Simultaneous Determination of Nicotinic Acid and Nicotinamide in Cooked Sausages

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A simple and sensitive method for determining simultaneously nicotinic acid and nicotinamide content in cooked sausages by ion-pair reversed-phase liquid chromatography is described. Samples are extracted with ultrapure water, centrifuged, deproteinized with zinc hydroxide, filtered, and chromatographed with UV detection at 261 nm on a 25 cm × 4 mm i.d. Spherisorb ODS-2 cartridge using as mobile phase a mixture consisting of 5 mM heptanesulfonic acid adjusted to pH 3.3 with phosphoric acid and acetonitrile (75:25, v/v). Both vitamins are measured on a reversed-phase column with a single ion-pair reagent. Precision of the method was 0.5 and 1.0% (within a day) and 2.3 and 4.5% (between days) for nicotinic acid and nicotinamide, respectively. The detection limit was 0.300 mg/100 g. The recovery was >92% of nicotinic acid and nicotinamide added to samples of meats. Twenty samples of six different products have been analyzed in duplicate. The mean value for nicotinic acid ranged between 0.908 and 1.267 mg/100 g of fresh weight and for nicotinamide between 1.968 and 2.880 mg/100 g of fresh weight.

Keywords: *Nicotinic acid; nicotinamide; sausages; ion pair reversed phase liquid chromatography*

INTRODUCTION

Niacin belongs to the water soluble vitamin group, being the generic term for two vitamers, nicotinic acid and nicotinamide. Nicotinamide is the active form, which functions as a constituent of coenzymes NAD and NADP. These coenzymes in their reduced form (NADH/NADPH) are central electron carriers in cells and participate in the metabolism of carbohydrates, fatty acids, and amino acids. Preformed niacin (NADH/NADPH) is widely distributed in foods of plant and animal origin, meat and meat products being important sources of this vitamin. All animal species are able to synthesize the metabolically active forms of niacin from an essential amino acid named tryptophan (Henderson, 1983).

Microbiological and chemical methods have been conventionally used for the estimation of niacin in foods. However, they have several disadvantages: they are time-consuming, they are not suitable for the differentiation of supplemented nicotinic acid or nicotinamide (Fontaine and Hörr, 1993), and chemical assay involves reaction with cyanogen bromide, which is a noxious and unstable reagent.

Because analysis by means of high-performance liquid chromatography (HPLC) avoids these problems, it has been used extensively for determining both forms of vitamin. However, the simultaneous determination of nicotinic acid and nicotinamide has been very difficult due to the different basicity and polarity of the two vitamers and/or interference problems, especially in the case of nicotinic acid, caused by other compounds extracted from the matrix. Reversed-phase techniques have been generally carried out; however, to modify the retention times of polar compounds on these columns, ion-pair reagents have to be used with different mobile phases (Toma and Tabekhia, 1979; Skurray, 1981; Kitada et al., 1982; Yoshida et al., 1982; Sakai et al., 1985; Takatsuki et al., 1987; Vidal-Valverde and Reche, 1991; Gigliotti and Daghetta, 1993). However, the results obtained in the above studies show the simultaneous detection of both vitamers was impossible, it being necessary to use different ion-pair reagents for the detection of each vitamer (Takatsuki et al., 1987). We therefore attempted to improve the method for the simultaneous detection.

The objective of this study has been to develop a rapid and reliable HPLC method with ultraviolet (UV) detection for the simultaneous quantitation of nicotinic acid and nicotinamide and to apply it to difficult matrices such as cooked sausages, trying to use only one ion pair for the measurement of both vitamers to simplify the assay and to reduce the analysis time.

EXPERIMENTAL PROCEDURES

Apparatus and Liquid Chromatographic Conditions. A model HP1090 high-performance liquid chromatograph (Hewlett-Packard) was used. The chromatographic column was a 25 cm × 4 mm i.d. stainless steel cartridge (Teknokroma) packed with Spherisorb ODS-2, 5 μm. A 10 μL volume of eluate was chromatographed, using a mixture of 75% heptanesulfonic acid solution (5 mM, pH 3.3) and 25% acetonitrile as mobile phase, isocratically pumped at a flow rate of 0.650 mL/min. The oven temperature was 35 °C. The measurements of nicotinic acid and nicotinamide were made at 261 nm. Quantification of the chromatograms was based on peak areas using external standard calibration curves.

Reagents. Nicotinic acid was obtained from Merck (Darmstadt, Germany) and nicotinamide from Sigma Chemical Co.

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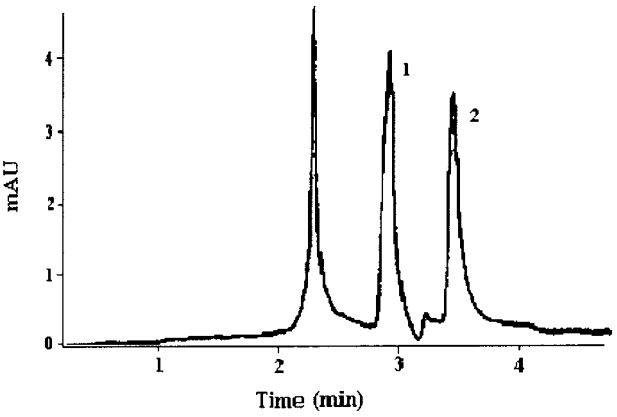

Figure 1. Chromatogram of (1) nicotinic acid (50 μg; 2.972 min) and (2) nicotinamide (50 μg; 3.515 min) standards. See text for chromatographic conditions.

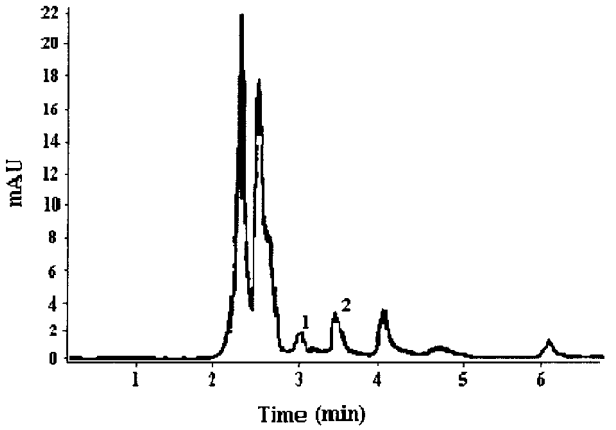

Figure 2. Chromatogram of (1) nicotinic acid (1.247 mg/100 g; 2.973 min) and (2) nicotinamide (2.416 mg/100 g; 3.536 min) determination in a cooked sausage of "chopped turkey". See text for chromatographic conditions.

Table 1. Calibration Data for Nicotinic Acid[a]

retention time (min)	amount (μg)	area
2.972	20.0	10.07
	50.0	25.98
	100.0	50.49
	200.0	105.69
	300.0	160.23

[a] $r^2 = 0.9998$; linear regression $= -0.955 + 0.535$ (amount).

(St. Louis, MO). Heptanesulfonic acid was also obtained from Sigma Chemical Co. Acetonitrile was of HPLC grade, and all other reagents were of analytical grade.

Samples. Six commercially purchased cooked sausages were analyzed: "lunch", "chopped pork", "chopped beef", "chopped turkey", "vitamined chopped", and "Sicilian mortadella". All of them are composed of meats, fat, water, sugars, salt, different spices, and some additives such as preservatives. They mainly differ in meat composition (greater or lesses quantities of pork, beef, and turkey meats, depending on the meat product) and grinding degree.

Sample Preparation. Niacin was extracted as given by the Takatsuki et al. (1987) method, modified for our purpose. Ten grams of finely ground sample was weighed in duplicate into two separate 50 mL beakers. Thirty milliliters of deionized water was added, and the mixtures were then homogenized by stirring at room temperature for 2 min. Contents were transferred to a 100 mL volumetric flask washing beaker twice with water and made up to volume with water. Samples were centrifuged for 5 min at 3000 rpm, and the upper solution was filtered through Albet No. 1305 filter paper. Twenty milliliters of filtered extract was brought to 25 mL in a volumetric flask, and to precipitate the proteins, 1 mL of saturated zinc sulfate solution and 1 mL of 1 N sodium hydroxide solution were added successively. The samples were brought to volume with water, mixed well, and allowed to stand for 30 min at room temperature. Solutions were filtered through Albet No. 1305 filter paper and refiltered through Millipore filters (0.45 μm) into amber vials for liquid chromatography analysis.

Procedure with Standard Solutions. A stock solution of 1000 μg/mL nicotinic acid and nicotinamide in ultrapure HPLC water generated by a Milli-RO4 coupled to a Milli-Q water purification system (Millipore, Bedford, MA) was prepared and stored in darkness in a refrigerator. Working standard solutions (50 and 500 μg/mL) were prepared daily before use. Aliquots of these solutions were treated as samples. The resulting peak areas of each vitamin were plotted against concentration (from 20 to 300 μg) for the calibration curves. The vitamin contents of the sample extracts were obtained by interpolation on the standard curve.

Table 2. Calibration Data for Nicotinamide[a]

retention time (min)	amount (μg)	area
3.515	20.0	8.74
	50.0	22.00
	100.0	43.94
	200.0	92.92
	300.0	143.42

[a] $r^2 = 0.9996$; linear regression $= -1.547 + 0.478$ (amount).

Table 3. Recoveries of Nicotinic Acid Added to Meat Product by HPLC

	before addition (mg/100 g)	amount added (mg/100 g)	amount found (mg/100 g)	recovery[a] (%)
chopped turkey	1.247	0.500	1.653	94.4
	1.254	0.500	1.631	93.1
	1.258	0.500	1.644	93.9
	1.245	1.000	2.106	93.6
	1.256	1.000	2.094	93.0
	1.243	1.000	2.130	94.6
mean $\pm$ SD	1.251 ± 0.006			93.8 ± 0.7

[a] Percent recovery = (amount found − mean value)/amount added × 100.

Table 4. Recoveries of Nicotinamide Added to Meat Product by HPLC

	before addition (mg/100 g)	amount added (mg/100 g)	amount found (mg/100 g)	recovery[a] (%)
chopped turkey	2.476	1.500	3.890	92.3
	2.520	1.500	3.894	92.6
	2.540	1.500	3.906	93.4
	2.480	2.500	4.884	95.2
	2.501	2.500	4.862	94.3
	2.511	2.500	4.857	94.1
mean $\pm$ SD	2.505 ± 0.024			93.7 ± 1.1

[a] Percent recovery = (amount found − mean value)/amount added × 100.

RESULTS AND DISCUSSION

For the liquid chromatographic conditions the choice of an adequate method is critical to allow the simultaneous analysis of nicotinic acid and nicotinamide in foods. Ion-pair reversed-phase chromatography using heptanesulfonic acid was tried with a Spherisorb ODS-2 cartridge stationary phase. Nicotinic acid and nicotinamide were optimally eluted with the simultaneous detection of both vitamers possible with a single mobile phase in a reasonably short time. Takatsuki et al. (1987) had to use different ion-pair reagents for the determi-

Table 5. Nicotinic Acid and Nicotinamide Contents in Cooked Sausages[a]

meat sample	*N*	water content (% ± SD)	nicotinic acid content (mg/100 g of FW ± SD)	nicotinamide content (mg/100 g of FW ± SD)
lunch	20	64.75 ± 0.63	1.061 ± 0.056	2.296 ± 0.187
chopped pork	20	70.70 ± 0.53	ND	2.650 ± 0.232
chopped beef	20	70.51 ± 0.57	ND	2.880 ± 0.183
chopped turkey	20	71.81 ± 0.45	1.267 ± 0.045	2.525 ± 0.130
vitamined chopped	20	70.60 ± 0.56	1.424 ± 0.084	8.694 ± 0.629
Sicilian mortadella	20	59.53 ± 0.72	0.908 ± 0.027	1.968 ± 0.140

[a] All samples were analyzed in duplicate. *N* is the number of different samples. FW indicates fresh weight. ND indicates not detectable.

nation of each vitamer with a similar method. Hamano et al. (1988) used cation exchange chromatography for their simultaneous detection. Other researchers studied only one compound.

Given the amphoteric nature of niacin, the retention times of the peaks are extremely sensitive to the pH of the mobile phase (McKee, 1982). The influence of pH on the retention times was studied (pH 3.3, 5.0, and 7.0), and results showed that the best separation of both vitamers was achieved with the lower pH. It was observed that the retention times of both compounds increased with decreasing pH. Peaks were clearly resolved with a Spherisorb ODS-2 cartridge stationary phase using a mixture of acetonitrile and 5 mM heptanesulfonic acid adjusted to pH 3.3 with phosphoric acid (25:75, v/v) as the mobile phase. Nicotinic acid was eluted in 2.969 min and nicotinamide was eluted in 3.510 min with a flow rate of 0.650 mL/min. With this method, retention times obtained are lower than those obtained by Hamano et al. (1988). Figures 1 and 2 show chromatograms of both vitamers in the standard and in a meat sample ("chopped turkey").

Standard curves were prepared between days (for 12 times over 3 months) to verify the applicability of this method to the quantitative evaluation of niacin in a sample. The calibration curves derived from the peak areas were linear in the working range from 20 to 300 μg, having linear regression coefficients ~0.9998 for nicotinic acid ~0.9996 for nicotinamide. Results were extremely satisfactory. Tables 1 and 2 show the calibration data for both forms of the vitamin. The detection limit was found to be 0.300 mg/100 g with a signal-to-noise ratio of 3. Sensitivity was adequate to allow the measurement of natural contents in the samples studied.

The precision expressed by the coefficient of variation was tested, the variation within a day being 0.5 and 1.0% when nicotinic acid and nicotinamide contents were determined in six aliquots of the same sample studied in parallel. The variation between days (10 samples analyzed in duplicate over 3 months) was 2.3 and 4.5% on average, respectively. The recovery test was made on a representative sample by adding two standard concentrations of nicotinic acid (Table 2) and nicotinamide (Table 3), resulting in recoveries ranging from 93.0 to 94.6% for nicotinic acid and from 92.3 to 95.2% for nicotinamide.

In the present study the nicotinic acid and nicotinamide contents were determined according to the HPLC method above developed in six commercially cooked sausages: "lunch", "chopped pork", "chopped beef", "chopped turkey", "vitamined chopped", and "Sicilian mortadella", collected weekly from a food factory during a 4 month period. Results obtained in the food samples studied appear in the Table 5 . Each value represents an average of 20 samples analyzed in duplicate. Niacin levels in the samples were constant during the period studied. In all samples except for "vitamined chopped" (with vitamins added), values ranged from 0.908 to 1.267 mg/100 g for nicotinic acid and from 1.968 to 2.880 mg/100 g for nicotinamide, so the values were not substantially different. They were considered to be within naturally occurring levels.

The preparation of an appropriate ion-pair reversed-phase chromatography method for the best extraction and simultaneous determination of nicotinic acid and nicotinamide in meat products has been interesting because previous procedures either studied only one compound or used different chromatographic conditions and ion-pair reagents for the determination of each vitamer. Hence, we attempted the simultaneous detection in one assay and with only one ion-pair reagent. Furthermore, rigorous control of the pH of the mobile phase ensured optimum peak within certain limits.

In conclusion, the proposed HPLC method provides rapid and efficient quantification of both vitamers and can be applied to the routine assay of such samples, the precision, accuracy, and sensitivity of this method being satisfactory.

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