after they were dried and this presence was confirmed by GC–mass spectroscopy. The benzoic acid was evidently formed from the oxidation of naturally occurring benzaldehyde during drying.

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Received for review May 22, 1978. Accepted July 31, 1978.

Analyses of Vitamin B_6 in Extractives of Food Materials by High-Performance Liquid Chromatography

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The B_6 vitamin components, pyridoxal, pyridoxine, and pyridoxamine, from extractives of fruits and vegetables were analyzed by use of high-performance liquid chromatography (LC). The B_6 components were obtained free from interfering materials by ion exchange. The 5′-phosphate esters of the B_6 components were hydrolyzed enzymatically with no adverse effects, thus permitting the determination of total B_6 content in the food samples. The LC columns (SCX) were developed with buffered phosphate solutions. Recovery of pyridoxine was quantitative; recovery of pyridoxal and of pyridoxamine was 60 and 85%, respectively. The consistency of the results and a precision of ±2% indicate this method is applicable to quantitative as well as qualitative analyses of these essential nutrients.

With the increasing interest in nutrition and emphasis on nutritive labeling we undertook to develop an accurate and rapid method suitable to fill these needs. Although the analysis of the B_6 vitamers (namely pyridoxal, pyridoxine, and pyridoxamine) using high-performance liquid chromatography (LC) is no longer new, the application has been only to pharmaceutical products or pure vitamin preparations (Cole et al., 1973). There is no information in the literature reporting on the successful use of LC for the determination of B_6 vitamins in foodstuffs.

B_6 vitamins occur naturally both in free and phosphate-bound forms (5′-phosphate, as coenzyme). In order to determine the total B_6 activity these bound forms must be cleaved. Therefore, it was necessary to ascertain the stability of the B_6 compounds when they are exposed to the rigorous hydrolysis treatment, which usually meant incubating the extracts at elevated temperatures and low pH for an extended period of time.

A second factor was the desirability of being able to use the extract prepared for the analyses of other vitamins such as riboflavin, thiamin, and ascorbic acid for the B_6 analysis without further handling. This would represent a substantial savings in time and effort of the analyst. If this was not feasible, then a third factor, the quantitative isolation of the desired B_6 components from the food extracts, would have to be developed.

The selection of correct column packing and developing solvent systems to achieve a rapid, high-resolution, and quantitative operation with the LC apparatus was of primary importance. Williams and Cole (1975) described the use of Aminex A-5 resin for the analysis of the B_6 isomers but did not apply this procedure to foodstuffs. Other investigators have used various ion-exchange resins such as Dowex AG50W to separate the pyridoxyl components, but have used either the usual bioassay (Toepfer and Lehmann, 1961) or fluorometric methods for the final quantitations (Chin, 1975; Gregory and Kirk, 1977). We report here a procedure which uses ion exchange to clean up food extracts, followed by vitamin B_6 quantitation with LC.

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EXPERIMENTAL SECTION

Apparatus and Materials. A Perkin-Elmer Model 601 LC unit equipped with an LC-55 variable wavelength detector was used for liquid chromatography.

Columns were stainless steel, 0.023 mm i.d. × 50 cm or 1 m, packed with Zipax SCX (Dupont).

The authentic B_6 vitamers (pyridoxal·HCl, pyridoxine·HCl, and pyridoxamine·H_2O·2HCl) were obtained from Calbiochem Laboratory.

Taka-diastase (Parke-Davis) was used as the phosphatase, papain, NF VIII (Difco Lab) as the protease enzymes. Dowex AG50W resins were obtained from Bio-Rad Laboratory.

Phosphate buffered solutions were prepared from reagent grade KH_2PO_4, and the pH was adjusted by using KOH or H_3PO_4 solutions.

Methods and Procedures. Standard stock solutions of authentic B_6 vitamers were made in glass distilled water. These solutions contained 0.1 mg (100 μg)/mL of each component and were diluted to lower concentrations with water. Samples of solutions containing individual B_6 vitamers as well as 1:1:1 mixtures of all three were injected onto the LC columns. The volume of samples injected onto the LC column was 20 μL. The lower limit of sensitivity of the detector was determined to be in the magnitude of 0.002 μg/20 μL (0.0002 aufs) equivalent to 100 μg or 0.1 mg/L.

Extracts of fruits and vegetables were prepared according to the procedure described by Pippen et al. (1975). The procedure was blended with equal weight of 95% ethanol. Twenty-five milliliter aliquots of each slurry were transferred to 100-mL actinic red volumetric flasks. Control B_6 mixtures of various concentrations were dissolved in 25 mL of water. All samples and controls were treated in the following manner. Sixty milliliters of 0.1

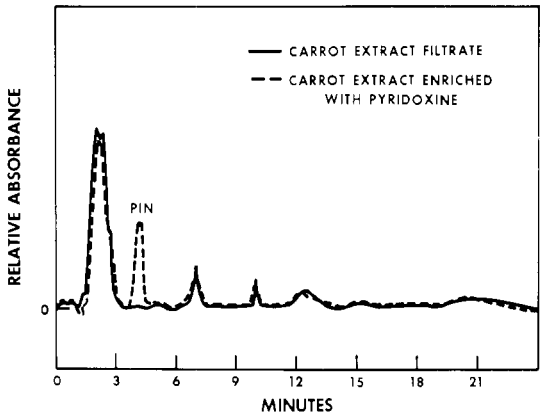

Figure 1. LC of carrot extracts: column 0.023 mm × 1 m Zipax SCX; flow rate, 45 mL/h; 40 °C; eluent, 0.1 M KH_2PO_4, pH 4.35.

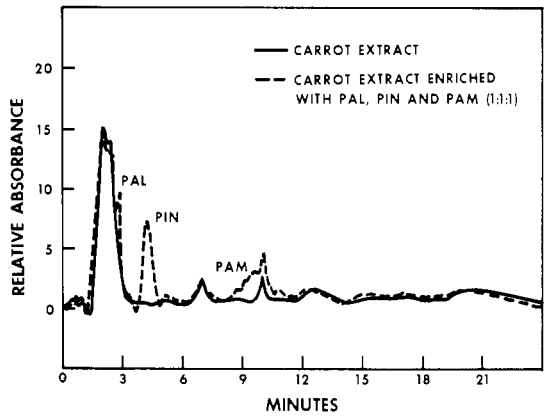

Figure 2. LC of carrot extracts with B_6 components: column 0.023 mm × 1 m Zipax SCX; flow rate, 45 mL/h; 40 °C; eluent, 0.1 M kH_2PO_4, pH 4.35.

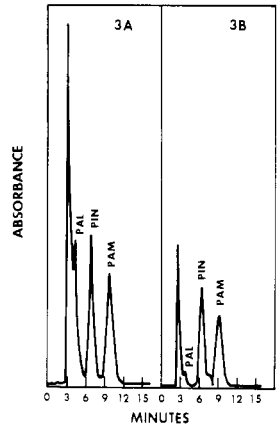

Figure 3. Elution of B_6 components from Dowex AG 50W with dilute NH_4OH: (A) first 25-mL aliquot; (B) third 25-mL aliquot.

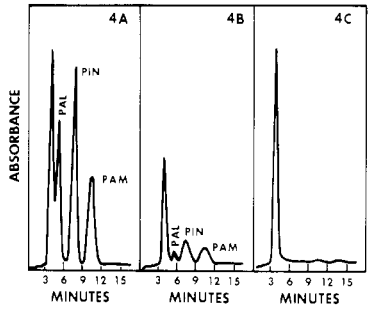

Figure 4. 0.05 N KOH elution of Dowex AG 50W containing same extracts as shown in Figure 3A and 3B: (A) first 25-mL aliquot; (B) third 25-mL aliquot; (C) 75 mL of eluate following "4B", concentrated 20 times.

N HCl was added and the mixture was heated on a hot water bath (ca. 97 °C) for 1 h, then allowed to cool to room temperature. Five milliliters of a solution containing 6% w/w each of diastase and papain in 2.5 M aqueous NaAcO was then added and thoroughly mixed, and the mixture (pH 4.5) was incubated overnight (16 h) at 37 °C. (An alternate rapid enzyme digest, incubating at 47 °C for 2 h gave equivalent results with no destruction of the B_6 vitamers). We used the overnight incubation because of its convenience in scheduling our sample preparations. After allowing the digested mixture to cool to room temperature, volume was made to 100 mL and gravity filtered through Whatman No. 1 or S&S 595 paper. The filtrates were clear and sometimes faintly colored depending on the material extracted.

Removal of interfering substances from sample extracts was accomplished by column chromatography using packings of either Dowex AG50W × 4 or AG50W × 8 in the K^+ forms. Aliquots of 25 mL of the filtered extract were pipetted onto the column containing 7.5 g of exchange resin. Columns were then washed with 200 mL of glass-distilled water. The wash waters were monitored using the LC procedure, but no B_6 components were detected. The B_6 vitamers were eluted with 0.05 N potassium hydroxide solutions. Fractions of 25 mL were collected. These fractions varied from neutral, initially, to alkaline. All fractions were acidified to about pH 6 with a minimum amount of 2 N H_3PO_4.

To verify the elution procedure, 20 μL of each 25-mL fraction was injected onto the LC column. Complete elution of B_6 components from the Dowex AG50W resin column was usually obtained within the third 25-mL fraction (total volume 75 mL). A minimum of 75 mL of eluate collected after the initial 75 mL and concentrated 20 times showed no B_6 vitamers.

The LC columns were conditioned by passing 0.1 N KH_2PO_4 at pH 4.35 through the columns for 24 h prior to use. Operational temperature was 40 °C and the flow rate, 45 mL/h. LC columns were developed isocratically with 0.1 N KH_2PO_4 solution at pH 4.35.

Absorbance of the emerging components was monitered at 210 nm with an LC-55 variable wavelength UV detector. Recorder sensitivity was 1.0 mvfs (0.02 aufs) and 0.1 mvfs (0.002 aufs) deflection. Average time per analysis, including column purging between runs, is in the order of 25–30 min.

RESULTS AND DISCUSSION

Filtrates of extracts were initially injected directly onto the LC column without further treatment to determine whether analyses for B_6 at this point was feasible. Figure 1 shows results obtained from a carrot extract and one enriched with 1 μg/mL of pyridoxine only. Subsequent extracts were enriched with 1:1:1 mixture of the B_6 vitamers at the same concentration for each (Figure 2). The pyridoxine and pyridoxamine components in the B_6 enriched extracts were clearly displayed on the LC curves. However, the pyridoxal peak was often obscured by one or two very large peaks preceding the pyridoxal peak. These results indicate the necessity of cleaning up the sample extracts.

Dowex AG50W × 4 and AG50W × 8 converted to the K^+ form were used successfully. Column of both resin types were handled in the same manner. Initially, after the ion-change columns were washed with distilled water, they were eluted with 0.1 N or 0.05 N NH_4OH in 25-mL aliquots into actinic glass flasks and made weakly acidic

Table I. Percent Recovery[a] of B_6 Vitamers from LC Analyses of Control and Sample Extractives[b]

	calcd concn, μg/mL	pyridoxal (PAL) found	%	pyridoxine (PIN) found	%	pyridoxamine (PAM) found	%
reagent control B_6 mixture	10.0	6.20	62	10.20	102%	8.70	87
	5.0	3.10	62	5.10	102%	4.25	85
	2.0	1.22	61	1.99	99.8	1.72	86
	1.0	0.60	60	0.997	99.7	0.851	85
	0.5	0.30	60	0.498	99.6	0.420	84
	0.1	0.06	60	0.0993	99.3	0.083	83
enriched apple extract	10.0	6.20	62	10.20	102%	8.62	86
	5.0	3.00	60	5.05	101%	4.30	86
	2.0	1.24	62	1.996	99.8	1.75	85
	1.0	0.61	61	0.999	99.9	0.845	84
	0.5	0.295	59	0.499	99.8	0.421	84
	0.1	0.059	59	0.0981	98.1	0.0832	83
enriched spinach extract	10.0	6.10	61	10.20	102	8.67	87
	5.0	3.05	61	5.10	102	4.30	86
	2.0	1.20	60	2.02	101	1.77	85.5
	1.0	0.59	59	0.990	99	0.841	84
	0.5	0.296	59	0.490	98	0.42	84
	0.1	0.058	58	0.098	98	0.0830	83

[a] Compared with reference vitamin B_6 mixtures of like concentrations. [b] Average values of a minimum of five analyses.

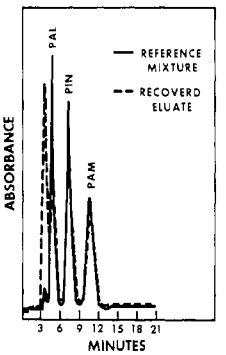

Figure 5. A typical LC chromatogram of eluates from Dowex of AG 50W: column 0.23 mm i.d. × 1 M Zipax SCX; flow rate, 45 mL/h, 40 °C; eluent, 0.1 M KH_2PO_4, pH 4.35.

with 2 N H_3PO_4. Pyridoxal (PAL) was mostly eluted with the first 25 mL of NH_4OH along with some of the pyridoxine (PIN) and pyridoxamine (PAM). The remainder of the PIN and PAM eluted out by the third 25-mL aliquot NH_4OH (Figure 3A and 3B). A large peak, interfering with the display of the PAL peak, was suspected as being caused by ammonium ion. This difficulty was greatly alleviated when 0.05 N KOH was substituted as the eluting solution (Figure 4). Potassium hydroxide (0.05 N) was then used throughout the experiment except at the beginning.

The chromatograms of B_6-enriched extracts of apple, pear, mango, spinach, chard, and squash and control B_6 superimposed on a standard B_6 reference curve of the same concentration (Figure 5) show that sample recovery is quantitative for pyridoxine (PIN), the most biologically important B_6 component. Recovery of PAL and PAM was consistantly 60 ± 2 and 85 ± 2%, respectively, throughout a range of concentrations from 0.10 μg through 10 μg/mL of enrichment (Table I). The loss of PAL and PAM appears to be in the failure of these vitamers to elute off the ion-exchange columns. Control samples injected on the LC columns without having been subjected to ion exchange did not show corresponding loss of PAL and PAM. The naturally occurring B_6 components are negligible as compared to the concentrations added (Figure 1).

These results show that the method we describe is suitable for quantitative as well as qualitative analyses of the three most important vitamin B_6 components from food extractives.

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