，口服液，菸鹼醯胺，直接注入法，氣相層析，定量分析。