Table IV. The Mean Effects of Ash Solution Preparation on the Analysis of and Recovery of Added Trace Elements, ppm

Plant material		Fe			Mn			Zn			Cu		
		Wet E[a]	Dry E[b]	Wet NE[c]	Wet E	Dry E	Wet NE	Wet E	Dry E	Wet NE	Wet E	Dry E	Wet NE
Alder leaves	native	153.4	139.0	135.5	419.0	409.2	418.3	57.0	53.0	54.6	10.8	10.4	10.4
	recovered[d]	80.1	75.7	72.7	80.5	83.2	79.4	40.8	41.3	36.4	40.0	39.6	36.3
Douglas fir needles	native	99.7	98.7	96.4	239.5	230.2	233.6	11.1	9.0	10.8	3.6	2.9	3.8
	recovered	82.1	75.5	70.8	81.5	74.8	79.5	41.7	39.3	38.2	40.8	26.8	38.0
Corn cobs	native	149.7	145.3	146.4	6.5	6.5	6.6	25.5	21.9	24.5	59.8	37.1	56.5
	recovered	79.0	72.0	77.6	80.0	78.4	79.2	40.0	36.0	40.0	41.0	10.8	41.7
Corn grain	native	25.1	25.2	25.4	7.1	7.2	6.8	25.2	27.1	24.8	6.6	6.7	7.5
	recovered	79.2	80.0	76.0	79.1	80.3	77.4	40.6	37.3	40.1	42.1	41.8	38.2
Cabbage leaves	native	126.6	115.0	113.0	44.6	44.2	40.7	48.3	45.4	46.0	13.8	13.3	12.3
	recovered	78.9	66.2	67.7	80.0	78.7	73.8	39.4	39.9	36.7	40.0	39.5	36.3
Alfalfa	native	206.5	189.2	179.5	23.1	21.9	20.2	30.8	26.6	28.9	14.5	5.7	13.3
	recovered	82.5	64.6	68.7	79.2	80.1	73.3	39.8	37.6	37.8	40.2	33.3	37.4
Mixed forage	native	120.0	115.7	109.0	151.2	152.0	142.1	34.8	31.3	33.8	13.9	13.3	12.6
	recovered	79.7	71.5	68.4	79.3	81.5	79.1	39.8	35.7	37.8	40.8	36.8	37.4
Relative SD, %		1.56	2.44	1.66	0.84	1.05	1.50	1.16	2.37	2.68	1.90	6.32	2.50
Mean recovery, %		100.3	90.3	89.6	99.9	99.5	96.7	100.8	95.4	95.3	102.4	81.6	94.8

[a] Wet ashed and trace elements extracted with PDTCA. [b] Dry ashed and trace elements extracted with PDTCA. [c] Wet ashed and trace elements were not extracted. [d] Trace elements were added to the plant tissue in amounts calculated to make the materials 80, 80, 40, and 40 ppm higher in Fe, Mn, Zn, and Cu, respectively.

diluted 1:2 and the ash solutions of the alder leaves were diluted 1:4. Recovery of Mn was poorest on cabbage leaves and alfalfa, the two materials which were relatively high in inorganic constituents and on which the determinations were made on undiluted ash solutions.

Some workers may feel that the proposed procedure is too time consuming for the analyses of a large number of samples and for samples on which the sampling error may exceed the analytical error. The proposed procedure can serve as a reference procedure for assessing errors involved in other methods. With slight modifications, the extracting procedure can be used to increase sensitivity manyfold by concentrating trace elements from dilute aqueous solutions and by redissolving the extracted trace elements in suitable organic solvents.

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Vitamin B_6: Gas–Liquid Chromatography of Pyridoxol, Pyridoxal, and Pyridoxamine

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The three major nonphosphorylated forms of vitamin B_6 have been separated by means of gas–liquid chromatography of their heptafluorobutyryl derivatives. The highly electronegative nature of the derivatives combined with the use of an electron capture detector provides a very sensitive and somewhat specific assay for these compounds. Data are presented which suggest that quantitative determination of pyridoxol is possible at least within the range of 1.0–10.0 ng and for pyridoxal and pyridoxamine within the range of 2.0–20.0 ng.

At present a number of methods are available for both qualitative and quantitative determination of vitamin B_6. The most extensively utilized method, microbiological growth stimulation, is reliable and extremely sensitive. This method, however, suffers from the disadvantage of requiring considerable physical manipulation and uncertain specificity.

Recent advances in the technique of gas–liquid chromatography present a potential means of assaying the major vitamin B_6 components with satisfactory sensitivity and specificity, in addition to a significant reduction of analysis time.

Due to the low volatility of vitamin B_6 it has been nec-

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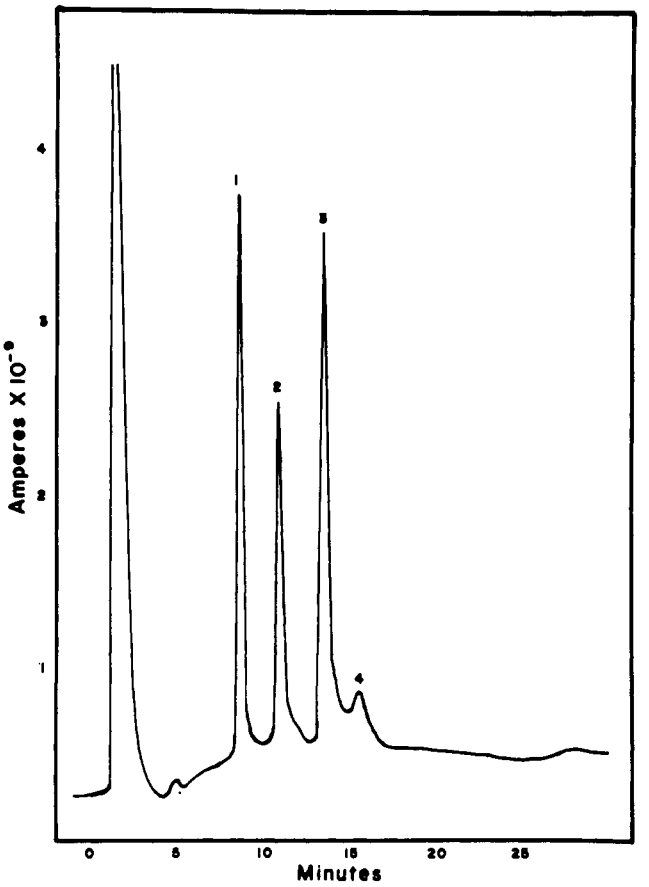

Figure 1. Chromatograph of Vitamin B_6. Peak 1, 18.0 ng of pyridoxine; peak 2, 36.0 ng of pyridoxal; peak 3, 36.0 ng of pyridoxamine; peak 4, unknown.

essary to convert these compounds to suitably volatile derivatives before analysis by gas–liquid chromatography becomes feasible.

Korytnyk *et al.* (1966) and Korytnyk (1967) investigated the gas chromatographic properties of the isopropylidene, acetyl, 3-*O*-benzyl, and trimethylsilyl derivatives of several compounds of the B_6 group. These derivatives were amenable to determination by flame ionization detection after separation on a column of silicone gum rubber, and peak areas of the acetates of pyridoxal and 4-pyridoxic acid lactone showed a linear increase in the range of 0.8–15.0 μg. This report stated that trimethylsilylation of B_6 phosphates resulted in single, sharp, and well-separated peaks. Prosser and Sheppard (1966) also reported satisfactory separation of the acetyl derivatives of pyridoxal, pyridoxol, and pyridoxamine, with subsequent detection by both flame ionization and β ionization detectors.

The trimethylsilyl derivatives of vitamin B_6 have been studied and utilized for analysis of these compounds by several investigators (Haskell, 1968; Richter *et al.*, 1967; Sennello *et al.*, 1967). These authors report good separation and detection of the vitamin derivatives in addition to simplicity and reproducibility of derivatization.

Imanari and Tamura (1967) prepared the trifluoroacetyl derivatives of pyridoxal methyloxime, pyridoxol, pyridoxamine, and pyridoxic acid lactone. These compounds were completely separated and exhibited a highly electronegative nature. The electron capture detector showed a good response to 0.1 ng of pyridoxal derivative and to 0.2 ng of pyridoxol and pyridoxamine derivative.

Sheppard and Prosser (1970) have described a method for determination of vitamin B_6 in pharmaceutical preparations. This method involves acetylation of the vitamin, followed by gas–liquid chromatography.

A similar method, also utilizing the acetyl derivatives of vitamin B_6, was described by Korytnyk (1970). In the present investigation we have made use of a new derivative of vitamin B_6 which combines the advantages of high volatility and electronegativity.

MATERIAL AND METHODS

Apparatus and Experimental Conditions. All analyses were carried out using a Bendix Model 2500 gas–liquid chromatograph fitted with a Ni^{63} electron capture detector. All peak areas were determined by use of an Infotronic CRS-101 electronic integrator. The column was 14 ft × ⅛ in. o.d. stainless steel packed with 3% SE-52 on 100–120 mesh Chromosorb W-HP. The column was conditioned for 18 hr at 300° with nitrogen at 15 ml/min.

The oven temperature was maintained at 165°, the injector at 200°, and the detector at 300°. The carrier gas was nitrogen, with a flow rate of 14 ml/min. Reagents used were pyridoxol, pyridoxal, and pyridoxamine, as the hydrochloride salts, and were obtained from Mann Research Laboratories, New York, N. Y.

Acetonitrile (silylation grade) and heptafluorobutyrylimidazole (HFBI) were obtained from Pierce Chemical Co., Rockford, Ill.

Benzene and methanol were analytical reagent grade. Benzene was dried with anhydrous sodium sulfate before use.

The derivatization reagent was prepared daily by mixing nine parts of acetonitrile with one part HFBI and storing in the cold.

Pyridoxol, pyridoxal, and pyridoxamine standards were prepared by dissolving an appropriate quantity of the hydrochloride salt in either water or methanol and storing in the refrigerator. These standards were found to be stable for at least 2 weeks.

Procedure. An appropriate quantity of the vitamin standard dissolved in methanol was pipetted into a small vial and placed in a vacuum oven at 45° until dry. The vial was then fitted with a Teflon–rubber septum and 0.01 ml of the derivatizing reagent was added. The vial was agitated to wet the inner surface and 0.99 ml of dry benzene added and mixed by gentle shaking. The reaction mixture was allowed to stand at room temperature for 10 min, and then was injected directly into the chromatograph.

In the cases where the vitamins were in water solution, the standards were placed in a vial, evaporated to dryness in the vacuum oven, dissolved in 0.5 ml of methanol, and heated for 1 hr at 90°. The methanol was evaporated and the sample treated as above.

It is important that materials such as water and alcohols be excluded from the reaction as these compounds react, producing extraneous peaks and low recoveries.

RESULTS

Figure 1 depicts a typical chromatogram obtained from 18.0 ng of pyridoxol (peak 1) and 36.0 ng each of pyridoxal and pyridoxamine (peaks 2 and 3).

Under the conditions used in this study, peaks 1, 2, and 3 exhibited retention times of 9.5, 11.7, and 14.3 min, respectively.

Linear response plots of individual vitamin derivatives are unaffected by the presence of the other derivatives when mixtures are chromatographed. These plots indicate that a shift in the linear dynamic range occurs at approximately 8.0 μl (Figure 2).

Reaction of the derivatizing reagent with the vitamins is practically instantaneous. The 10-min reaction period before injection was found to be desirable to allow time for crystallized materials to attain a size which will not clog the syringe.

Mass spectroscopic studies indicate that the derivatives of pyridoxol, pyridoxal, and pyridoxamine have molecular weights of 757, 559, and 756, respectively. These molecu-

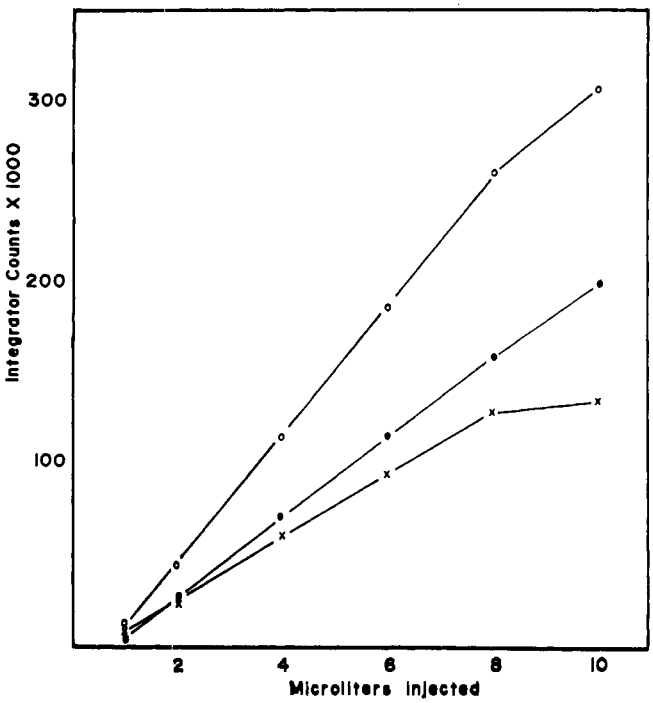

Figure 2. Standard curves of Vitamin B_6 moieties. O, pyridoxine; X, pyridoxal; ●, pyridoxamine. Pyridoxine, 1.0 ng/μl; pyridoxal and pyridoxamine, 2.0 ng/μl.

lar weights are consistent with the expectation that the vitamin molecules are esterified at all available sites. These data indicate that pyridoxol and pyridoxamine contain three esterified HFB moieties, while pyridoxal contains only two.

DISCUSSION

Because of the nonvolatile nature of pyridoxol, pyridoxal, and pyridoxamine, volatile derivatives must be synthesized before gas–liquid chromatography may be utilized for either qualitative or quantitative determination of these materials.

Heptafluorobutyrylimidazole reacts rapidly with all hydroxyl and primary amine groups in the vitamin molecules. The heptafluorobutyryl derivatives of pyridoxol and pyridoxamine are sufficiently volatile and stable to be assayed by gas–liquid chromatography. Pyridoxal, however, does not produce a sufficiently volatile HFB derivative and must be treated with methanol prior to reaction with HFBI. The methanol treatment results in formation of the methyl acetal (Nurnberg, 1961) which, when subsequently treated with HFBI, may be chromatographed as described.

Reaction mixtures containing the derivatives can be kept at room temperature for 4–6 hr if moisture is excluded and a slight excess of derivatizing reagent is present. A large excess of derivatizing reagent is to be avoided since it will produce a very broad "solvent peak" and frequently results in other minor peaks which may interfere with quantitation of the vitamin components.

Chromatography of the vitamin B_6 group, as described here, frequently results in the appearance of minor peaks such as peak 4 (Figure 1). The identity and/or origin of these peaks has not been determined.

Although not reflected in the data presented here, previous experiments indicated that the detection limit for the vitamin B_6 derivatives was approximately 10 pg of the vitamin base.

It was also found that certain other liquid phases could be utilized for separation of the B_6 derivatives. Among those which show adequate resolution and efficiency are 5.0–7.5% OV-1, 5% polyphenyl ether (6 ring), and 3% OV-17, all coated on 100–120 mesh Chromosorb W-HP. In most cases these phases produced sharp symmetrical peaks having rather short retention times. The rapid elution characteristics of these columns resulted in considerable interference from the solvent–reagent peaks.

In summary, a method is described which can be utilized as a rapid means of resolving and quantitating mixtures of pyridoxol, pyridoxal, and pyridoxamine.

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