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Application News

No. AD-0237

Food Analysis / LCMS-8045

Simultaneous Quantitative Analysis of Five Vitamers of B3 and B6 in Banana by LC/MS/MS

❑ Introduction

Vitamin B3 and B6 are water-soluble vitamins that are essential micronutrients to maintain good health. Vitamin B3 helps to lower cholesterol level and improves skin function while vitamin B6 plays an important role in the nervous system. These two vitamins are mainly obtained from meats, vegetables and fruits. Banana is an excellent source of both vitamins. Many of the available methods analyse vitamins B3 and B6 separately using HPLC coupled with fluorescence detector which can be time-consuming. Here, we developed a sensitive LC-MS/MS method to simultaneously analyse 5 vitamers of B3 and B6 namely, nicotinic acid, nicotinamide, pyridoxamine, pyridoxal and pyridoxine using LCMS^{TM}-8045 with acid hydrolysis as the sample extraction method.

❑ Experimental

Standard preparation

Nicotinic acid, nicotinamide, pyridoxamine, pyridoxal, pyridoxine and ammonium formate were obtained from Sigma Aldrich. Nicotinamide-d4 was obtained from Isosciences. Formic acid was obtained from Fischer and methanol was obtained from Kanto. The 5 vitamin standards were dissolved in Milli-Q® water and nicotinamide-d4 in methanol at concentrations of 1000 ppm as stock solutions. The stock solutions of vitamins were diluted into working standards of 0.5, 1, 5, 10 and 50 ppb with nicotinamide-d4 as internal standard at 10 ppb using 20 mM ammonium formate in Milli-Q® water with 0.1% formic acid as the diluent.

Sample preparation

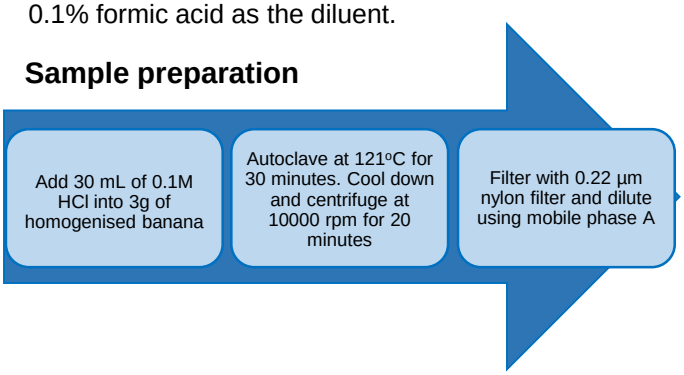

Figure 1: Sample preparation method of 5 compounds of vitamins B3 and B6 in banana samples

Analytical conditions

Shimadzu LCMS-8045 was used in this analysis. The LC-MS/MS conditions are shown in Table 1.

Table 1: LC-MS/MS conditions of vitamin B3 and B6 analysis

LC Conditions

Column	Shim-pack^{TM} GIST C18-AQ, 100mm × 2.1 mmid, 3µm
Flow Rate	0.3 mL/min
Mobile Phase	A) 20 mM ammonium formate in Milli-Q® water with 0.1% formic acid B) Methanol
Elution Mode	**Gradient elution, LC Time Program** B% : 0% (0.0 – 0.5 min) ➡30% (3.0 min) ➡90% (3.1 to 4.5 min) ➡0% (4.6 to 8.0 min)
Oven Temperature	40 ºC
Injection Volume	5 µL

MS/MS Conditions

Interface	ESI Heated
MS Mode	Positive mode
Block Temperature	400ºC
DL Temperature	250ºC
Interface Temperature	350ºC
Nebulizing Gas Flow	Nitrogen, 3.0 L/min
Drying Gas Flow	Nitrogen, 10.0 L/min
Heating Gas Flow	Nitrogen, 10.0 L/min

❑ Results and Discussion

Method development

Automated MRM optimisation of the 5 vitamins and nicotinamide-d4 was carried out using the LabSolutions workstation. Two MRM transitions for every compound were chosen as quantifier and confirmation ion. The MRM transitions used are shown in Table 2 below.

Table 2: MRM transitions of 5 vitamin compounds and nicotinamide-d4 internal standard

Name	Mode	MRM 1	MRM 2
Nicotinic acid	ESI (+)	124.10>80.05	124.10>53.05
Nicotinamide	ESI (+)	123.10>80.05	123.10>53.00
Pyridoxamine	ESI (+)	169.20>152.05	169.20>134.05
Pyridoxal	ESI (+)	168.20>150.05	168.20>94.00
Pyridoxine	ESI (+)	170.20>152.00	170.20>134.05
Nicotinamide-d4	ESI (+)	127.10>84.05	127.10>56.05

Performance evaluation of established LC-MS/MS method

A calibration curve with concentration range of 0.5 ppb – 50 ppb was established with R^2 greater than 0.9995. Each calibrant was injected thrice and the average area was used to plot the calibration curve (Figure 3). Internal calibration method was applied to nicotinamide while the other four compounds use external calibration method.

The limit of detection (LOD) and limit of quantitation (LOQ) ranged from 0.07 – 0.2 ppb and 0.2 – 0.6 ppb respectively. Repeatability (n=6) was also evaluated at vitamins concentration of 10 ppb (Table 3).

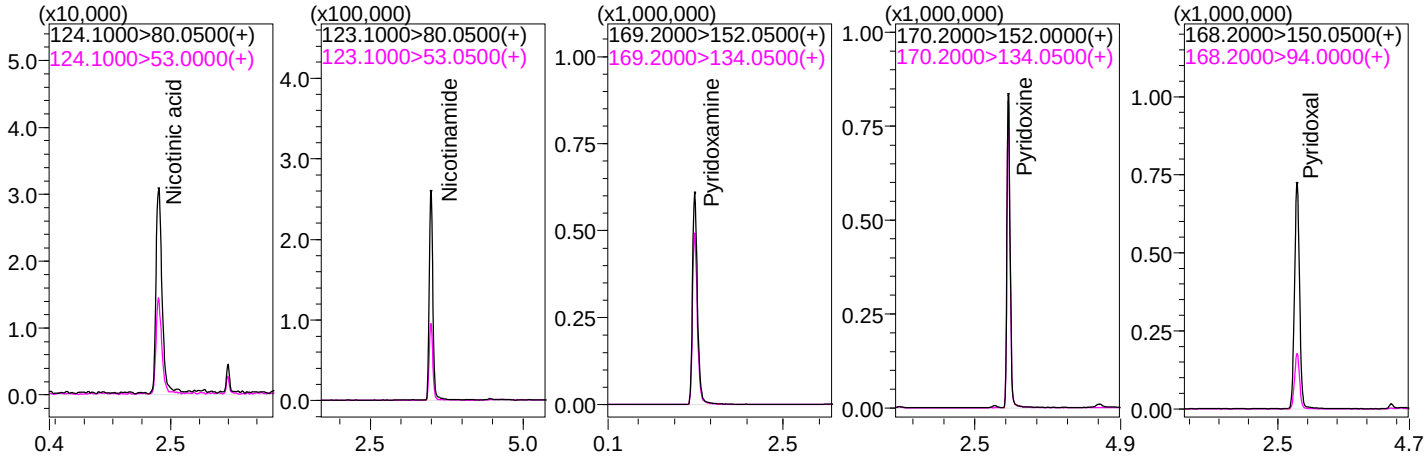

Figure 2: MRM chromatograms of 5 vitamin compounds at concentration of 10 ppb

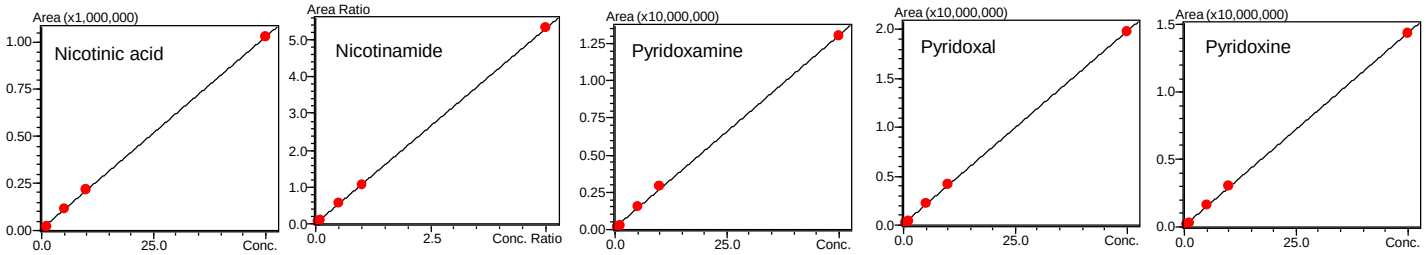

Figure 3: Calibration curves of the 5 vitamin compounds. Nicotinamide uses internal calibration method, and the other four compounds use external calibration method.

Table 3: Linearity, LOD, LOQ and repeatability results for 5 vitamin compounds

No	Name	RT (min)	Range (ppb)	Linearity	LOQ (ppb)	LOD (ppb)	% RSD (n=6)
1	Nicotinic acid	2.336	1-50	0.9999	0.60	0.20	1.85
2	Nicotinamide	3.514	0.5-50	0.9999	~0.25	~0.08	2.10
3	Pyridoxamine	1.358	0.5-50	0.9996	~0.20	~0.07	0.44
4	Pyridoxal	2.857	0.5-50	0.9998	~0.20	~0.07	0.51
5	Pyridoxine	3.089	0.5-50	0.9999	~0.20	~0.07	0.95

Recovery and matrix studies of 5 vitamins in banana samples

A recovery test was performed using banana samples as the matrix. The 5 vitamins were spiked in banana samples at concentration of 0.2 mg/100g.

The recovery and matrix effect test were performed twice to obtain reliable results. Each duplicate was injected thrice and the average area was used for the calculation of recovery and matrix effect (Table 4).

To improve the lower recovery and matrix effect of nicotinamide, nicotinamide-d4 was used as internal standard.

Table 4: Average recovery of 5 vitamins in infant banana (n=2 x 3).

Compound	Recovery (%)	Matrix effect (%)
Nicotinic acid	102.3	101.0
Nicotinamide	74.5	54.9
Pyridoxamine	104.4	119.3
Pyridoxine	91.7	96.1
Pyridoxal	62.2	99.8

Quantitation of 5 vitamins in banana samples

The established method was evaluated using three banana samples bought from local supermarket. Each banana sample was extracted twice and each duplicate was injected thrice to calculate the average content of vitamins. The results obtained are tabulated in Table 5 and MRM chromatograms of banana samples are shown in Figure 4.

Table 5: Results of quantitation of 5 vitamins in banana samples (mg/100g)

Name	Sample A	Sample B	Sample C
Nicotinic acid	0.104	0.113	0.109
Nicotinamide	0.437	0.458	0.427
Pyridoxamine	0.241	0.241	0.213
Pyridoxal	0.026	0.024	0.023
Pyridoxine	0.090	0.101	0.103

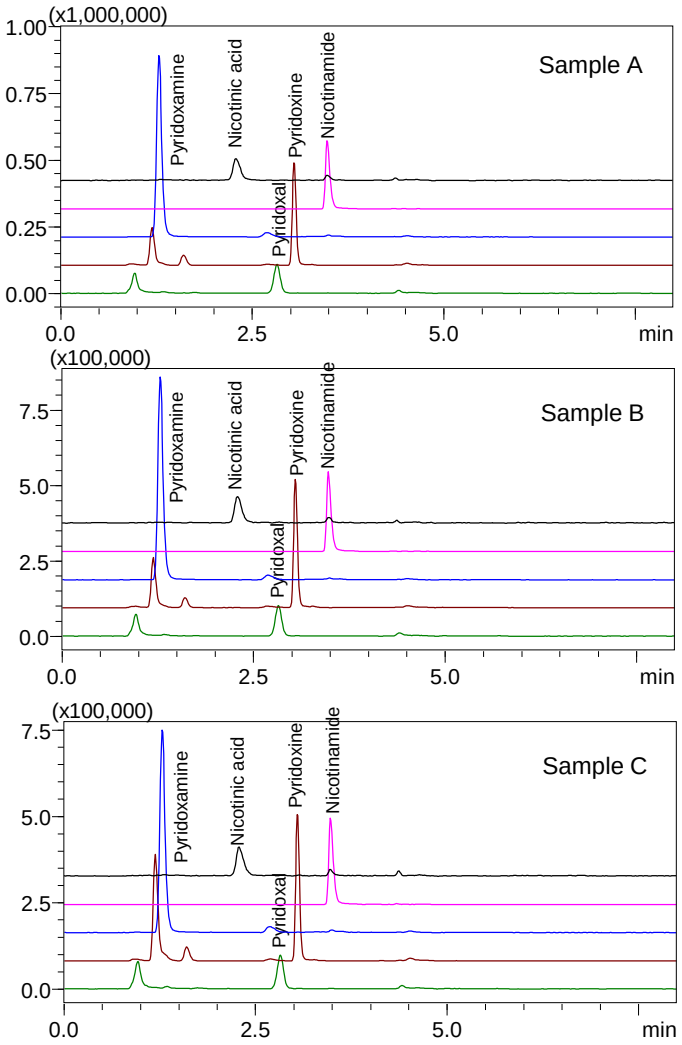

Figure 4: MRM chromatograms of 3 banana samples
*** The intensity of nicotinic acid is magnified by 5x**

❑ Conclusion

A sensitive and selective simultaneous LC-MS/MS method was established for quantitation of 5 vitamers of B3 and B6. Nicotinamide-d4 was used as the isotopic internal standard for nicotinamide while the other 4 compounds use external standard calibration.

Good linearity with R^2 greater than 0.9995 was achieved. The LOQ and LOD ranged from 0.2 - 0.6 ppb and 0.07 – 0.2 ppb respectively. Three banana samples bought from local supermarket were analysed and the results were tabulated in Table 5.

❑ References

1. *Amaro, C., Flores, C., Dias, M., and Lidon, F. (2014) Pyridoxine analysis by high performance liquid chromatography and validation in fortified milk powder. Acta Alimentaria 43, 297–305.*
2. *Kall, M. A. (2003) Determination of total vitamin B6 in foods by isocratic HPLC: a comparison with microbiological analysis. Food Chemistry 82, 315–327.*

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