Sulfosalicylic Acid as an Extraction Agent for Vitamin B_6 in Food

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Procedures are described for the extraction and preparation of extracts from several different food samples for vitamin B_6 analysis. These procedures have been combined with previously described high-performance liquid chromatographic (LC) methods to accurately quantitate vitamin B_6 vitamer contents of various samples. Chromatographs of extracts from all foods were devoid of interfering compounds. Recoveries of B_6 vitamers added to samples were 95–105% for all vitamers except pyridoxine phoshate where the recovery was 85%. With the exception of breakfast cereal, higher amounts of vitamin B_6 were found in foods than would be expected from previously published results. These extraction procedures combined with high-performance LC methods to quantitate B_6 vitamers provide a powerful tool to routinely quantitate B_6 in biological systems.

There has been increasing interest in past years in development of automated methods for the analysis of nutrients in foods (Stewart, 1979; Gregory and Kirk, 1978). Since high-performance liquid chromatography (LC) has the capability of being easily automated, it has proven to be a useful tool in such analyses. For example, high-performance LC methods were recently described (Vanderslice et al., Vanderslice and Maire, 1980) which have the capability of detecting and quantifying all forms of vitamin B_6 in clean samples (Figure 1). Vitamin B_6 compounds have been abbreviated, according to published recommendations (IUPAC–IUB Commission on Biochemical Nomenclature, 1970) as follows: pyridoxal phosphate, PLP; pyridoxamine phosphate, PMP; pyridoxine phosphate, PNP; pyridoxal, PL; pyridoxamine, PM; pyridoxine, PN. To be useful for the analysis of complex systems such as food, it is necessary to precede such analyses with an extraction procedure which will quantitatively remove vitamin B_6 from the food sample and present the vitamers in a relatively clean milieu. The purpose of the present paper is to describe two extraction procedures which have the following characteristics: (1) quantitative recovery of all forms of vitamin B_6 from various food samples; (2) simple and mild treatments; (3) compatability with recently developed high-performance LC analytical methods in that they yield relatively clean extracts for analyses; (4) capability of future automation.

There are a number of excellent reviews on extraction procedures for B_6 (Storvick et al., 1964; Toepfer and Polansky, 1964; Storvick and Peters, 1964; Sauberlich, 1970; Loo and Cort, 1972; Bell, 1974). For the most part, acid extraction procedures are used which range from autoclaving for several hours with hydrochloric acid (Toepfer and Polansky, 1964) to mild heating for 5 min in a metaphosphoric acid solution (Wada et al., 1957; Loo and Cort, 1972). For quantitation of the various vitamers of B_6, both bound and free, care must be taken that the phosphate forms are not destroyed by phosphatases, enzymes found in most tissues (Turner, 1961), or by mineral acids. The hydrochloric acid and autoclaving procedures invariably hydrolyze the phosphate forms of the B_6 vitamers, whereas treatment of samples with metaphosphoric or perchloric acid appears to preserve the phoshate forms while still releasing the bound vitamins from other food components (Loo and Cort, 1972; Bain and Williams, 1960). Most previously described extraction procedures have proven to be unsatisfactory for our purposes in that either the phosphate forms are destroyed or the methods are too complicated to be suitable for automation. The simple, mild procedures, such as those with metaphoshoric acid, offer possibilities for automation but appear to be incompatible with our high-performance LC separation and analytical schemes.

A few years ago, Mondino et al. (1972, 1975) reported a deproteination procedure using sulfosalicylic acid (SSA) which has proven to be ideal for the determination of amino acids and related compounds in plasma. The method is simple and is now routinely used in automated chromatographic work on amino acids (Wannemacher and Dinterman, 1977). The results of the present study indicate that SSA can serve as the basis of an excellent and simple extraction procedure for the recovery of all forms of vitamin B_6 from foods and the resulting method yields clean samples for quantitative analysis by column chromatography.

Chromatographic traces and B_6 vitamer contents are presented for five different foods. The traces are free of interfering compounds; all vitamer forms present are observed, and the results for spiked and unspiked samples indicate recoveries close to 100%.

EXPERIMENTAL SECTION

Reagents. Hexane and methylene chloride were of high-performance LC quality. Hydrochloric acid and ferric chloride were of analytical-grade purity. All were obtained from Fisher Chemical Co. Sequanal-grade sulfosalicylic acid was obtained from Pierce Chemical Co., while semicarbazide and 3-hydroxypyridine came from Aldrich Chemical Co. With the exception of pyridoxine phosphate, all pure forms of the vitamers were obtained from Sigma Chemical Co. Pyridoxine phosphate was prepared by the method of Peterson and Sober (1954). All aqueous solutions were prepared with water which was distilled and then resin purified to at least 15-megaohm resistance. All glassware was detergent washed and then rinsed thoroughly 6 times, three with tap water and three with distilled water. All pipets were acid washed and rinsed with tap water, followed by distilled water.

Sample Purification System. The apparatus used to prepare samples is a high-performance LC system and is shown schematically in Figure 2. It was assembled by using a high-pressure reciprocating pump (Milton Roy, Model 396) with a depulsing system (Stewart, 1977), one sample-injection valve, and one reverse-flow valve (Valco Model AH 60), a 6 mm × 30 cm glass column (Glenco) packed with 200–400 mesh Dowex AG 2-X8 anion-exchange resin, and a fluorescence detector (Aminco fluorometer) with a Corning 7-54 primary filter and a Wratten 2E secondary filter. The components of the high-per-

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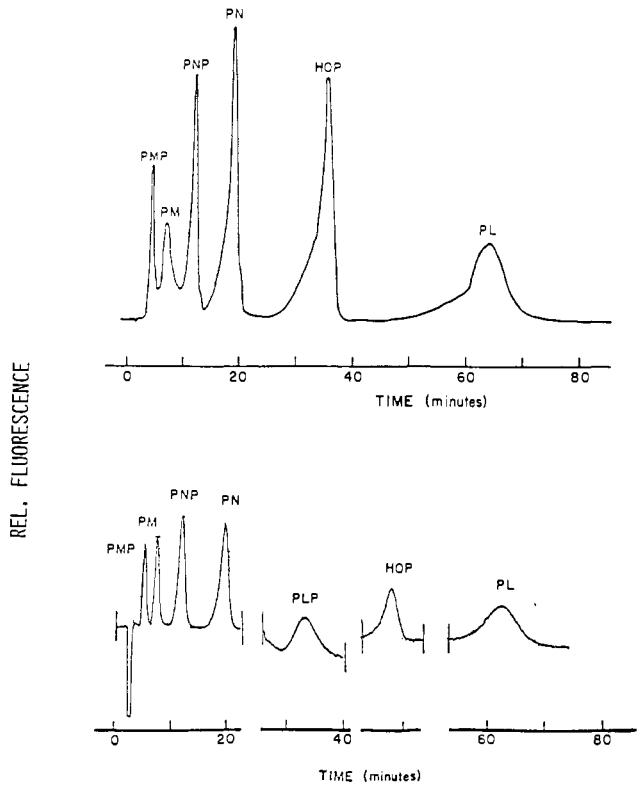

Figure 1. Chromatographic traces illustrating previous separations of B_6 vitamers. The upper figure is from the 1979 method (Vanderslice et al., 1979) where the sensitivity limits ranged from 25 to 100 ng/mL. PLP is not shown in this trace. The lower figure is from the 1980 method (Vanderslice and Maire, 1980) where the sensitivity limits ranged from 0.1 to 0.5 ng/mL. Breaks in this trace are due to changes in excitation and emission wavelengths. Information is printed during these breaks. The integrator is programmed to compensate for base-line changes.

formance LC system were connected by AWG 26 thin-wall Teflon tubing in conjunction with Chromatronic fittings (Durrum Corp., Palo Alto, CA). The electrical output of the detector was monitored on a strip chart recorder (Kipp and Zonen, Model BD 40).

B_6 Vitamer Analysis. The two high-performance analytical apparatus have been described elsewhere (Vanderslice et al., 1979; Vanderslice and Maire, 1980); a Shimadzu CR1A Chromatopac integrator has replaced the minigrator used in our earlier work (Vanderslice et al., 1979) as the peak area integrator. Basically, both systems will separate all six vitamers in ~60 min. The operating range of the first system (Vanderslice et al., 1979) is 10 μg–10ng for all vitamers except for pyridoxal phosphate where the range is 100 μg–100 ng. The second system (Vanderslice and Maire, 1980) is much more sensitive and has lower limits of 0.1 ng for all vitamers except for pyridoxal phosphate where the lower limit is 0.5 ng. The content of each vitamer is calculated from an appropriate standard curve and from the recovery of an internal standard which is added to the original sample and behaves throughout both procedures in the same manner as the B_6 vitamers. Eventually impurities from the food samples may accumulate on the analytical columns, causing the pressure to rise. It is, therefore, recommended that the analytical columns be stripped weakly by washing through with 0.1 M HCl.

Extraction Procedure 1. This is the original procedure which has been used in conjunction with the 1979 analytical system to determine the B_6 content of a variety of breakfast cereals (Vanderslice et al., 1980). The procedure is shown in Figure 3. First, a known amount of the internal standard, 3-hydroxypyridine (HOP), is added to 1 g of sample which is then homogenized with 10 mL of a 5% (w/v) sulfosalicylic acid solution for 5 min in a 50-mL Sorvall omnimixer. The sample is then centrifuged at 7500*g* for 20 min at 4 °C. If the resulting supernatant liquid is clear, it is filtered through a 0.45-μm Millipore filter in a Swinnex holder, evaporated to three-fourths its volume at 75 °C under a stream of nitrogen, and finally vortexed for 1 min with an equal volume of hexane. If, on the other hand, the supernatant from the centrifugation step is cloudy, it is first evaporated to three fourths its volume in a nitrogen evaporator, and centrifuged for 20 min in a clinical centrifuge (1500*g* max). The clear supernatant is then filtered through a 0.45-μm Millipore filter as described above, and the filtrate is vortexed with an equal volume of hexane. The resulting mixture is allowed to separate for 20 min and the water layer is drawn off and filtered through a 0.22-μm Millipore filter. An aliquot of the resulting solution, usually 0.36 mL, is injected into the sample purification system, to be described, and 6–10 mL of eluant is collected. An aliquot, usually 0.5 mL, of this eluant is then injected into the high-performance LC analytical system (Vanderslice et al., 1979) for final separation and quantitation of the B_6 vitamers.

Extraction Procedure 2. After the cereal samples were analyzed, the above procedure was modified for use with other food samples. The modified procedure was subsequently found to be useful for cereals. It is much simpler than the preceding one and has also been used for all food

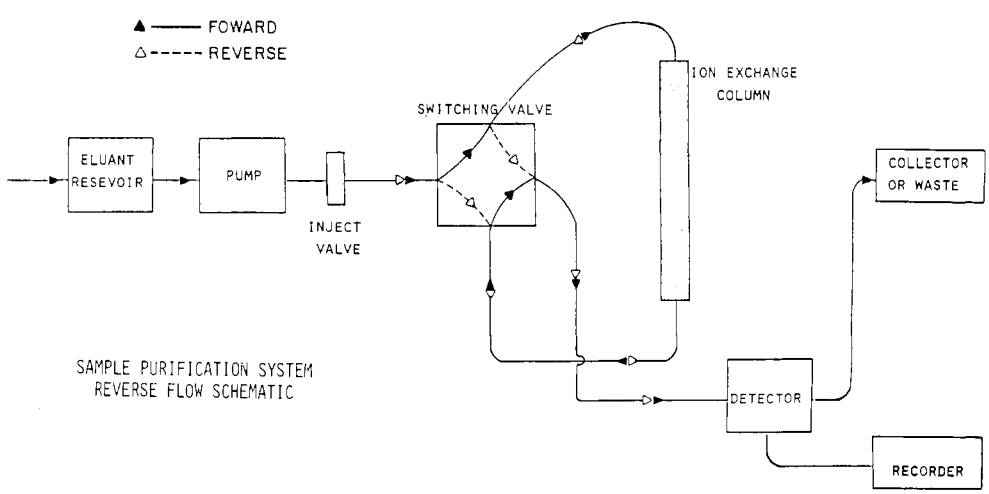

Figure 2. Schematic of the sample purification system illustrating reverse flow for column cleanup.

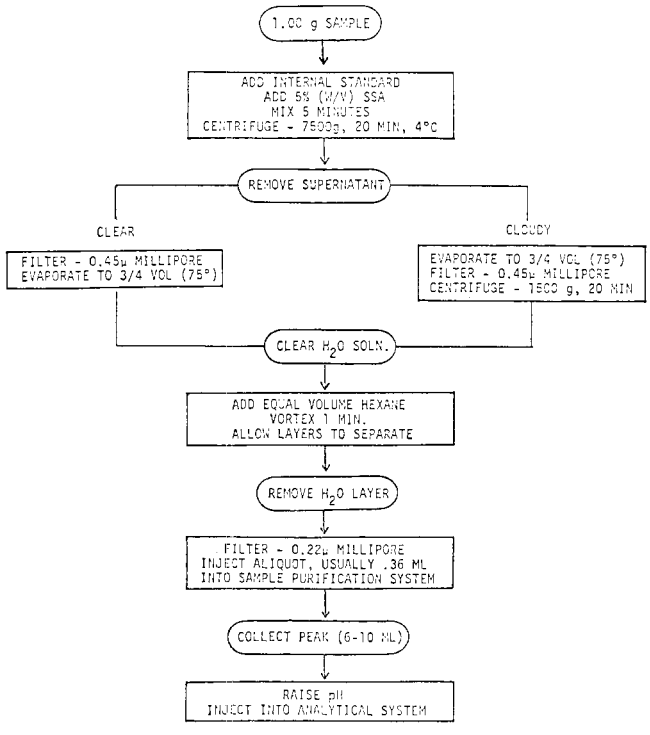

Figure 3. Block diagram of the extraction method used on cereals.

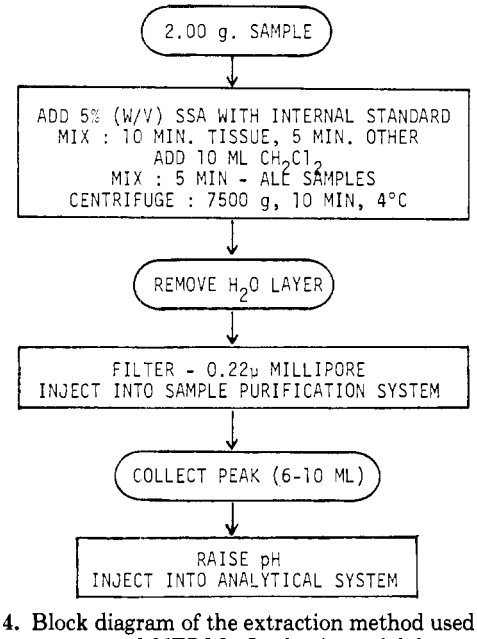

Figure 4. Block diagram of the extraction method used on pork, hamburger, carp, and NFDM. It also is useful for cereals.

samples reported here other than the breakfast cereal. It is shown in Figure 4. To a 2-g sample is added 10 mL of a 5% (w/v) solution of SSA together with a known amount of the internal standard. The mixture is ground in an omnimixer (10 min for tissue; 5 min for milk). Ten milliliters of methylene chloride is added and the entire mixture is then reground for 5 min. The resulting mixture is centrifuged at 7500*g* for 10 min at 4 °C; the water layer is removed and filtered through a 0.22-μm Millipore filter, and an aliquot, usually 0.36 mL, of the resulting solution is introduced into the sample purification system. The remainder of the procedure is then identical with the preceding one.

Sample Purification. The one problem encountered with sulfosalicylic acid is that it binds very tightly to the analytical anion-exchange column—so much so that it is difficult to remove and changes the elution times of the vitamers after a series of runs. SSA is also highly fluorescent, and when it eventually does elute, it creates a high fluorescent background. Hence, it must be removed prior to the final analysis. This is easily accomplished by the sample purification system described here and shown in Figure 2.

The sample is injected into the forward flowing stream (0.1 M HCl), illustrated by the black triangles in Figure 2. The stream passes through a six-port valve into the ion-exchange column. The effluent from the column passes through the same six-port valve, then through a detector, and finally to a collector or to waste. The SSA is bound to the column while the vitamers pass through and appear at the detector in ~2 min. As soon as the vitamers appear at the detector, the effluent is collected, usually 6–10 mL, for analysis. This solution, which is acidic, is made neutral, $6.5 \leq pH \leq 7.5$, with the addition of 1 M NaOH (otherwise, the elution patterns of the vitamers are changed) and an aliquot, usually 0.5 mL, is injected onto the analytical column.

Approximately 15–20 samples can be injected onto the sample purification column before it becomes saturated with SSA. When the SSA must be cleaned from this column, the flow is reversed by means of the six-port valve, and a solution of 0.7 M NaCl, 0.1 M HCl, and 2% $FeCl_3$ is pumped backward through the column and to waste. $FeCl_3$ is added to this solution since the ferric ion is chelated by SSA to form an intense purple color which not only visually indicates how saturated the column is but also indicates when the SSA has been completely removed. It is good practice to allow the column to run 20–30 min after all visible SSA–Fe^{3+} chelate has been removed. This assures that no trace amounts of SSA are retained on the column. The backwashing procedure takes ~3 h to clean a completely saturated column. The flow is subsequently returned to its original direction and the column washed with 0.1 N HCl for 30 min. This removes any excess Fe^{3+} which would cause the SSA to move more rapidly down the column and reduce sample capacity. Eventually, some Fe^{3+} accumulates on the column and SSA movement can be monitored visually. When the pale-purple color approaches the bottom of the column, it is time to backwash and clean the column. The flow rates in both forward and reverse directions are normally kept at 1.2 mL/min.

RESULTS AND DISCUSSION

Solutions prepared with known amounts of the different vitamers and run through the entire extraction and analytical procedure gave total recoveries, with the exception of PNP, of 93% with a relative standard deviation (RSD) of 3%. Samples of breakfast cereal, nonfat dry milk (NFDM), carp, pork, and hamburger with known amounts of vitamers added gave total recoveries of 95%, 85%, 95%, 95%, and 95%, respectively, with a RSD of 3%. Test runs on the internal standard, HOP, gave identical recoveries. Most of the loss occurs just after the first centrifugation step and filtration. Here, the solids retain some moisture and the vitamers are distributed in this solvent. If the solids are washed, the vitamers recovered, and the resulting solutions analyzed for B_6 vitamer content, then all total recoveries, including HOP, were greater than 95%. In fact, it is not necessary to be concerned with this since internal standard behaves in exactly the same way as the vitamers throughout the extraction, cleanup, and analytical procedures. Thus, any loss in processing is automatically compensated for by a similar loss in internal standard. It is on the basis of the known concentration of this internal standard that the concentrations of vitamers in food sam-

Table I. B_6 Vitamer Contents of Selected Foods with SSA Extraction

food	sample	PMP	PM	PNP	PN	PLP	PL	total
pork (broiled)	(1) original analysis, μg/g	3.890		0.092	0.172	2.110		6.264
	(2) original analysis, nmol/g	15.71		0.37	1.02	8.54		25.64
	(3) vitamer added, μg/g	1.469	1.312	1.314	1.490	1.570	1.393	
	(4) (1) + (3), μg/g	5.359	1.312	1.406	1.662	3.680	1.393	14.81
	(5) analysis of (4)	5.119	1.255	1.220	1.653	3.620	1.536	14.40
	(6) % (5)/(4)	96	96	87	99	98	110	97
carp (fresh, frozen)	(1) original anlaysis, μg/g	0.357				4.022		4.379
	(2) original analysis, nmol/g	1.44				16.27		17.71
	(3) vitamer added, μg/g	1.408	1.096	0.858	1.333	1.485	1.061	
	(4) (1) + (3), μg/g	1.765	1.096	0.858	1.333	5.507	1.061	11.61
	(5) analysis of (4)	1.783	1.134	0.727	1.376	5.257	1.088	11.36
	(6) % (5)/(4)	101	103	85	103	95	102	96
hamburger (lean, fresh)	(1) original analysis, μg/g	1.477			0.107	2.116		3.70
	(2) original analysis, nmol/g	5.96			0.63	8.56		15.15
	(3) vitamer added, μg/g	1.389	1.081	0.847	1.315	1.465	1.047	
	(4) (1) + (3), μg/g	2.866	1.081	0.847	1.422	3.581	1.047	10.84
	(5) analysis of (4)	2.865	1.049	0.708	1.379	3.463	1.095	10.56
	(6) % (5)/(4)	100	97	84	97	97	104	97
NFDM (Giant)	(1) original analysis, μg/g	1.348	0.818		0.207	1.443	2.192	6.008
	(2) original analysis, nmol/g	5.44	4.85		1.23	5.84	13.13	30.49
	(3) vitamer added, μg/g	1.372	1.120	0.821	1.424	1.604	1.249	
	(4) (1) + (3), μg/g	2.720	1.938	0.821	1.631	3.047	3.441	13.598
	(5) analysis of (4)	2.539	1.899	0.705	1.642	3.237	3.334	13.356
	(6) % (5)/(4)	93	98	86	101	106	97	98
NFDM (Carnation)	(1) original analysis, μg/g	1.359	0.935		0.146	2.850	2.407	7.697
	(2) original analysis, nmol/g	5.49	5.54		0.86	11.53	14.42	37.84
	(3) vitamer added, μg/g	1.399	1.142	0.837	1.453	1.635	1.273	
	(4) (1) + (3), μg/g	2.758	2.077	0.837	1.599	4.485	3.680	15.436
	(5) analysis of (4)	2.553	1.931	0.726	1.606	4.207	3.493	14.516
	(6) % (5)/(4)	93	93	87	100	94	95	94
cereal[a]	(1) original analysis, μg/g				22.97			22.97
	(2) original analysis, nmol/g				136			136
	(3) vitamer added, μg/g				8.37			8.37
	(4) (1) + (3), μg/g				31.34			31.34
	(5) analysis of (4)				31.31			31.31
	(6) % (5)/(4)				100			100

[a] Only PN content was measured; therefore, did not spike with all vitamers.

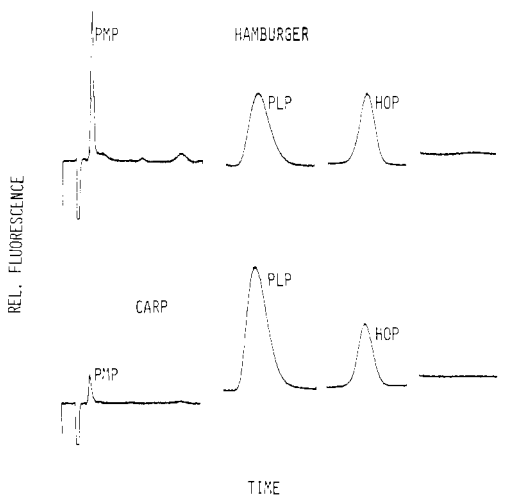

Figure 5. Actual chromatographic trace of extracted hamburger and carp samples. Integrator instructions and data printout have been deleted.

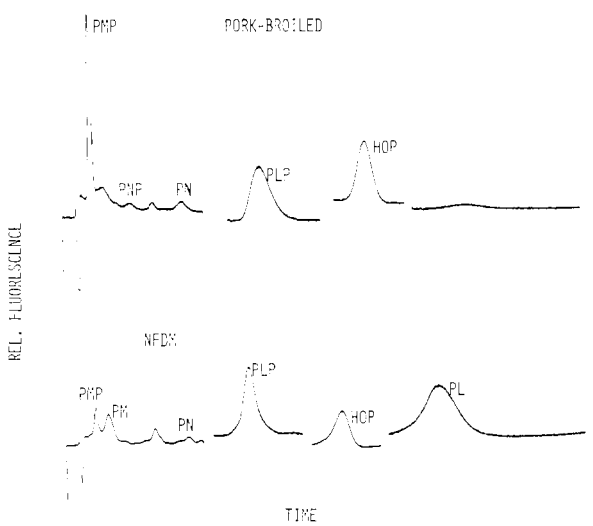

Figure 6. Actual chromatographic trace of extracted pork and NFDM samples. Integrator instructions and data printout have been deleted.

ples are now routinely calculated.

The B_6 vitamer content of the different foods was analyzed with the procedures described in this paper. Both spiked and unspiked samples were run on each sample. Typical chromatographic traces obtained from the different samples are shown in Figures 5–7. It is seen that the traces are clean and free from interfering substances near the vitamer peaks. The elution times for the different vitamers are identical with those obtained with pure standards. The concentrations of the vitamers obtained by these procedures are given in Table I for both spiked and unspiked samples. Recoveries (relative to that of HOP) from spiked samples for all vitamers are close to 100% of predicted, with the exception of PNP, where recovery is 85%. In this case, the standard is thought to be impure, which would lead to an incorrect calibration curve; PNP was not further purified since it constitutes

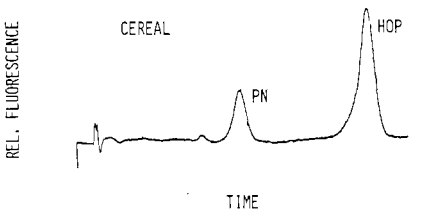

Figure 7. Typical cereal trace. Integrator instructions and data printout have been deleted.

a small proportion of the total vitamin B_6 content of the samples investigated.

The results for the unspiked samples given in Table I are of interest. The values obtained for PMP and PLP are higher than one might expect on the basis of the earlier microassay work of Polansky and Toepfer (1969). On the other hand, the values of PN are smaller than expected. Whereas agreement between the vitamin B_6 values in breakfast cereals obtained by the present methods and with a microradioassay method (Guilardi, 1980) using AOAC extraction procedures (Association of Official Analytical Chemists, 1980) is within 5%, the AOAC microassay procedure performed by commercial labs sometimes differed by greater than 30% (Vanderslice et al., 1980). A thorough comparison is underway featuring the three different methods (the present one, the microradioassay, and the standard AOAC procedure) in an attempt to explain any discrepancies.

With the present procedures, eight food samples can be analyzed in a working day by one person. The ultimate goal is to automate as much of the procedure as possible, which should lead to even better reproducibility and more analyses per day.

In conclusion, the described chemical assay procedure is a versatile one which can handle different food groups and give reproducible and reliable results for B_6 vitamer content in a reasonable amount of time.

In addition, laboratory results indicate that SSA will also serve as an agent for extraction of other water-soluble vitamins.

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