Determination of Available Niacin in Legumes and Meat by High-Performance Liquid Chromatography

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A high-performance liquid chromatographic (HPLC) method was developed for determining the available niacin in legumes (chickpeas, lentils, beans, green beans) and meat. Samples were subjected to both acidic and enzymatic extraction. The sample extracts were purified through an acidic Dowex 1-X8 resin. A reversed-phase liquid chromatography column with tetrabutylammonium bromide dissolved in a buffer solution was used as the mobile phase in the analysis. Chromatographic conditions specific to each sample were determined to avoid interference by other substances. The niacin was detected at 254 nm.

INTRODUCTION

Pellagra, first described by Casal in 1735 in Asturias (Spain), is a nutritional disease brought about by deficient tryptophan and niacin in the diet. Niacin is present in foods in several forms. As nicotinic acid, nicotinamide, and the nicotinamide of the nucleotide coenzymes NAD and NADP, it is available to man. It may also be present as nicotinoyl esters (niacytin) (Bender and Bender, 1986) or NADH (Wall and Carpenter, 1988), which are not metabolically available. In addition, the tryptophan from the proteins in the diet is metabolized to quinoleic acid, which can act as a precursor in the synthesis of nicotinamide nucleotides (Bender and Bender, 1986).

Determination of the available niacin in foods is one of the first steps in establishing a diet sufficient in this vitamin. Chemical, microbiological, and HPLC methods of analyzing niacin exist. Chemical assay depends on reaction with cyanogen bromide to yield a derivative of pyridinium. This reaction is not specific to niacin but is common to all substituted pyridines, including bound niacin. Two organisms are widely used in microbiolgical assays; one, *Leuconostoc mesenteroides*, has a specific requirement for nicotinic acid but is not suitable for measuring nicotinamide, while the other, *Lactobacillus plantoides*, can be used to measure not only the two vitamers nicotinic acid and nicotinamide but also other derivatives of nicotinic acid that have no vitamin activity in man (Goldsmith and Miller, 1967). As a result, analysis of niacin by chemical and microbiological methods tends to yield higher niacin values.

Analysis of niacin in food by means of HPLC has generally been carried out by using a reversed-phase column and ion-pair reagents in the mobile phase. When the purpose of the analysis is to determine the available niacin in foods, an acid hydrolysate is preferred, since an alkaline procedure would release nonavailable niacin. One of the first papers on the HPLC analysis of niacin in foods was by Toma and Tabeckia (1979) for rice and rice products. Single-column methods, using UV detection, suffer the drawbacks of low sensitivity and the presence of interfering compounds (Finglas and Faulks, 1987). A method of column switching has been developed in an attempt to overcome this problem (Snyder and Kirkland, 1979), and it was applied successfully by van Niekerk et al. (1984) to analyze a range of foods, including spaghetti, rice, maize, soybeans, and mushrooms.

Finglas and Faulk (1984) used gradient elution programs to analyze niacin in raw and cooked potatoes. The HPLC values obtained were approximately 50% lower than those given in food tables, which were obtained by microbiological assay. The system could not be extended to other vegetables because of the presence of other interfering compounds. Van Niekerk et al. (1984) pointed out that HPLC techniques afforded an advantage over microbiological methods, because part of the niacin in the acid extract was not present as free niacin but did exist in a form available only to the microorganisms. Other authors (Trugo et al., 1985; Finglas and Faulks, 1987) have also concluded that HPLC offers clear advantages over existing methods of chemical or microbiological assay for determining the available niacin in food systems.

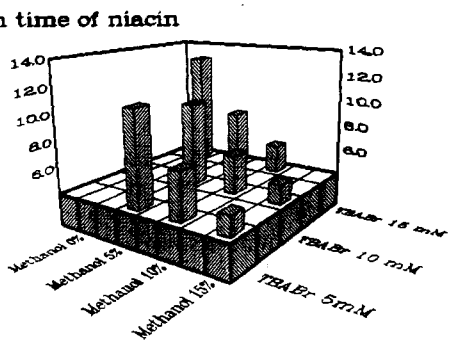

Figure 1. Influence of tetrabutylammonium bromide (TBABr) and methanol concentration in the mobile phase on the retention time of niacin.

The object of this paper is to propose an HPLC method with a single column and UV detection that can be applied to different foods. The method overcomes the problems relating to interference and sensitivity mentioned above.

EXPERIMENTAL PROCEDURES

Apparatus. A Waters Associates liquid chromatograph equipped with a M-510 pump, a U6K injector, and a 440 Model UV absorbance detector, provided with a filter at 254 nm, was used. The detector signal was recorded on a Houston Instrument Omniscribe recorder. A precolumn 3.2 mm i.d. × 4.0 cm packed with C_{18} Porasil B (Waters Associates) was also used. The chromatographic column was a 3.9 mm i.d. × 30 cm stainless steel column packed either with μBondapack C_{18} (10 μm) (Waters Associates) or with ODS2 Spherisorb (10 μm) (Spheris).

Reagents. The mobile phase (pH 4.72) was prepared by dissolving tetrabutylammonium bromide (Fluka) (0.005 mol/L)

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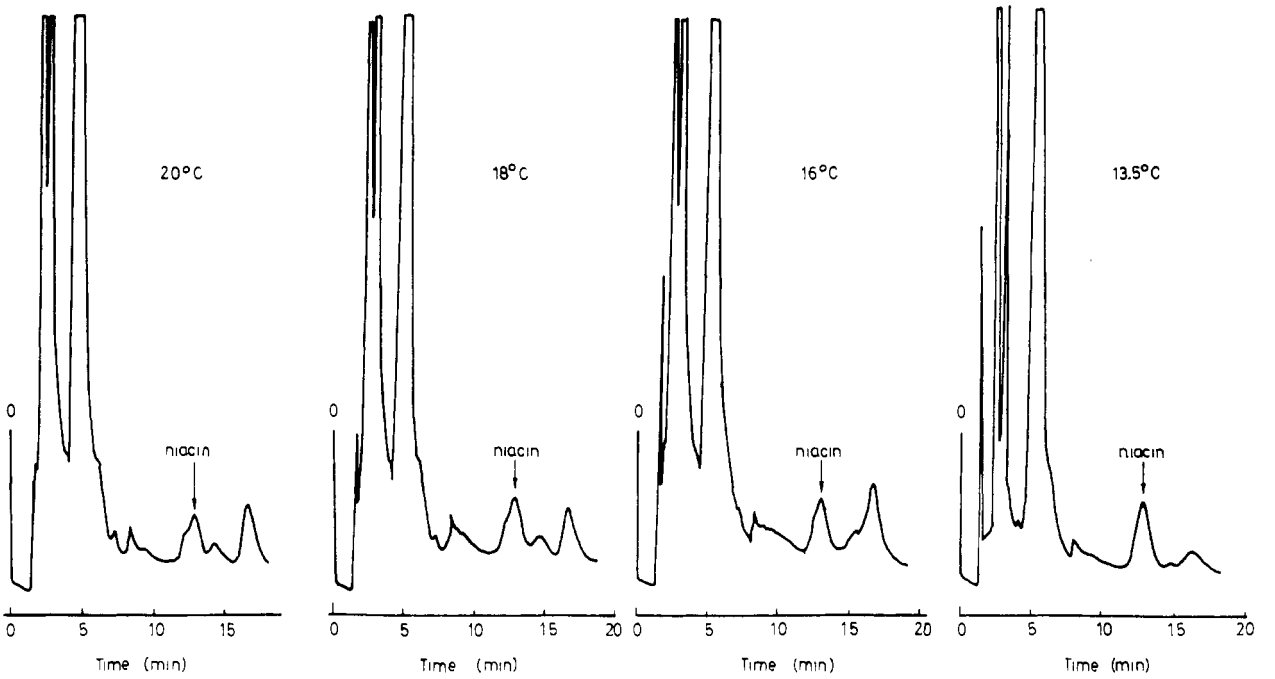

Figure 2. Effect of column temperature on the shape of niacin peaks in a lyophilized sample of green beans. Absorbance range 0.005; flow rate 1.4 mL/min; detection at 254 nm.

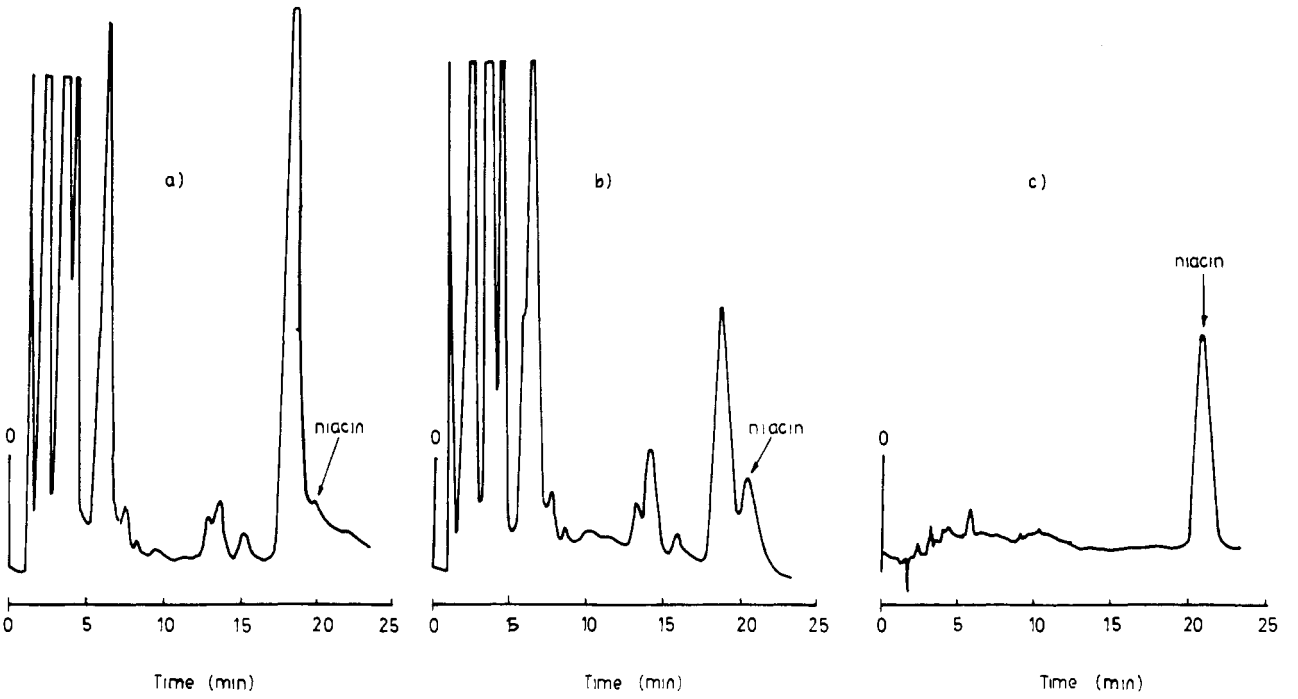

Figure 3. Effect of pH of the mobile phase on the retention time of niacin in a chickpea sample: (a) niacin peak for a chickpea sample at a mobile phase pH of 5.5; (b) the same sample after spiking with nicotinic acid standard; (c) the niacin standard. ODS2 Spherisorb (10 μm) column; absorbance range 0.01; flow rate 1.5 mL/min; detection at 254 nm.

in a mixture (1:9 v/v) of methanol and 0.01 mol/L sodium acetate buffer (pH 4.66).

Standard stock solution of nicotinic acid (Sigma) was prepared by dissolving 40 mg of niacin in 500 mL (80 μg/mL).

The takadiastase enzyme suspension was prepared by dissolving 6 g of takadiastase (Serva) in 100 mL of distilled water.

Extraction of Food Samples. Sufficient ground materials (1–10 g, equivalent to 10–100 μg of niacin) were hydrolyzed with 0.1 N HCl (30 mL) and 6 N HCl (1 mL) in an autoclave at 121 °C for 15 min. The pH of the solution was then adjusted to 4.0–4.5 with 2 N sodium acetate; 5 mL of freshly prepared aqueous enzyme solution (6% takadiastase) was added, and the samples were incubated at 48 °C for 3 h. The sample solution, once cooled, was filtered through Whatman No. 40 filter paper and diluted to 100 mL with distilled water.

Purification of Sample Extracts. The extract was readjusted to pH 4.7 ± 0.02 with 2 N sodium acetate. An aliquot of this extract containing 1.2–3.6 μg was passed through an ion-exchange column (1.0 i.d. × 3.0 cm) packed with Dowex 1-X8 (BDH, 18–52 mesh) in acid form. The column was washed with distilled water (30 mL) followed by 0.15 N HCl (30 mL). Evaporation of the acid solution under vacuum yielded the purified extract. The residue was taken up with a mixture (1:9 v/v) of methanol and 0.1 mol/L sodium acetate buffer of the required pH (4.77–5.0) (see Table I) to obtain a final range of pH 4.7–4.9, optimum to carry out a correct separation.

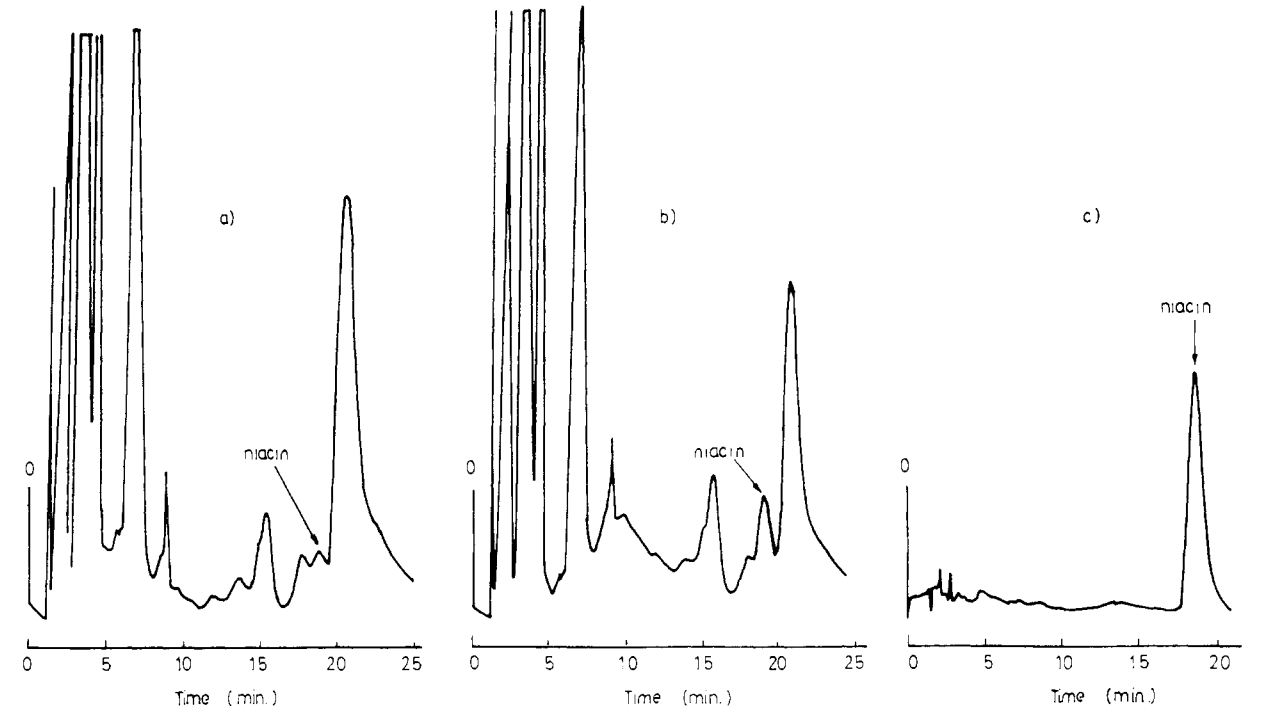

Figure 4. Effect of pH of the mobile phase on the retention time of niacin: (a) niacin peak for a chickpea sample at a mobile phase pH of 5.04; (b) the same sample after spiking with nicotinic acid standard; (c) the niacin standard. ODS2 Spherisorb (10 μm) column; absorbance range 0.01; flow rate 1.5 mL/min; detection at 254 nm.

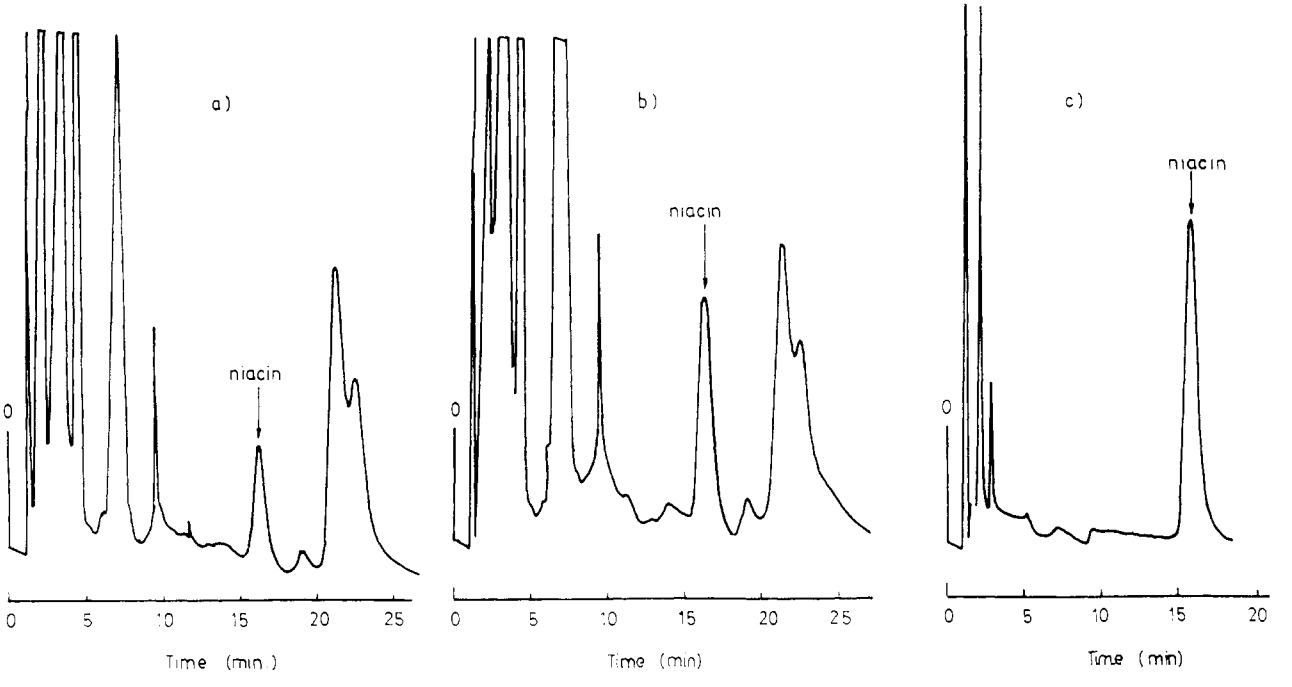

Figure 5. Effect of pH of the mobile phase on the retention time of niacin: (a) niacin peak for a chickpea sample at a mobile phase pH of 4.72; (b) the same sample after spiking with nicotinic acid standard; (c) the niacin standard. ODS2 Spherisorb (10 μm) column; absorbance range 0.01; flow rate 1.5 mL/min; detection at 254 nm.

Procedure with Standard Solutions. Standard solutions of nicotinic acid (80 μg/mL) were prepared. Four aliquots of the standard solution (ranging between 40 and 120 μg) were subjected to the extraction and purification procedures described above. Aliquots of 50–100 μL of these processed solutions were injected and the data used to obtain the calibration curve by plotting peak height vs concentration. Linear least-squares analyses were performed to determine the best fitting line. The niacin content of the sample extracts was obtained by interpolation on the corresponding standard curve.

RESULTS AND DISCUSSION

Samples consisted of beans, chickpeas, lentils, lyophilized green beans (from two different batches) and lyophilized pork muscle (from two different batches). Table I summarizes the available niacin content in these food samples.

By use of standard niacin solutions, processed in the same manner as the food samples, a regression line ($y = a + bx$) ($a = -0.108$, $b = 0.071$) was obtained by HPLC.

Table I. Available Niacin Content and Chromatographic Conditions of Samples Analyzed by HPLC

foods	niacin,[a] mg/100 g wet matter	niacin, mg/100 g dry matter	column temp, °C	pH of buffer sample injection
niacin[b]			g	4.77
chickpeas	1.29 ± 0.06	1.33	23.0–24.0	4.94
beans	1.15 ± 0.16	1.19	18.0	4.94
lentils	1.19 ± 0.11	1.23	23.0–24.0	4.94
green beans[c]	4.66 ± 0.09	4.70	13.5	5.00
green beans[d]	4.39 ± 0.31	4.46	13.5	5.00
pork muscle[e]	11.26 ± 0.50	11.52	18.0	4.94
pork muscle[f]	10.84 ± 0.82	11.02	18.0	4.94

[a] Means of 5–9 replicates. [b] Standard niacin submitted to the extraction and purification procedure. [c,d] Different batches of lyophilized samples. [e,f] Different batches of lyophilized samples. [g] As indicated for each food sample.

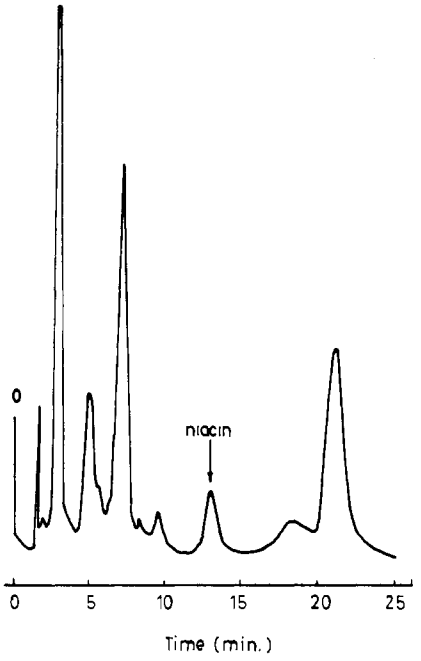

Figure 6. Chromatogram of lyophilized pork muscle sample. Absorbance range 0.005; flow rate 1.4 mL/min; detection at 254 nm.

The correlation coefficient was 0.999, and the standard estimation error (s) was 0.063. The detection limit ($3s/b$) obtained (Miller and Miller, 1986) was 2.66 ng.

According to the literature (Toma and Tabeckia, 1979; Skurray et al., 1981; van Niekerk et al., 1984; Dawson et al., 1988), acid hydrolysis may be followed by enzymatic hydrolysis, although some workers have used acid hydrolysis alone to determine the available niacin (Kral, 1983). Enzymatic hydrolysis was absolutely necessary in the case of the legume samples, since the hydrolysate was extremely thick, probably because of the high starch content. For the sake of consistency, it was also applied to the rest of the samples.

Samples from food matrices usually require a purification step prior to chromatographic determination. Omission of this purification step led to consistent overlap of the niacin peak with the peaks from the food matrix, a finding previously reported by McKee et al. (1982) and Finglas and Faulks (1987).

Several purification methods were tested. Standard solutions of niacin were first used to check the recovery. Once an acceptable level of recovery had been achieved, food samples were employed to study the performance of the purification methods. A recovery level of 60% was

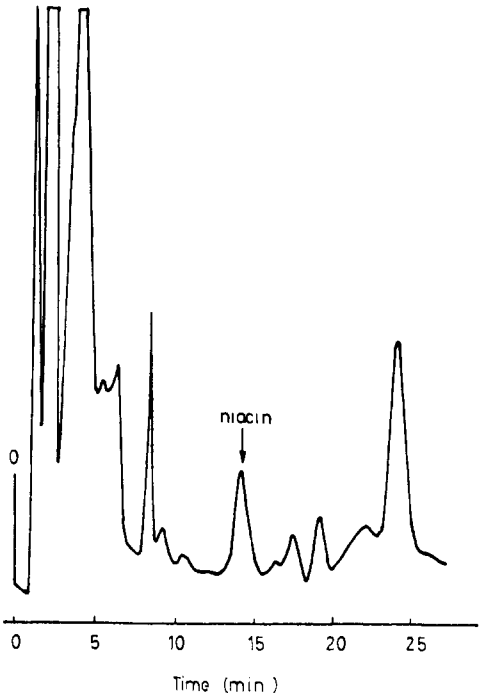

Figure 7. Chromatogram of niacin in a bean sample. Absorbance range 0.01; flow rate 1.5 mL/min; detection at 254 nm.

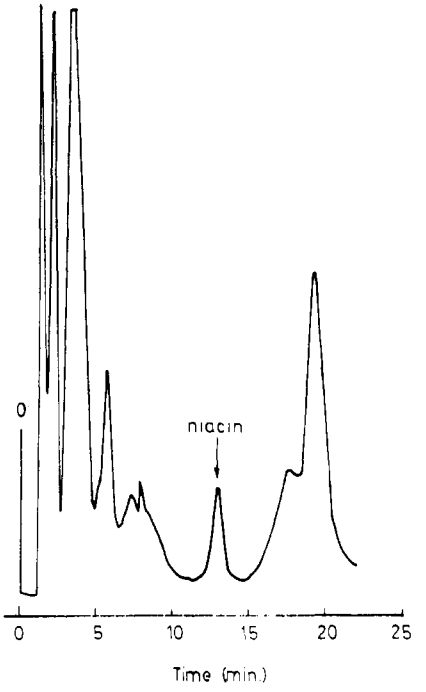

Figure 8. Chromatogram of niacin in a lentil sample. Absorbance range 0.01; flow rate 1.5 mL/min; detection at 254 nm.

obtained by using a C_{18} cartridge (Waters Associates) as a cleanup device, as recommended by Toma and Tabeckia (1979) and Trugo et al. (1985). A CN cartridge (Waters Associates) yielded higher recoveries (98%) but failed to remove interfering substances. Oxidation with $KMnO_4$, as recommended by Tyler and Shagro (1984), was also ineffective for the samples employed.

Ion-exchange columns were an additional choice for purification. Amberlite CG_{50} (Fluka, 100–200 mesh) gave good recoveries with the standard solutions but failed to achieve a satisfactory purification of the food samples. An AG1-X8 acetate resin (100–200 mesh, Bio-Rad) column, used by Tyler and Shagro (1980) for the purification of

cereals, was not available to the authors. The use of Dowex 1-X8 acetate resin (18–52 mesh) gave modest recoveries (65–70%) for the standard solutions. The recovery level climbed to 98% when conditioning of the resin was carried out with 1 N HCl. Nevertheless, purification of the food samples was dependent on column length. Optimum purification was achieved by using 3-cm columns. Longer columns resulted in poorer separations.

The choice of an appropriate stationary phase is critical to successful analysis of the niacin in foods. Methods based on ion-exchange columns have been used only occasionally. To obtain an acceptable separation, such a column was coupled to a reversed-phase column by means of a switching device (van Niekerk et al., 1984). Given the polar nature of niacin, normal phase chromatographic systems would require long analysis times. The low affinity for the stationary phase of reversed-phase chromatographic systems causes this vitamin to move with the solvent front or to yield poorly defined peaks, usually with severe tailing (Hengen and de Vries, 1985). Initial results using 5–10% methanol in water as the mobile phase with a μBondapack C_{18} column resulted in a very small K' for the niacin peak and severe overlapping. Ion suppression and ion pairing are two possible ways of modifying the retention times of polar substances in reversed-phase systems. Longer retention times were required to separate niacin from the rapid eluters in the food samples. Sulfonic acid salts have been employed by different workers as counterions (Sood et al., 1977; Kirchmeier and Upton, 1978; Toma and Tabeckia, 1979; Skurray, 1981; Sukai et al., 1985). Sodium hexanosulfonate and sodium heptanosulfonate were added, either separately or mixed together, to a methanol/acetic acid/water mobile phase in a μBondapack C_{18} column, with little effect on the K' of the niacin standard (0.5–0.6). The retention times for the niacin were still very short, and the vitamin eluted with overlapping impurities from the food sample extracts.

The addition of tetrabutylammonium bromide (TBABr) to a methanol/water mobile phase or the increase of the methanol component in the mobile phase in a μBondapack C_{18} column resulted in a net reduction in the retention time of the niacin (see Figure 1). Good separation of the niacin in the food sample extracts was achieved with a μBondapack C_{18} or ODS-2 column, but under the conditions described herein the column life of the former column was very short.

The shape and retention time of the niacin peak were extremely sensitive to the pH of the mobile phase (McKee et al., 1982) and consequently to column temperature because of the amphoteric nature of niacin. Each different food required a trial and error procedure to ascertain the column temperature for optimum separation (see Table I). Figure 2 shows the effect of column temperature on the shape of the niacin peak from a lyophilized green bean sample. A column temperature of 13.5 °C was selected as optimum for the niacin determination with this sample. The sample was also spiked with added nicotinic acid, which produced the expected increase in all peak areas. Rigorous control of the pH of the sample and the mobile phase gave chromatograms with well-defined niacin peaks that were clearly resolved from neighboring peaks, as shown by Figures 3–5 for the chickpea extract. The identity of the peak was confirmed by the chromatograms of the spiked samples shown in Figures 3b, 4b, and 5b and the chromatograms of the pure standard shown in Figures 3c, 4c, and 5c. An optimum pH of 4.72 for the mobile phase was selected for the determination of the sample of chickpea extract.

The available niacin content of the various foodstuffs and the optimum column temperature for each analysis are shown in Table I and in Figures 6–8.

A determination of a mixture of niacin, riboflavin, and thiamin standards was also carried out by using ODS-2 Spherisorb (10 μm) or μBondapack C_{18} columns with different mobile phases. However, interference by the peaks from the food matrices precluded the same type of simultaneous determination of the three vitamins in these food samples that had been carried out previously for multivitamin blends (Sood et al., 1977; Kirchmeier and Upton, 1978). In this case, the determination of each of these vitamins required a specific procedure for the cleanup step.

In conclusion, the purification method used provided a clean separation of niacin from interfering matrix peaks, and the chromatographic conditions established herein improved previous results obtained for foodstuffs (van Niekerk et al., 1984; Dawson et al., 1988) and required shorter analysis times than the column switching method (van Niekerk et al., 1984). Proper manipulation of sample pH and column temperature for each food sample ensured optimum peak shape and, within certain limits, control over the retention time of niacin with respect to neighboring interfering peaks.

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