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Note

Quantitative separation of B-6 vitamers in selected foods by a gas–liquid chromatographic system equipped with an electron-capture detector

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Gas chromatography (GC) is one of the most important and widely used separation techniques to date. Recent advances in the techniques of GC offer potential for the detection and quantitative determination of the various forms of vitamin B-6 in foods and similar materials with satisfactory specificity, sensitivity, convenience, and reduction in analysis time. Several investigators[1–3] have used trimethylsilylation for the analysis of the various B-6 vitamers. Imanari and Tamura[4] examined the GC separation of the trifluoroacetyl derivatives of pyridoxol (also called pyridoxine, PN), pyridoxamine (PM), methyloxime of pyridoxal (PL), and pyriodoxic acid lactone. Sennello and Argoudelis[5] have used N,O-bis-(trimethylsilyl)-acetamide as a derivatizing reagent in the GC determination of vitamin B-6. GC separations of acetylated derivatives of PL, PN, and PM have been reported by Sheppard and Prosser[6], Prosser *et al.*[7], and Korytnyk[8]. The heptafluorobutyryl derivatives of PL, PN, and PM have also been separated with GC by Williams[9]. Patzer and Hilker[10] have recently used a new reagent N-methyl-bis-trifluoroacetamide (MBTFA) for the formation of vitamin B-6 derivatives which offers the advantage of being a rapid, clean, and simple analytical procedure; the hydrochlorides of PL, PN, and PM were used; the detection minimum was at least 250 ng using a flame ionization detector (FID).

Preliminary studies in our laboratory indicated that enhanced sensitivity in the detection of B-6 vitamers in standard solutions was obtained when the electron-capture detector (ECD) was utilized as compared to the FID. We utilized MBTFA as a derivatizing reagent and a GC system equipped with a ^{63}Ni ECD (GC–ECD) for the separation and quantitation of PL, PN, and PM. Preliminary studies demonstrating the application of this technique to the separation and quantitation of naturally occurring PL, PN, and PM in selected foods are also described.

EXPERIMENTAL

Hydrochloride forms of PL, PN, and PM (Sigma, St. Louis, MO, U.S.A.) were used as standards. MBTFA, obtained from Pierce (Rockford, IL, U.S.A.), was used as the derivatizing reagent. MBTFA was used to trifluoroacylate primary and secondary amines, hydroxyl, and thio groups under mild, non-acidic conditions[11]. Pesticide grade absolute ethanol and ethyl acetate were glass-distilled before use.

A stock solution containing a mixture of the three B-6 vitamers (1000 ng/μl each of PL, PN, and PM) were prepared in deionized–distilled water and protected from light. Trifluoroacetylation of the B-6 forms was carried out using a modification of the method of Patzer and Hilker[10]. A 50-μl volume of the aqueous mixture of the three B-6 vitamers was introduced into each of two 1 ml reactivials and dried under a gentle stream of nitrogen at 65°C with the use of a No. 18800 Reactitherm heating module equipped with a No. 18804 Reacti-block (Pierce).

Absolute ethanol, 50 μl, was added to each vial to convert PL to its hemiacetal in order to distinguish it from PN after derivation[3]. The vials were covered with PTFE, silicone discs and sealed with open top screw caps. The contents of the vials were refluxed at 85°C for 30 min, cooled to room temperature, and then the ethanol was evaporated under nitrogen at 65°C. MBTFA, 50 μl, was added to the contents of each vial; refluxing with closed tops was carried out at 130°C for 20 min. The contents of the vials were allowed to cool to room temperature and 450 μl of ethyl acetate were added to bring about a 1:10 dilution of the derivatized mixture so that a concentration of 100 ng/μl of each B-6 vitamer was obtained. The contents were mixed using a vortex for 0.5 min to ensure homogeneity. The derivatized B-6 compounds were further diluted with ethyl acetate to obtain concentrations of 0.01 to 100.0 ng/μl of each of the B-6 vitamers. Volumes of 1.0 μl were then injected directly into the gas chromatograph and a calibration curve prepared. All injections were done in duplicate.

Analyses of food extracts

Brand names of selected foods were purchased at a local grocery store; these included Rainbo enriched white bread, Carnation instant non-fat dry milk, and Green Giant sweet peas. Aqueous slurries of the foods (1:2; solid–water) were prepared by homogenization.

The B-6 vitamers in the homogenates were solubilized and released by acid hydrolysis followed by enzymatic treatment using alpha-amylase (EC 3.2.1.1), pepsin (EC 3.4.23.1), and papain (EC 3.4.22.2); all enzymes were obtained from Sigma Chem. Co., St. Louis, MO. To 50 ml (bread or milk) or 25 ml (peas) slurry, 30 ml 0.2 *M* hydrochloric acid were added; samples were placed in a boiling water bath for 1 h with constant stirring using a magnetic stirring bar. Flasks were then cooled to room temperature and 2 ml alpha-amylase solution (3 g in 2.5 *M* sodium acetate), 2 ml pepsin solution (3 g in 2.5 *M* sodium acetate), and 1 ml 1% papain solution were added; then samples were incubated at 37°C for 16 h in a shaker water bath. Samples were then filtered through No. 1 filter paper using a Buchner funnel. The filtrate was then passed through an ion-exchange column (AC-50W-X8, 100–200 mesh, Bio-Rad Labs., Richmond, CA, U.S.A.) in order to further remove contaminants.

Aliquots (150 μl) of the food extracts were derivatized under the same conditions as for the B-6 standards. Calibration curves with external standards were prepared to accompany each set of chromatographic determinations. This allowed the GC analyst to compensate for sensitivity changes that occurred during normal GC operations.

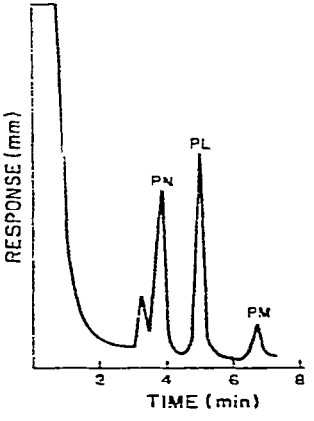
GC operating conditions

GC was carried out using a MT-220 (Microtek Instruments, Baton Rouge, LA, U.S.A.) gas–liquid chromatograph fitted with a ^{63}Ni ECD. The detector voltage was set at 10^2 and the sensitivity at 1/32. Several different columns and operating conditions including that of temperature were tried and those described below were found to be the best for the quantitative separation of the B-6 vitamers. The column was 1.54 m × 2 mm I.D., glass, packed with 10% SP-2100 on Supelcoport 80–100 mesh (Supelco, Bellefonte, PA, U.S.A.). The column temperature was maintained at 125°C, the injection port was operated at 205°C, and the detector temperature was set at 350°C. The carrier gas was nitrogen with a regulator pressure of 40 p.s.i.g. and a flow-rate of 30 ml/min.

RESULTS AND DISCUSSION

A representative chromatogram of B-6 standards separated by GC–ECD is shown in Fig. 1. The separation of all three B-6 compounds was completed in less than 8 min. Trifluoroacylation of PL and PM gives rise to single peaks; whereas, trifluoroacylation of PN gives rise to two peaks —a major peak with a retention time of approximately 3.8 min and a minor peak with a retention time of approximately 3.2 min which sometimes appeared as a shoulder to the major peak. Korytnyk[3] had reported that trimethylsilylation of PN generally yielded 2 peaks. The variation in areas of the two peaks was dependent on the time the vitamer was exposed to the trimethylsilylation mixture. In the present study, variation in areas of the two peaks resulting from formation of the MBTFA derivative of PN was observed. The peak with the longer retention time was always the predominant peak. The calibration curve for PN was plotted using the sum of the peak heights. This gave a satisfactory linear plot.

The log response *vs.* log ng standard calibration curves for PN and PL were linear between 0.01 and 0.5 ng; whereas the curve for PM was linear between 0.01 and 5.0 ng. The minimum detectable quantity for all three vitamers was 0.01 ng. Several factors affect the operation of the gas chromatograph such as purity and dryness of the carrier gas, sensitivity of the ECD, temperature, column bleed, column conditioning times, and electrical noises also often contribute to variability in the signal output in GC–ECD techniques.



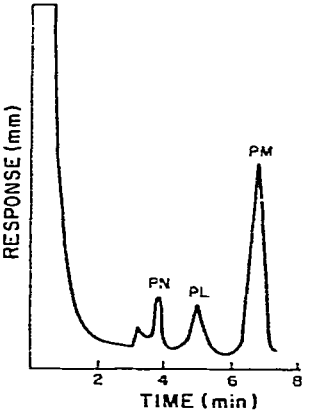


Fig. 1. Separation of B-6 standards by GC–ECD. Conditions: Microtek MT-220; column, 1.54 m × 2 mm I.D., glass, packed with 10% SP-2100 on Supelcoport 80–100 mesh; column, 125°C, injection port, 205°C, detector, 350°C; carrier gas, nitrogen at 30 ml/min; ^{63}Ni ECD.

Fig. 2. Separation of B-6 vitamers in milk by GC–ECD. Conditions as for Fig. 1.

GC–ECD analyses of food extracts

A representative chromatographic pattern of separated B-6 vitamers in derivatized extracts of milk is shown in Fig. 2. Similar patterns were obtained for extracts of bread and peas. Identification of the B-6 vitamers was accomplished by matching peak patterns and retention times of the B-6 vitamers from the foods with those of pure standards chromatographed in the same set of determinations. In general, good separations with little interference were obtained.

The B-6 vitamer content of these selected foods as measured by GC–ECD techniques is shown in Table I. Published values for GC analyses of B-6 vitamer content were not available for comparison. The values which we obtained using GC–ECD methods are higher than reported values as ascertained by microbiological assay[12].

TABLE I

B-6 VITAMER CONTENT OF SELECTED FOODS AS MEASURED BY GC–ECD

$\bar{X} \pm$ S.D. for duplicate analyses of two separate extractions.

Food	*mg/100 g wet wt.*			
	PM	*PL*	*PN*	*Total B-6*
Enriched bread	0.41 ± 0.11	0.34 ± 0.07	1.04 ± 0.24	1.79 ± 0.41
Non-fat dry milk	0.95 ± 0.08	0.41 ± 0.20	4.58 ± 0.64	5.94 ± 0.91
Sweet peas	0.99 ± 0.32	0.39 ± 0.01	5.33 ± 1.32	6.71 ± 1.00

The short separation time of the MBTFA derivatives of PL, PN, and PM as well as the exceptional sensitivity of the ECD suggest the potential use of this GC–ECD method for the detection and quantitation of B-6 vitamers in food. Additional precision could be obtained by use of an internal standard such as deoxypyridoxine. Further studies should involve the use of mass spectrometry for additional confirmation of peak identities.

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