Journal of Chromatography, 466 (1989) 307–317
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 182

GAS CHROMATOGRAPHIC DETERMINATION OF NICOTINAMIDE IN MEATS AND MEAT PRODUCTS AS 3-CYANOPYRIDINE

AKIO TANAKA*, MASAO IIJIMA, YOSHINORI KIKUCHI, YOJI HOSHINO and NORIHIDE NOSE

Saitama Institute of Public Health, Kamiokubo-Higashi, 639-1, Urawa, Saitama (Japan)

(First received August 26th, 1988; revised manuscript received December 13th, 1988)

SUMMARY

The determination of nicotinamide as 3-cyanopyridine after dehydration by heptafluorobutyric anhydride (HFB) was performed by gas–liquid chromatography (GLC) with flame ionization detection (GLC–FID) and a column of 5% OV-17 on Chromosorb W AW DMCS at 130°C. Determination was possible with 3–100 μg of the dehydrated reaction mixture. The procedure for determining nicotinamide in various meats and meat products involves direct analysis by GLC–FID without a clean-up stage; the detection limit is 5 ppm and the recovery ranged from 93.4 to 104.6% (average 98.0%). Various possible interferents in the samples did not interfere in the production or determination of 3-cyanopyridine. The procedure is suitable for routine use. The dehydrated derivative of nicotinamide was confirmed as 3-cyanopyridine by combined gas chromatography–mass spectrometry and infrared spectrometry.

INTRODUCTION

Nicotinamide is one of the B vitamins and serves as a precursor in the biosynthesis of the coenzymes NAD and NADP, which are involved in many enzymatic reactions and metabolic pathways. Nicotinamide deficiency manifests itself as the disease pellagra, which, although common in corn-eating areas two centuries ago, is now rarely seen except in occasional cases of alcoholism. On the other hand, nicotinamide is used as a vitamine-enriching agent and in several countries an official tolerance has been established. Several investigators have reported the use of nicotinamide as a colour-producing agent or for maintaining the colour quality of meat, leading to temporary poisoning with symptoms of facial or cutaneous flushing and itching[1–4]. The development of a simple, rapid and accurate method for the determination of nicotinamide in meat samples is therefore of interest.

The various methods used for determining nicotinamide in foods include colour reactions and absorption measurement[5], microbiological assay[6], high-performance liquid chromatography (HPLC)[2,4,7,8] and gas chromatography (GC)[1,3]. However, several of these methods are not suitable for the determination of nicotinamide

0021-9673/89/$03.50

because they involve the determination of total nicotinic acid after the decomposition of nicotinamide and require complex pretreatments[5–8]. Further, the simultaneous determination of nicotinamide and nicotinic acid using HPLC reported by Yoshida *et al.*[2] is influenced by ascorbic acid.

Determinations of nicotinamide in foods by gas chromatography have been described. Aoyama *et al.*[3] studied the direct determination of nicotinamide by gas–liquid chromatography (GLC) with flame ionization detection (FID). This method is simple but has poor sensitivity. Miyama and Imaida[1] studied the determination of nicotinamide as methyl nicotinate after separation by ion exchange and decomposition of nicotinamide, followed by methylation in the presence of methanol and hydrochloric acid and then GLC–FID. This method lacks specificity, which is crucially important for the determination of nicotinamide because of the determination of nicotinic acid after decomposition of nicotinamide, and also requires a long time for the methylation stage. However, we found that 3-cyanopyridine is over six times more sensitive than nicotinamide in GLC; it can be prepared quantitatively by the instantaneous dehydration of nicotinamide with heptafluorobutyric anhydride (HFB).

Nicotinamide in foods was extracted with acetonitrile without the need for a clean-up stage. The proposed method is simple and selective and offers a practical means of determining nicotinamide in various meats and meat products. The recovery of nicotinamide added to various samples was satisfactory.

EXPERIMENTAL

Reagents and apparatus

Nicotinamide (Tokyo Kasei Kogyo, Tokyo, Japan) was dried at 100°C for 5 h under vacuum immediately before use. A stock solution was prepared by dissolving nicotinamide in acetonitrile to give a concentration of 100 μg/ml. HFB (Wako, Osaka, Japan) stage was of specially pure grade. The internal standard solution for GC was prepared by dissolving 100 μg of 1,2,4-trichlorobenzene in 1 ml of diethyl ether. 3-Cyanopyridine (Tokyo Kasei Kogyo) was of specially pure grade and was used without further purification.

The column packing materials for GLC, *viz.*, Chromosorb W AW DMCS, Advance, DEGS, OV-17, OV-330, PEG-20M, SE-30 and XE-60, were of high purity and were obtained from Nihon Chromato (Tokyo, Japan). All other reagents and solvents were of high purity from Wako.

For identification of the dehydration product of nicotinamide, a Shimadzu GC-MS QP-1000A combined gas chromatograph–mass spectrometer and an infrared spectrometer were used. For GC, a glass tube (2 m × 3 mm I.D.) packed with OV-17 on Chromosorb W AW DMCS was used; the carrier gas (helium) flow-rate was 30 ml/min and the column temperature was 130°C. The conditions for mass spectrometry (MS) were as follows: separator temperature, 180°C; ion source temperature, 210°C; trap current, 60 μA; electron energy, 70 eV; and accelerating potential, 3.5 keV.

The 3-cyanopyridine for IR analysis was prepared as follows. A 0.3-g portion of nicotinamide was placed in a 10-ml test-tube with a ground-glass stopper, then 2 ml of diethyl ether and 0.1 g of HFB were added. The mixture was allowed to react for 10 min at room temperature with occasional shaking. After reaction, 0.5 ml of water (*ca.* pH

8.0) was added and the mixture was shaken gently for 5 min and centrifuged at 1400 *g* for 2 min. The supernatant solution was applied to the column (10 cm × 1.0 cm I.D., prepared with 2 g of activated alumina topped by 0.5 g of anhydrous sodium sulphate) and eluted with diethyl ether. A 10-ml volume of effluent was collected and then evaporated at room temperature. The residue was kept in a desiccator for 1 day, then mixed with a suitable amount of dried potassium bromide. The IR spectra of both nicotinamide and 3-cyanopyridine were measured with a Shimadzu IR-435 recording spectrophotometer.

Preparation of 3-cyanopyridine

A suitable amount of nicotinamide (1–100 μg) or extract dissolved in 2 ml of acetonitrile was placed in a Pyrex test-tube (11.5 cm × 15 mm I.D.), and the solvent was evaporated to dryness under reduced pressure at 40°C for 10 min in a water-bath. To the dried residue were directly added 0.5 ml of internal standard solution followed by 20 μl of HFB, and the reaction was allowed to proceed in a test-tube fitted with a ground-glass stopper at room temperature for 5 min with occasional shaking. A 3-μl volume of the final solution was injected into the gas chromatograph.

Gas–liquid chromatography

A Shimadzu GC-7A gas chromatograph with a flame ionization detector was used for all analyses. The column consisted of a glass tube (2 m × 3 mm I.D.) packed with 5% of OV-17 on Chromosorb W AW DMCS (80–100 mesh) and was conditioned and operated at 130°C; the detector and injector temperatures were 190°C and the flow-rate of the carrier gas (nitrogen), hydrogen and air were 50, 50 and 800 ml/min, respectively.

Calibration graph

A series of working standard nicotinamide solutions were prepared by dilution of the stock solution with acetonitrile. Aliquots were placed into a Pyrex test-tube to give 3, 10, 30, 50, 70 and 100 μg of nicotinamide, and the solvent was removed by evaporation at 40°C for 10 min in a water-bath. After dehydration by addition of internal standard solution and HFB according to the procedure described above, 3-μl aliquots of the resulting solutions were injected into the GC column at 130°C. As shown in Fig. 1, the retention time (3.6 min) of the 3-cyanopyridine relative to that of 1,2,4-trichlorobenzene was 0.53. The minimum detectable amount of 3-cyanopyridine in this method was *ca.* 2.0 μg. The peak-height ratio of 3-cyanopyridine to 1,2,4-trichlorobenzene was plotted against the amount of nicotinamide analysed; a typical calibration graph is shown in Fig. 2.

Preparation and analysis of various meats and meat products

An accurately weighed sample (generally about 10 g) of finely ground sample was placed in the 300-ml stainless-steel container of a homogenizer, 80 ml of acetonitrile were added and the mixture was homogenized at high speed for 10 min. The extracted solution was filtered and diluted accurately to 100 ml with acetonitrile. A 2-ml volume of the filtrate was placed in a 5-ml test-tube fitted with a ground-glass stopper and the solvent was evaporated to dryness under reduced pressure at 40°C for 10 min in a water-bath. To the dried residue were directly added 0.5 ml of the internal

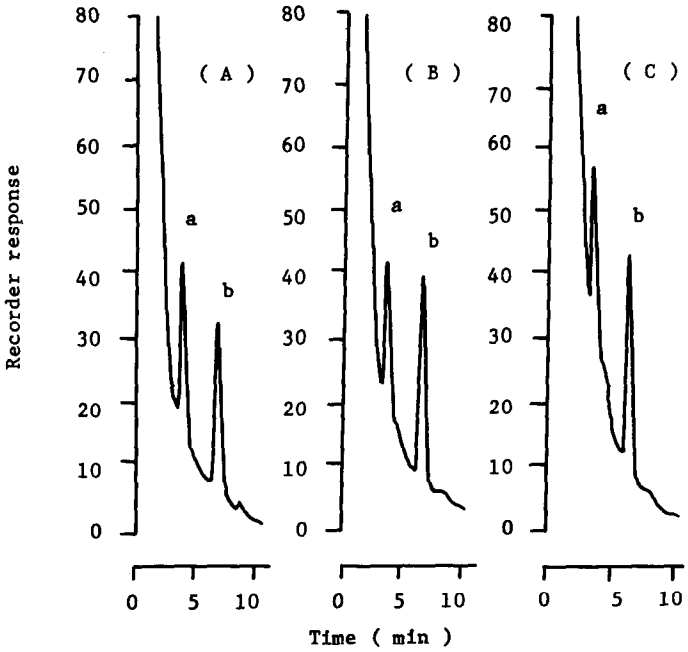

Fig. 1. Gas chromatograms of the dehydration product of (A) a standard reaction mixture to which nicotinamide was added at level of 50 ppm, (B) chicken meat and (C) tuna meat. The sample size was 3 μl. Shimadzu GC-7A gas chromatograph with FID. Column, 5% OV-17 on Chromosorb W HP (80–100 mesh) (2 m × 3 mm I.D.); column temperature, 130°C; injector and detector temperatures, 190°C; flow-rates of nitrogen carrier gas, hydrogen and air, 50, 50 and 800 ml/min, respectively. Peaks: a, 3-cyanopyridine; b, 1,2,4-trichlorobenzene.

standard solution followed by 20 μl of HFB. The mixture was allowed to react as described above and analysed by GC under the described conditions.

The contents of nicotinamide in foods were determined by GLC as described above using the internal standard method and comparison with the calibration graph.

RESULTS AND DISCUSSION

Standard assay

For the GLC assay using the described procedure, there was a linear relationship between peak height and amount of nicotinamide. As shown in Fig. 2, the calibration graph was linear from 3 to 100 μg of nicotinamide and the average relative standard deviations of five determinations were 4.5% for 3 μg, 3.8% for 10 μg, 2.1% for 30 and 50 μg and 2.4% for 70 and 100 μg; the reproducibility was considered to be satisfactory.

Dehydration of nicotinamide

A chromatogram of the dehydrated derivative of nicotinamide is shown in Fig. 1A; the retention time was 3.6 min. The optimum amount of reagent and the optimum time were investigated by using HFB, and the results are shown in Figs. 3 and 4. For 100 μg of nicotinamide, at least 29.8 μg of HFB in 0.5 ml of diethyl ether were required.

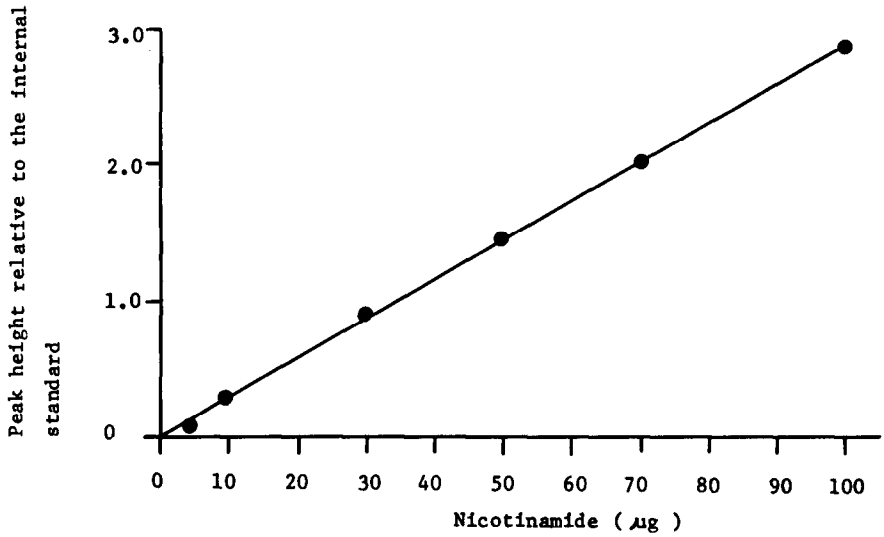

Fig. 2. Calibration graph for nicotinamide. Dehydration of nicotinamide was carried out at room temperature for 5 min. The sample size for GLC was 3 μl; the column temperature was 130°C and the nitrogen flow-rate was 50 ml/min. The abscissa shows the nicotinamide content of the reaction mixture and the ordinate the detector response measured as the peak height relative to that of the internal standard (1,2,4-trichlorobenzene; 100 ng per μl of reaction mixture).

The reaction proceeded fairly rapidly and when HFB solution in diethyl ether (40 μl/ml) was added to the solid residue of nicotinamide, the yield of the dehydrated product reached 100% within 3 min, and in practice 20 μl of reagent and a reaction time of 5 min were used.

To obtain good dehydration reactivities towards nicotinamide, aliquots of 20 μl of various acid anhydride reagents were added to 100 μg of nicotinamide dissolved in

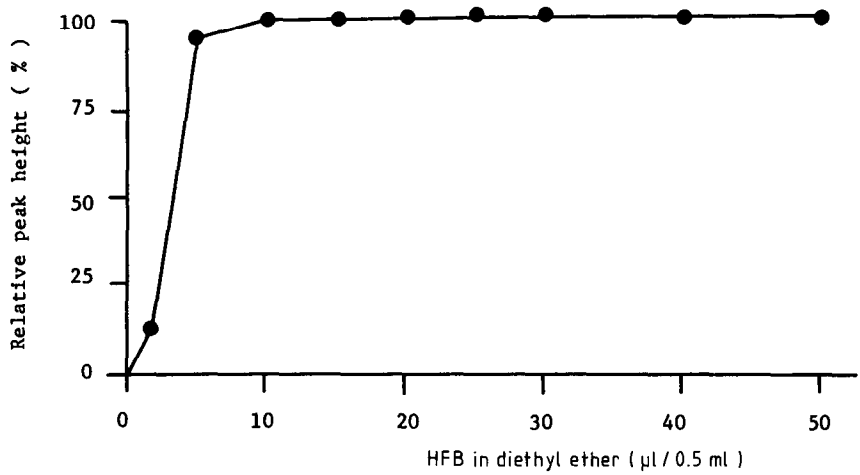

Fig. 3. Effect of amount of HFB on the dehydration of nicotinamide. To 100 μg of nicotinamide was added HFB in 0.5 ml of diethyl ether at room temperature and the product was analysed by GLC after 5 min.

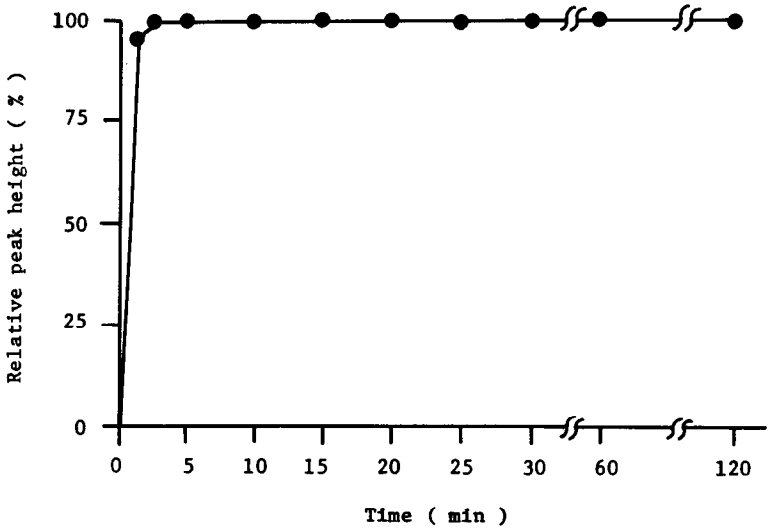

Fig. 4. Time course of formation of the dehydration product after addition of HFB to nicotinamide. To 100 μg of nicotinamide was added HFB in 0.5 ml of diethyl ether at room temperature and the product was analysed by GLC.

TABLE I

EFFECT OF VARIOUS ACID ANHYDRIDE REAGENTS ON DEHYDRATION OF NICOTINAMIDE

20 μl of acid anhydride reagent were added to 100 μg of nicotinamide dissolved in 0.5 ml of diethyl ether. Reaction and GLC conditions as in the described procedure.

Acid anhydride	*Relative peak height (%)*
Acetic anhydride	5.2
Trifluoroacetic anhydride	75.1
Propionic anhydride	29.9
Pentafluoropropionic anhydride	94.8
Butyric anhydride	63.7
Heptafluorobutyric anhydride	100.0

TABLE II

EFFECT OF SOLVENT ON DEHYDRATION OF NICOTINAMIDE

Reaction and GLC conditions as in the described procedure. Each reaction mixture (0.5 ml) contained nicotinamide (100 μg) and HFB (20 μl).

Solvent	*Relative peak height (%)*
Diethyl ether	100.0
Acetonitrile	74.8
Acetone	41.8
Ethyl acetate	27.4
Benzene	2.7
Hexane	2.7
Methanol	0.5

0.5 ml of diethyl ether. The relative yields obtained from the gas chromatograms are shown in Table I. It is assumed that the reaction is influenced by the presence or absence and number of fluorine atoms in the acid anhydride structure. The most suitable reagent for the dehydration of nicotinamide was HFB, and it gave good chromatogram (Fig.1A). Ethyl acetate, acetone, diethyl ether, acetonitrile, benzene, hexane and methanol were tried as reaction solvents in the dehydration of nicotinamide. The most suitable was diethyl ether and the least suitable was methanol, as shown in Table II. We chose diethyl ether because of its good solvent properties for 3-cyanopyridine and 1,2,4-trichlorobenzene.

Gas chromatographic sensitivity

Columns containing Advance (2%, w/w), DEGS (5%, w/w), OV-17 (5%, w/w), OV-330 (5%, w/w), PEG-20M (5%, w/w), SE-30 (5%, w/w) and XE-60 (3%, w/w) on Chromosorb W AW DMCS were tested. All of the columns showed the peak of 3-cyanopyridine; particularly good peak characteristics and sensitivity were achieved with OV-17 under the conditions described above. A low temperature and a long column were preferable for the GLC of the 3-cyanopyridine. At 130°C, a 2.0-m column containing OV-17 on Chromosorb W AW DMCS gave a good gas chromatogram; the retention times of 3-cyanopyridine relative to that of the internal standard was 0.53. This GC determination of nicotinamide after dehydration with HFB was six times more sensitive than the direct determination reported by Aoyama *et al.*[3]. After dehydration, the reaction mixture should be injected into the gas chromatograph as soon as possible; at room temperature the sample was stable for at least 5 h, but after 24 h the content of 3-cyanopyridine had decreased to 96.3%.

Influence of evaporation of the solvent on the recovery of nicotinamide and 3-cyanopyridine

Prior to dehydration of nicotinamide it was necessary to evaporate the acetonitrile in order to change to diethyl ether as the preferred reaction solvent. A 2-ml volume of acetonitrile containing 100 μg of nicotinamide was evaporated under reduced pressure at 40°C for 10 min. No loss of nicotinamide during or after the evaporation was observed. On the other hand, when the evaporation was performed at room temperature in order to eliminate excess of HFB after dehydration, a significant decrease in the amount of 3-cyanopyridine was observed. The losses of 3-cyanopyridine as a function of evaporation time at room temperature were 9.3% after 2 min, 21.2% after 5 min, 35.7% after 10 min and 68.3% after 20 min. Therefore, the dehydrated sample was injected directly into the GC column without evaporation.

Interferences

Nicotinamide can be extracted from foods with acetonitrile[1–4] and this simple and rapid extraction permits the determination of nicotinamide in foods by GLC without effects from interfering substances. To investigate the effects of various possible interferents, 100-μg portions of nicotinamide were added to various amounts of substances, and each mixture was analysed by direct dehydration without clean-up. As shown in Table III, none of them had much effect on the determination. Another possible interferent is the water derived from the samples, but the addition of 1–10 g of water to 100 μg of nicotinamide did not affect the determination. It could be presumed

TABLE III

INFLUENCE OF VARIOUS SUBSTANCES ON RECOVERY OF NICOTINAMIDE

Each amount of substance was added to 100 μg of nicotinamide dissolved in 0.5 ml of diethyl ether. Reaction and GLC conditions as in the described procedure.

Substance	*Amount added (μg)*	*Recovery of nicotinamide (%)*
Uracil	50	98.5
	100	97.1
Cytosine	50	94.2
	100	92.7
Adenine	50	95.3
	100	94.6
Guanine	50	94.7
	100	95.3
Xanthine	50	99.7
	100	107.1
5'-Inosinic acid	50	101.2
	100	105.0
Adenosine 5'-(tetrahydrogentriphosphate) (ATP)	50	99.2
	100	98.0
Nicotinic acid	50	97.0
	100	100.3
Sorbic acid	50	99.1
	100	101.0
Butylhydroxyanisole	50	101.0
	100	99.7
Butylhydroxytoluene	50	97.5
	100	95.5
Benzoic acid	50	99.1
	100	100.0
p-Hydroxybenzoic acid	50	98.9
	100	99.7

TABLE IV

RECOVERY (%) OF NICOTINAMIDE ADDED TO VARIOUS MEATS AND MEAT PRODUCTS

Each result is the average of five determinations.

Sample	*Amount of nicotinamide added (μg/g)*			
	10	*20*	*50*	*100*
Chicken	98.4	97.6	98.0	99.1
Pork	97.3	98.7	104.6	98.0
Beef	93.4	95.7	94.7	96.4
Tuna	100.3	101.6	98.1	99.6
Ham	94.7	95.3	98.0	101.1

TABLE V

COMPARISON OF GC AND HPLC METHODS WITH THE PROPOSED METHOD FOR THE DETERMINATION OF NICOTINAMIDE IN VARIOUS MEATS AND MEAT PRODUCTS

Each sample was purchased commercially and each result is the average of four determinations.

Sample	*Nicotinamide (ppm)*		
	GC method[1]	*HPLC method*[2]	*Proposed method*
Chicken	98.6	88.6	96.6
Pork	45.2	44.6	41.8
Beef	20.5	17.4	25.4
Tuna	131.8	121.3	119.0
Ham	32.3	28.4	31.0

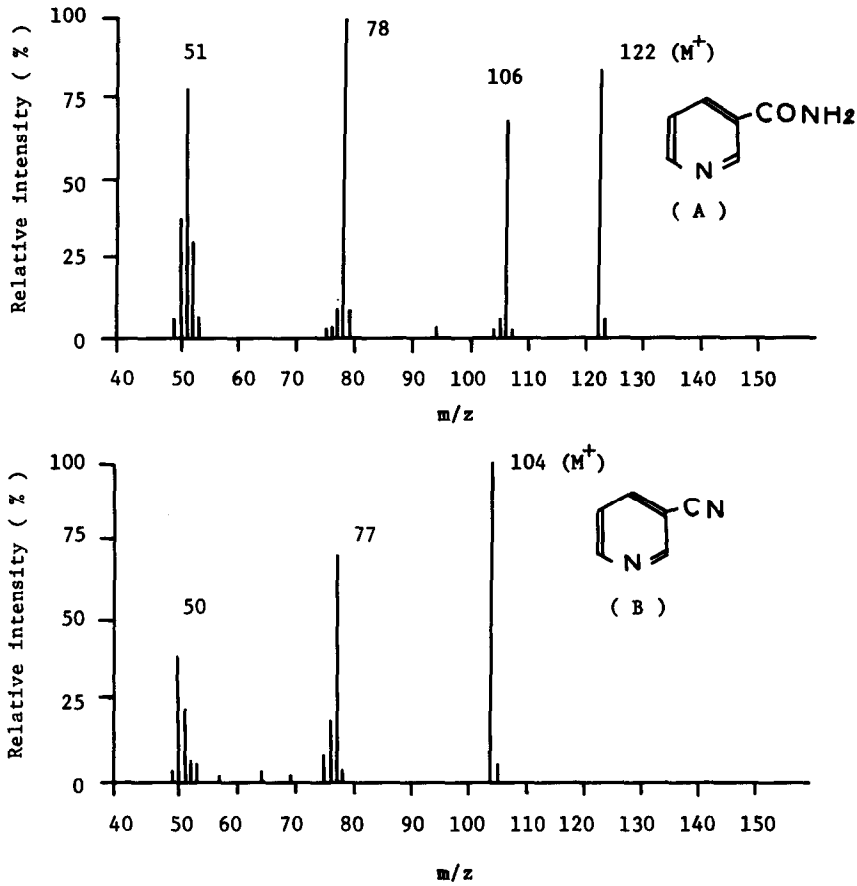

Fig. 5. Mass spectra of (A) nicotinamide and (B) its dehydration product.

that the water was eliminated during or after the evaporation of the acetonitrile extract of nicotinamide. Therefore, further dehydration of the acetonitrile extract was not necessary.

The method described was tested on the analysis of chicken and tuna meat and the results of the analysis of the sample digest by GLC after the formation of 3-cyanopyridine are shown in Fig. 1B and C. The dehydrated extract obtained from both meats gave gas chromatograms with good peak characteristics.

Recoveries and application

Nicotinamide (10, 20, 50 and 100 μg) added to 10-g samples of chicken, pork, beef, tuna and ham chopped and then ground in a porcelain pestle and mortar was determined by the proposed method. Five determinations were carried out at each concentration. As shown in Table IV, the average recovery was 98.0% and ranged from 93.4% at the lower concentrations to 104.6% at the higher concentrations. The detection limit was 5 ppm.

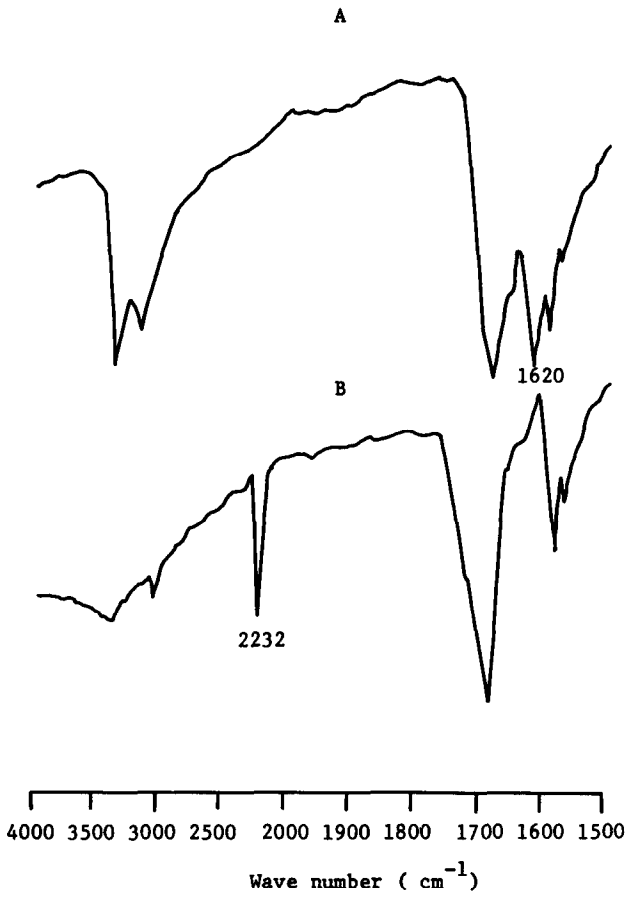

Fig. 6. IR spectra of (A) nicotinamide and (B) its dehydration product in potassium bromide.

Further, the proposed method was compared with another GC method[1] and an HPLC method[2]. The results from the different procedures correlated well (Table V) and it is concluded that the proposed method is suitable for routine use.

Identification of the dehydration product of nicotinamide

The mass spectrum of the product derived from the dehydration of nicotinamide by HFB was identical with the standard spectrum of nicotinamide, with ion peaks at m/z 122 (M^+), 106 ($M^+ - NH_2$), 78 ($-CO$) and 51 ($-HCN$), as shown in Fig. 5A. The mass spectrum corresponding to the peak obtained by dehydration of nicotinamide is shown in Fig. 5B, with m/z 104 (M^+), 77 ($M^+ - HCN$) and 50 ($-HCN$). The parent peak (m/z 122) for nicotinamide and that at m/z 104 for the dehydration product correspond to the molecular weight of each compound. The shift of the peaks from m/z 104 to 77 for the dehydration product could be ascribed to removal of a cyano group. Further, the elution time (3.6 min) and the mass fragmentation pattern of the hydrated product agreed with those of the 3-cyanopyridine standard. Partial characteristics of the IR spectra of nicotinamide and its dehydration product are shown in Fig. 6. In the spectrum of the dehydration product, the absorption at 1620 cm^{-1} resulting from the carbonyl group in the nicotinamide spectrum has disappeared, and a medium absorption at 2232 cm^{-1} resulting from a cyanide group is observed.

These experiments confirmed that the dehydration product of nicotinamide was 3-cyanopyridine.

REFERENCES

1 K. Miyano and M. Imaida, *Annu. Rep. Osaka Inst. Public Health*, 13 (1982) 37.
2 K. Yoshida, Y. Yamamoto and M. Fujiwara, *Shokuhin Eiseigaku Zasshi*, 23 (1982).
3 M. Aoyama, M. Tunoda, N. Inoue and A. Hasebe, *Lecture Accumulation of 21st Hygienic Chemistry Technical Conference of Japan*, 1984, p. 40.
4 M. Oishi, K. Onishi, H. Nakamura and S. Sakai, *Annu. Rep. Tokyo Metr. Res. Lab. Public Health*, 35 (1984) 252.
5 R. Sawamura, M. Uchiyama, N. Imura, T. Kawamura, Y. Sayado, Y. Nakazawa and A. Hamada (Editors), *Standard Methods of Analysis for Hygienic Chemists, Japan*, Pharmaceutical Society, Tokyo, 1980, p. 215.
6 R. Sawamura, M. Uchiyama, N. Imura, T. Kawamura, Y. Sayado, Y. Nakazawa and A. Hamada (Editors), *Standard Methods of Analysis for Hygienic Chemists, Japan*, Pharmaceutical Society, Tokyo, 1980, p. 223.
7 R. B. Tona and M. M. Tabekhia, *J. Food Sci.*, 44 (1978) 263.
8 T. A. Tayler and R. R. Shrag, *J. Liq. Chromatogr.*, 3 (1980) 269.