THE USE OF PTERIN-6-CARBOXYLIC ACID FOR THE INDIRECT DETERMINATION OF FOLIC ACID IN ENRICHED CEREAL GRAIN PRODUCTS BY HPLC WITH FLUORESCENT DETECTION

by

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CHAPTER 1

INTRODUCTION

The study of folic acid has historically proven difficult for several reasons:

1. The multiplicity of oxidation states and conjugated forms of the molecule
2. The instability of folic acid and
3. The minute amounts found in nature

For these reasons, the chemical and biological importance of folic acid and its derivatives were not fully understood until the late 1940's.

The name "folic acid" is generally used in reference to the compound N-(4-(((2-amino-4-hydroxy-6-pteridinyl)methyl)amino)benzoyl) glutamic acid, however, it is often used in the literature as a generic label for the broad group of related compounds including the poly-γ-glutamyl and reduced derivatives. Various more specific names have been proposed for folic acid, as pterolyglutamic acid and folicin, but none have achieved broad acceptance.

A chronology of the events which led to the elucidation of the chemical composition of folic acid, and consequently its relation to the many derivatives with similar but unique properties, is given in Table 1.

TABLE 1

A CHRONOLOGY OF THE EVENTS WHICH LED TO THE CHARACTERIZATION OF FOLIC ACID

Synonym	Year	Source	Activity	Reference
Vitamin M	1932	Yeast and liver extracts	Relieved anemia, leukopenia and granulocytopenia in monkeys	1
Vitamin B_c	1938	Fuller's earth adsorbate from liver extract	Corrected chick hyperchromic macrocytic anemia	2
	1944	Enzymatic digestion products of Vitamin B_c	Growth factor for both L. casei and S. fecalis	3
Factor U	1938	Present in bran, yeast, and alfalfa	Corrected deficient state in chicks	4
Norit Eluate Factor	1939	Eluate from passage of liver extract through Norit	Growth factor for L. casei and S. fecalis	5
	1941		Essential growth factor for chicks	6
	1943	Norit eluate from yeast	Fully active for L. casei, half as active for S. fecalis	7
Folic acid	1941	Purified spinach leaf extract	Growth factor for S. fecalis	8
SLR Factor	1943	Fermentation liquors from various cultures	Growth factor for S. fecalis	9
Pteroylglutamic acid	1946	Purified liver extract	Elucidation of chemical structure	10
Citrovorum factor	1949	Liver, peptone, and yeast extract	Growth factor for L. citrovorum (Pediococcus cerevisiae)	11

Folic acid as a vitamin. The utilization of folic acid by man, and its requirement as an essential nutrient, were difficult to demonstrate because of the basal levels produced by the intestinal flora and the minute amounts needed. However, Wills and Bilimoria (1) were able to induce a deficient anemic state in monkeys by maintaining them on a regimen similar to that consumed in reported cases of human tropical macrocytic anemia. Full recovery was observed after treatment with liver and yeast extracts. Spies et al. (12) were later able to successfully treat macrocytic anemia in many by parenteral injection and oral administration of crystalline L. casei factor. This then firmly established the vitamin value of folic acid for man. Subsequently, successful treatments of pernicious anemia, nutritional anemia in pregnancy, pellegra, and sprue were reported in the literature.

Folic acid metabolism. The metabolic role of folic acid revolves around its ability to serve as a donor of single carbon units in the biosynthesis of many biologically important molecules. Specifically, folic acid has been shown to transfer these units in the conversion of glycine to serine; in the methylations of ethanolamine to choline, homocysteine to methionine, nicotinamide to N^1-methylnicotinamide, and uracil to thymine; and in the introductions of C^2 and C^8 in purines and the amidine carbon in histidine biosynthesis (13,14).

Natural occurrence of folic acid. Folic acid occurs in low levels natively in most foods. A summary of the extremes of folic acid concentration in major food categories is given in Table 2, and in cereal grains and their products in Table 3. Because of the low levels of naturally occurring folates and their physiological importance as essential nutrients, the following daily requirements have been suggested (Table 4).

TABLE 2

EXTREMES OF OCCURRENCE OF FOLIC ACID BY FOOD GROUP (15)

Source	Free Folic Acid (ng/g)	Total Folic Acid (ng/g)
Meat		
Chicken Liver	----	3770
Lamb Stew Meat	4	19
Vegatables		
Spinach	310-1100	490-1150
Turnips	----	43
Fruit		
Avacados	176	60-570
Apples	----	5
Dairy Products		
Cottage Cheese	----	210-460
Egg White	----	6

TABLE 3

NATIVE FOLIC ACID CONTENT OF CEREAL GRAINS AND GRAIN PRODUCTS (15)

Source	Free Folic Acid (ng/g)	Total Folic Acid (ng/g)
Wheat		
Whole Kernel	8-30	270-510
Whole Wheat Flour	---	380
Bran	240-470	1000
White Flour	---	81
White Bread	---	150
Barley		
Whole Kernel	210	500
Brown Rice		
Whole Kernel	109	220
White Oats		
Whole Kernel	130-260	230-660
Oat Meal	78	305
Rye		
Whole Kernel	138	344
Rye Bread	---	198
Yellow Corn		
Whole Kernel	5	236
Corn Meal	24	65
Corn Flakes	28	55

TABLE 4

RECOMMENDED DAILY ALLOWANCE FOR FOLIC ACID (16)

	Folic Acid (ug/day)
Adults	400
Man	400
Woman	400
Pregnant	800
Lactating	600
Children	
Less than 1 yr	50
1-3 yr	100
4-6 yr	200
7-9 yr	300
10 yr and above	400

Bread and flour have been recommended by the National Research Council as an appropriate vector for folic acid enrichment (16). Because of the instability of folic acid and the variations in flour storage conditions and additives, an efficient method of monitoring levels of folic acid is essential.

The purpose of this research, then, was to derive a rapid, sensitive method for detection of folic acid, and determine its applicability to the analysis of folic acid in enriched grain products.

CHAPTER II

REVIEW OF THE LITERATURE

Chemical properties of folic acid. Folic acid, as most pterin derivatives, is insoluble in non-polar organic solvents. It is slightly soluble in glacial acetic acid, phenol, methanol, and in water when it exists as the unionized form (17). In the ionic state, however, it is three orders of magnitude more soluble in water, 15mg dissolving in 1ml (18).

The instability of the folic acid molecule is greatly enhanced by the presence of oxygen. In a 1.0 N aerobic sodium hydroxide solution, the molecule is rapidly cleaved between C^9 and N^{10} (Figure 1) to pterin-6-carboxylic acid and p-aminobenzoylglutamic acid (19). Heating the same solution anaerobically at 120°C results in very little cleavage but extensive racenization. Heating folic acid in a 1.0 N sulfuric acid solution in the absence of oxygen yields a mixture of pterin-6-carboxylic acid, 6-methylpterin, and p-aminobenzoylglutamic acid (19). Folic acid is also cleaved in a 0.5 N sulfurous acid solution yielding the unstable pterin-6-carboxaldehyde, which upon exposure to alkaline pH rapidly undergoes a Cinnizzaro-type rearrangement (20).

Reductive cleavage of folic acid to 6-methylpterin and p-aminobenzoylglutamic acid is accomplished by adding zinc powder to a dilute acidic solution (20).

Folic acid is also photolytically cleaved by ultraviolet light. This occurrs in a progressive manner, the molecule being cleaved to pterin-6-carboxaldehyde and p-aminobenzoylglutamic acid initially, the former then being oxidized to pterin-6-carboxylic acid, and finally decarboxylated under very intense radiation to pterin (21). All of these compounds are inactive for the commonly used assay organisms and, therefore, caution is required in protecting

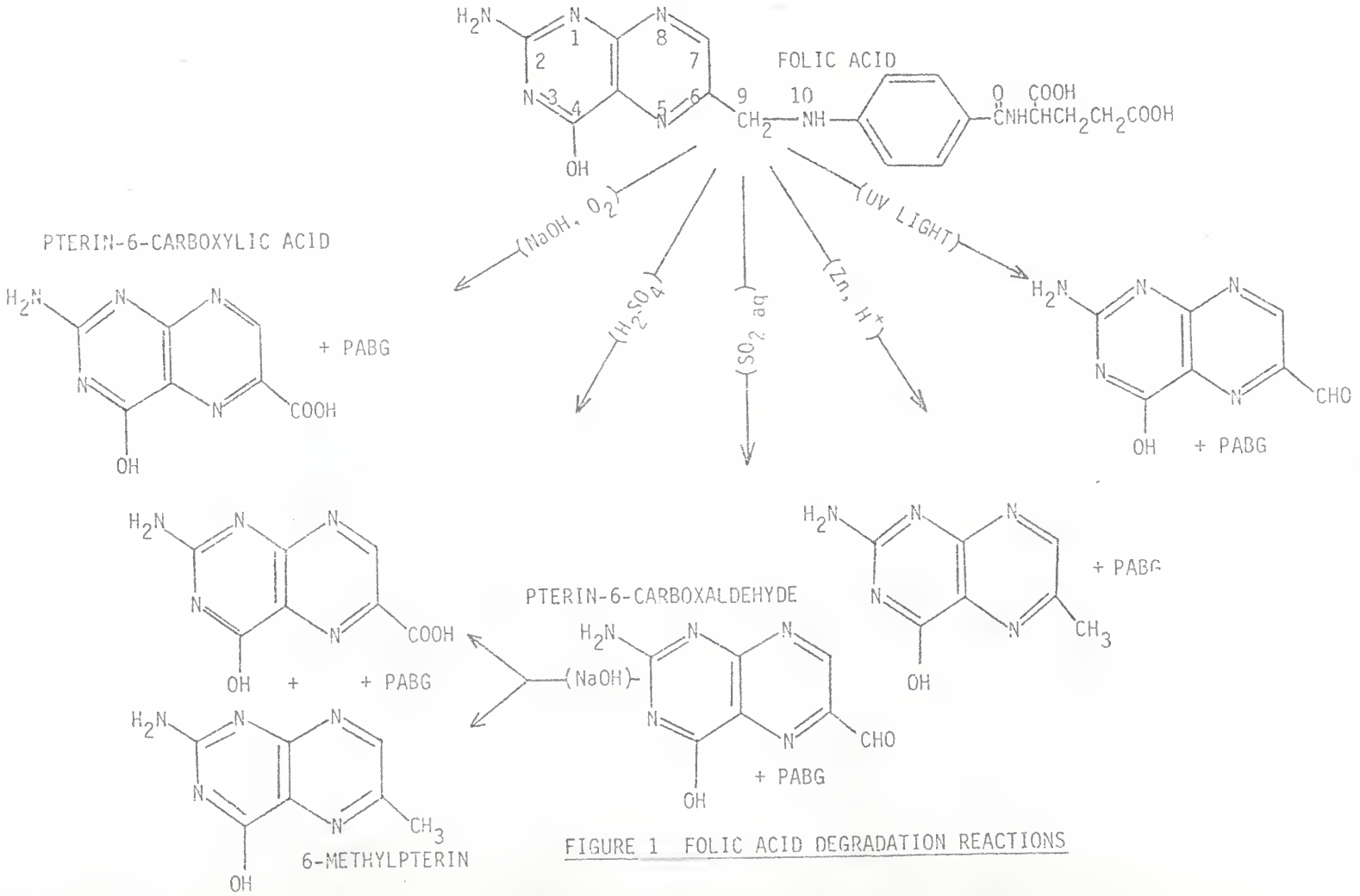


FIGURE 1 FOLIC ACID DEGRADATION REACTIONS

extract solutions from light.

Flourescence of folic acid. Conflicting reports on the fluorescent nature of folic acid may be found in the literature. Uyeda and Rabinowitz, and Duggen et al. have reported a fluorescence emission maximum between 450 and 460nm with an excitation maximum at 363nm, whereas Wright et al. reported no fluorescence could be observed for a folic acid solution (22, 23, 24). It was suggested (22) that the basis for this discrepancy was the difference in pH values of the solutions studied. However, Rouseff (25) has recently demonstrated, by use of dual detectors with a high performance liquid chromatographic separation system, that regardless of sample purity a fluorescent peak eluted outside of the folic acid peak. This then suggests that the fluorescence observed by earlier researchers was attributable to an analytical artifact.

Methods of folic acid determination. The methods of folic acid determination fall mainly into two categories -- those based on its biological activity and those based on its chemical behavior. Most of the methods in current use are logical extensions of the early descriptive observations on the nature of the folic acid molecules ability to stimulate the growth of microorganisms.

The biological methods of analysis have two major advantages, the ability to detect as little as 25pg/ml in the microbiological assay organism.

The microbiological assay, employing the organisms Lactabascillus casei, Streptococcus fecalis, or Pediococcus cerevisiae, is generally performed as described by Tepley and Elvehjem (27) often with minor modifications. The major disadvantages of this method include the selectivity for certain molecular forms of the folic acid group (Table 5), the necessity to continually transfer the culture to maintain viability, the shelf life of stock media preparations, the requirement for meticulously clean glassware, the necessity for aseptic

TABLE 5

GROWTH RESPONSE OF ASSAY ORGANISMS TO FOLATE DERIVATIVES (47)

Compound	P. cerevisiae	S. fecalis	L. casei
Pteroate	-	+	-
N^{10}-formylpteroate	-	+	-
N^{5}-formylpteroate-H_4	-	+	-
Pteroylglutamate (Folic acid = F)	-	+	+
N^{10}-formylF	-	+	+
FH_2	-	+	+
N^{10}formylFH_2	-	+	+
FH_4	+	+	+
N^{5}-formylFH_4	+	+	+
N^{10}-formylFH_4	+	+	+
N^{5}-formiminolFH_4	+	+	+
N^{5-10}-methyleneFH_4	+	+	+
N^{5}-methylFH_4	-	-	+
Pteroyldiglutamate	-	+	+
N^{5}-formylpteroyldiglutamateH_4	+	+	+
Pteroyltriglutamate	-	-	+
N^{10}-formylpteroyltriglutamate	-	-	+
N^{5}-formylpteroyltriglutamateH_4	+	-	+
Pteroylheptaglutamate	-	-	-

conditions and the time factor involved in a single determination. However, the time factor has recently been reduced somewhat by an automated method which measures the rate of reduction of 2,3,5-triphenyltetrazolium hydrochloride instead of turbidity as an index of growth (29).

Folic acid assay with higher animals is accomplished by maintenance on folate dificient diets, often accompanied by elimination of folate producing intestinal organisms with insoluble sulphonamides (30). Once a deficient state has been induced, folic acid activity can be determined by plotting the dosage-response curve of hematopoeisis or weight gain (28,31). Although this method has the advantage of measuring only the molecular forms available to the respective animal, the extremely long assay time, the lack of sensitivity, and the expense limit its usefulness.

Chemical methods of folic acid determination. The category of chemical methods of analysis may be further subdivided into two classes. These are, determinations based on chemical modification of the folic acid molecule and estimating the concentration indirectly; and methods dependant upon chemical and physical differences between folic acid (and its derivatives) and the matrices that they are found in.

The first method reported utilized the reductive cleavage of folic acid with zinc dust at acid pH liberating free p-aminobenzoylglutamic acid (32). This was quantitated by the Bratton-Marshall method of coupling the free aryl amine with N-(1-naphthyl)ethylenediamine dihydrochloride, forming a spectrophotometrically analyzable azo dye (33). The sensitivity of this method, accurately measuring as little as 5µg/ml, is the main limitation.

A much more sensitive fluorometric assay was reported by Allfrey et al., which employed oxidative cleavage of folic acid by the incremental addition of potassium permanganate to extract solutions (34). The concentration of folic

acid was determined by comparison of the change in sample fluorescence with a standard plot. This method is capable of detecting 10ng/ml but does not provice similar functionalities, such as tryptophan and tyrosine, which also fluoresce under the oxidative conditions.

Paper chromatographic separation followed by bioautographic or ultraviolet spectrophotometric determination has been used extensively in the analysis of folic acid (35,36,37). DEAE and TEAE cellulose columns and thin layer chromatographic preparations have also proven useful in the separation, identification, and quantitation of folic acid and its derivatives (38,39). Although these methods have contributed greatly to an understanding of the folic acid molecule, classical chromatography is limited by the large time factor necessary to achieve adequate separation. In addition, their usefulness as a quantitative measure of folic acid is restricted by the detection system employed.

Recently, the rapidly developing technique of high performance liquid chromatography has been applied to the analysis of folic acid. This offers the resolution of the earlier chromatographic methods with the additional advantages of greatly increased speed of analysis and sophistocated in-line detection systems. Branfman and McComish (40) demonstrated that folic acid could be separated from its derivatives by paired-ion chromatography in the reverse phase. Folic acid has also been resolved from a mixture of water soluble vitamins by reverse phase chromatography with (41) and without (42) ion-pairing agents in the mobile phase. Clifford and Clifford (43), using a 10^{-4}m X 3m capillary column packed with Pellonex SAX (a strong anion exchange resin) were able to separate folic acid from its derivatives in extracts of food. However, their reported detection limit of 0.035 nmole per 10ul in-

jection, corresponding to a folic acid extract concentration of 1.54 mg/ml, renders this method inapplicable for the analysis of nearly all foods (see Tables 2 and 3) without the inclusion of a ten-fold concentration step. All methods reported, then employed UV detection which is insufficiently sensitive for the analysis of folic acid in grain products enriched or unenriched.

Other, less broadly applicable methods of folic acid determination have been described. These include polarographic analysis (44), enzymatic interconversion (45), and radio-enzymatic determination (46).

CHAPTER III

MATERIALS AND METHODS

The folic acid used in derivitization experiments was purchased from Sigma Chemical Company. U.S.P. folic acid reference standards stored under P_2O_5 in a dessicator were used for quantitation of the microbiological and chromatographic assays. Solvents used in the chromatographic experiments were distilled twice, degassed for five minutes with a Megason ultrasonic, and filtered through an XX10-04700 Millipore vacuum filtration system fitted with a 0.45um porosity filter.

The chromatographic system consisted of a Rheodyne model 7120 sample injector, a Waters Associates M6000 pump, and a 4.1mm ID x 25cm stainless steel column slurry packed with uBondapack C_{18} under 8,000 psi pressure. Fluorescence detection was accomplished with an Aminco model J4-7440 Fluoro-Colorimeter equipped with a 9ul continuous flow cell. A Hewlett Packard model 1041B 254nm fixed wavelength detector was used for ultraviolet detection and quantitation was performed by a Hewlett-Packard model 3385A data handling system.

The absorbance spectrum for folic acid at pH 5.5, 6.5, 7.5 and 8.5 at a concentration of 1.6ug/ml was determined on a Beckman model DB-G spectrophotometer. This was done to ascertain the optimum wavelength and pH for folic acid detection by ultraviolet absorption.

The response of folic acid retention time to varying concentrations of methanol in 0.01 M phosphate buffer at pH 7.5 was studied to determine the ease of manipulation of the peak for future separation from matrix interferences. Injections of 10ul of 5ug/ml folic acid were made using 3,4,5,6,15 and 30% methanol mobile phases. A retention time of 3.10 minutes was obtained for folic

acid using the 15% methanol mobile phase, and since this gave a sharp baseline resolved peak, it was used to establish the detection limits at 254nm. It was also determined from the spectral data that maximum folic acid absorption at this wavelength occurred at pH 8.5, and therefore, the mobile phase was adjusted accordingly. Other conditions maintained during the determination of the detection limit for folic acid at 254nm included a detector attenuation of 64, slope sensitivity and attenuation settings on the data handling system of 0.50 and 4 respectively, and a flow rate of 1ml/min.

Fluorescent derivatization of the folic acid molecule. To discover the most rapid and complete method of cleavage of the folic acid molecule to its fluorescent pterin derivatives (see Figure 1), several reaction conditions were tried.

Cleavage at the C^9- N^{10} bond to a mixture of pterin-6-carboxylic acid and 6-methylpterin was attempted under both aerobic and anaerobic conditions using 50ug/ml folic acid in 0.50 N H_2SO_4. Toluene was layered over the anaerobic sample and it was bubbled with N_2 for 10 minutes. The aerobic sample was vigorously vortexed for 5 minutes, after which both samples were sealed and placed over steam. Aliquots were withdrawn at 30 minute intervals and chromatographed. It was found that cleavage did in fact occur, but at a lethargic pace so HCl was substituted for H_2SO_4 and the experiment repeated to determine whether the type of acid would affect this rate.

Oxidative cleavage of the folic acid molecule to pterin-6-carboxylic acid was then attempted under various oxidizing conditions. These included adding 1ml of folic acid (100ug/ml) to each of several tubes containing either 1ml H_2O, 1ml 0.5 N H_2SO_4, 1ml 30% H_2O_2, 1ml 0.5 N NaOH, 1ml 0.47 M $KMnO_4$, 1ml of 0.5 N H_2SO_4 diluted with 30% H_2O_2, or 1ml of 0.47 $MKMnO_4$ in 0.5 N NaOH. The tubes were sealed and set in steam, and samples were removed every 30

minutes for chromatographic analysis.

Fluorescent excitation and emission spectra were then determined on the produce resulting from oxidation of folic acid with $KMnO_4$ at alkaline pH, with a Aminco-Bowman model 4-8202 Spectrophotofluorometer. The UV spectrum of the pterin-6-carboxylic acid and p-aminobenzoylglutamic acid mixture was also determined on a Gilford Spectrophotometer 2400. The presence of p-aminobenzoylglutamic acid was also accounted for by incorporating pure p-aminobenzoylglutamic acid in the blank.

The limit of fluorescent detection of the oxidation product was then determined by analyzing a series of standards ranging in concentration from 100ng/ml to 10ng/ml. These were made by adding the appropriate concentration of freshly prepared folic acid to a screw cap culture tube and diluting to 8.5ml. To this, 1ml of 0.25 N NaOH in 0.125 M KH_2PO_4 and 0.5ml of 0.47 M $KMnO_4$ were added. The reaction tubes were then sealed, thoroughly mixed, and steamed for 10 minutes. After cooling on the lab bench for 5 minutes, 1ml of 30% ascorbic acid solution was added to decolorize the reaction mixture and the vessels were then centrifuged at maximum speed on a Damon model 6000 centrifuge. The supernatant was then quantitatively transferred to a 25 ml volumetric and diluted with distilled water to the mark.

Oxidative cleavage of the folic acid molecule when extracted from a flour matrix was performed by adding 0, 0.25, 0.50, 0.75, 1.00, 2.00, and 3.00ml of 0.47 M $KMnO_4$ to alkaline flour extracts. The extracts were prepared by mixing 2g samples of flour enriched to 1ug/g with folic acid in 2ml of 0.25 N NaOH in 0.125 M KH_2PO_4 solution and 4ml of H_2O. These tubes were warmed to 55^{o}C in an incubator for 30 minutes on a stirring plate. The samples were then centrifuged at 5,000 rpm at 5^{o}C for 15 minutes. To the

supernatant, the above listed aliquots of $KMnO_4$ were added with enough H_2O to make a total addition of 3ml. The tubes were then vortexed, steamed for 5 minutes, 1ml of 30% ascorbic acid added, and recentrifuged. The supernatant was quantitatively transferred to a 25ml volumetric and diluted to the mark with water.

Chromatographic conditions were optimized for the separation of pterin-6-carboxylic acid using several packing materials and solvent systems. The column packing materials tested included Lichrosorb RP2 and RP8, and uBondapak C_{18}. Solvent systems tried were 2 to 30 percent methanol:water, the use of hexanesulfonic acid as an ion pairing reagent, and 0.01 M KH_2PO_4 in water at pH 6, 7, and 8. Since pterin-6-carboxylic acid eluted as a single, well resolved peak with a retention time of 7 minutes when 0.01 M KH_2PO_4 pH6 was used as the mobile phase with the uBondapak C_{18} column, this system was employed for the remainder of the experimentation.

Efficiency and reproducibility of extraction was then determined on an enriched, first midds, hard wheat flour preparation. The flour was enriched by adding 0.1mg of folic acid to 10.0g of flour. This mixture was tumbled for 4 hours at 40 rpm in a fluted 1 quart Ball jar, after which 90.0g of flour were added and the total preparation tumbled for 32 hours more. The extraction and oxidation were performed as described above except that 2.0ml of 0.47 M $KMnO_4$ was added to each sample.

The spectrum of application of this method was assessed by oxidizing extracts of unenriched durum flour and corn meal; and enriched soft wheat flour, corn meal, light toast, bread, yellow cake, corn meal mush, durum flour, and cooked spaghetti. The procedure followed was that described above. The reverse phase chromatographic analysis was accomplished using a

0.01 M KH_2PO_4 mobile phase at pH 6.0 and a flow rate of 1ml/min. In all determinations, a 100ul injection was made and the results quantitated by the Hewlett-Packard model 3385A data handling system calibrated with an external standard.

Microbiological assays were used for comparison in the application study, and were accomplished according to section 43.0097 of the Approved Methods of the AOAC with minor modifications, using S. fecalis ATCC 8043 as the assay organism. Samples (1.000g) were extracted with 25ml of 0.05 N NH_4OH in propylene centrifuge tubes by autoclaving at 20 psi and 121°C for 10 minutes. Starch gels formed from this extraction were broken up by homogenizing in a blender with 50ml of additional distilled water. The extracts were then filtered through Watman No. 4 qualitative filter paper, rinsing the filtrate twice with about 10ml of distilled water each time, the pH adjusted to 7.0 and diluted to 100ml in a volumetric. The extracts were then diluted 1:2 to give a final concentration of folic acid between 1 and 10ng/ml.

Assays were then performed by preparing duplicate culture tubes containing 1 and 2 ml of the diluted extract. The volume of each tube was brought to 5ml and the vessels were capped loosely and autoclaved to 10 minutes more. To each culture tube, 5ml of double strength Bacto Folic Acid Assay Medium (Difco) which was previously sterilized, was aseptically added.

Each vessel was then innoculated with 1 drop of an innoculum which was prepared by pelleting an overnight culture which had been grown in a limited folate medium, and resuspending it in 100ml of sterile H_2O. The assay tubes were then incubated at 37°C for 18 hours, and the growth measured turbidimetrically at 540nm with a Spec 20. The absorbance of each sample was compared to that given by standards and the concentration of folic acid in the original sample calculated.

CHAPTER IV

RESULTS AND DISCUSSION

The absorbance spectrum for folic acid as determined is illustrated in Figure 2. This corresponds closely to spectral data found in the literature (18,48,49). The effect of variations in pH on the absorbance of folic acid over the range of 5.5 to 8.5 (the range most compatable with HPLC systems) is summarized in Table 6. The optimum pH for folic acid determination was determined to be pH 8.5.

The dependance of folic acid retention time, when chromatographed on a C_{18} uBondapack column, on the amount of methanol in the phosphate mobile phase is summarized in Figure 3. This hyperbolic response greatly simplified its separation from matrix interferences in the preliminary UV determinations when folic acid was chromatographed as the intact molecule. However, it was also found that the folic acid peak was quite responsive to fluctuations in room temperature.

The detection limits for folic acid at 254nm were determined under the conditions previously described, which gave a minimum retention time with baseline resolution, and consequently a maximum peak heighth. Since the detection limits are defined as that concentration which gives a detector signal of twice the noise level, these results (Figure 4) must be considered as a best case analysis.

Since it was determined (see Table 6) that the absorbance of folic acid at 280nm, very near the λmax, was between 2.3 and 2.6 times greater than at 254nm depending upon pH, the detection limit at the former wavelength can be calculated to be between 73ng/ml and 79ng/ml using a 100ul injection. It was, therefore, demonstrated that even under optimized conditions the sensitivity

FIGURE 2

ABSORBANCE SPECTRUM FOR FOLIC ACID (pH 7.0, 10ug/ml)

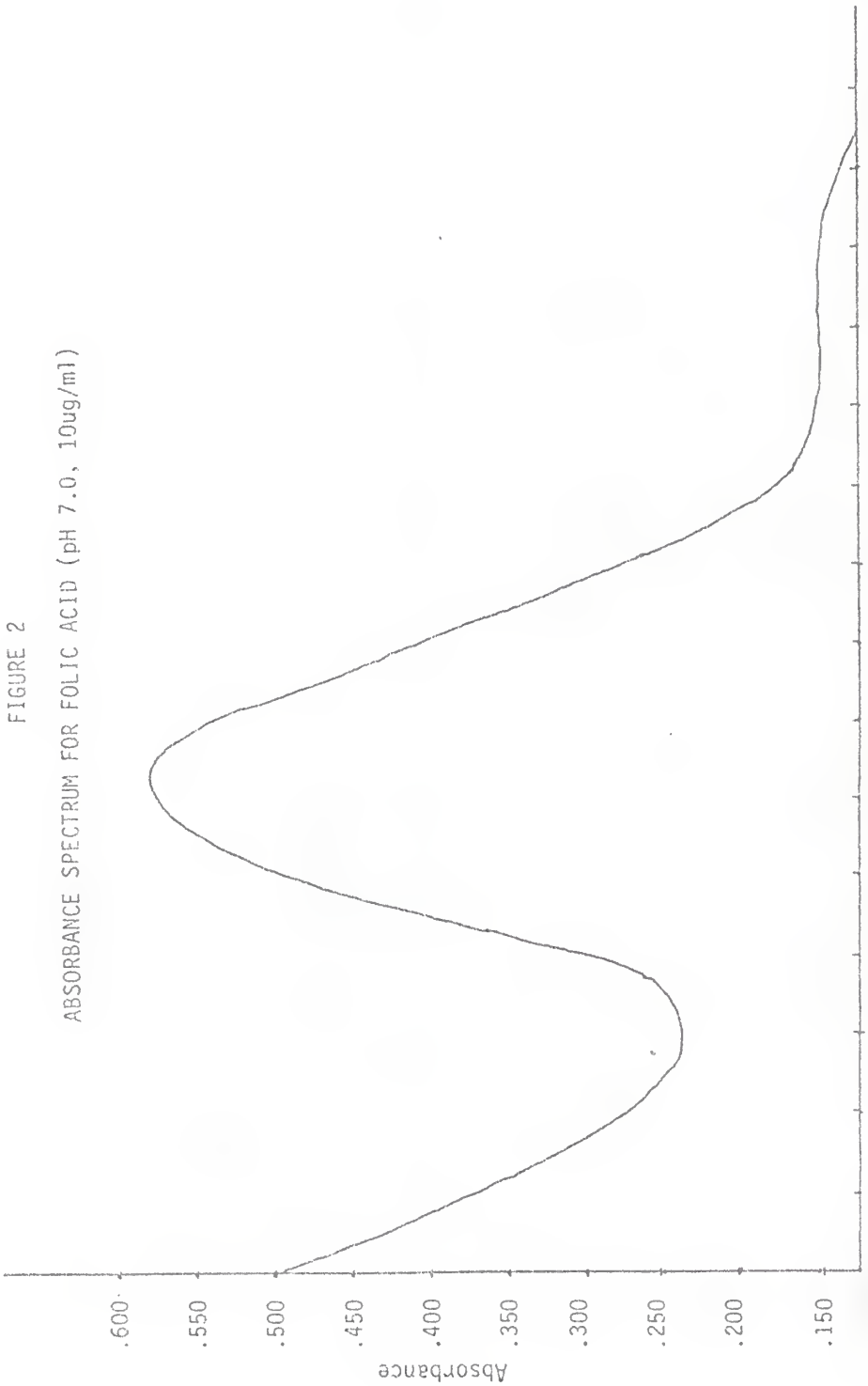


TABLE 6

THE RESPONSE OF FOLIC ACID ABSORBANCE TO VARIATIONS IN pH

pH	λmin (nm)	Absorbance at λmin	Absorbance at 254nm	λmax (nm)	Absorbance at λmax	Absorbance at 280nm
5.5	252	.0325	.0350	282	.0940	.0930
6.5	249	.0325	.0360	280	.0950	.0950
7.5	249	.0325	.0340	282	.0900	.0900
8.5	249	.0375	.0410	281	.0940	.0940

FIGURE 3

THE RESPONSE OF FOLIC ACID RETENTION TIME TO THE CONCENTRATION OF METHANOL IN THE 0.01 M PHOSPHATE MOBILE PHASE pH 7.5

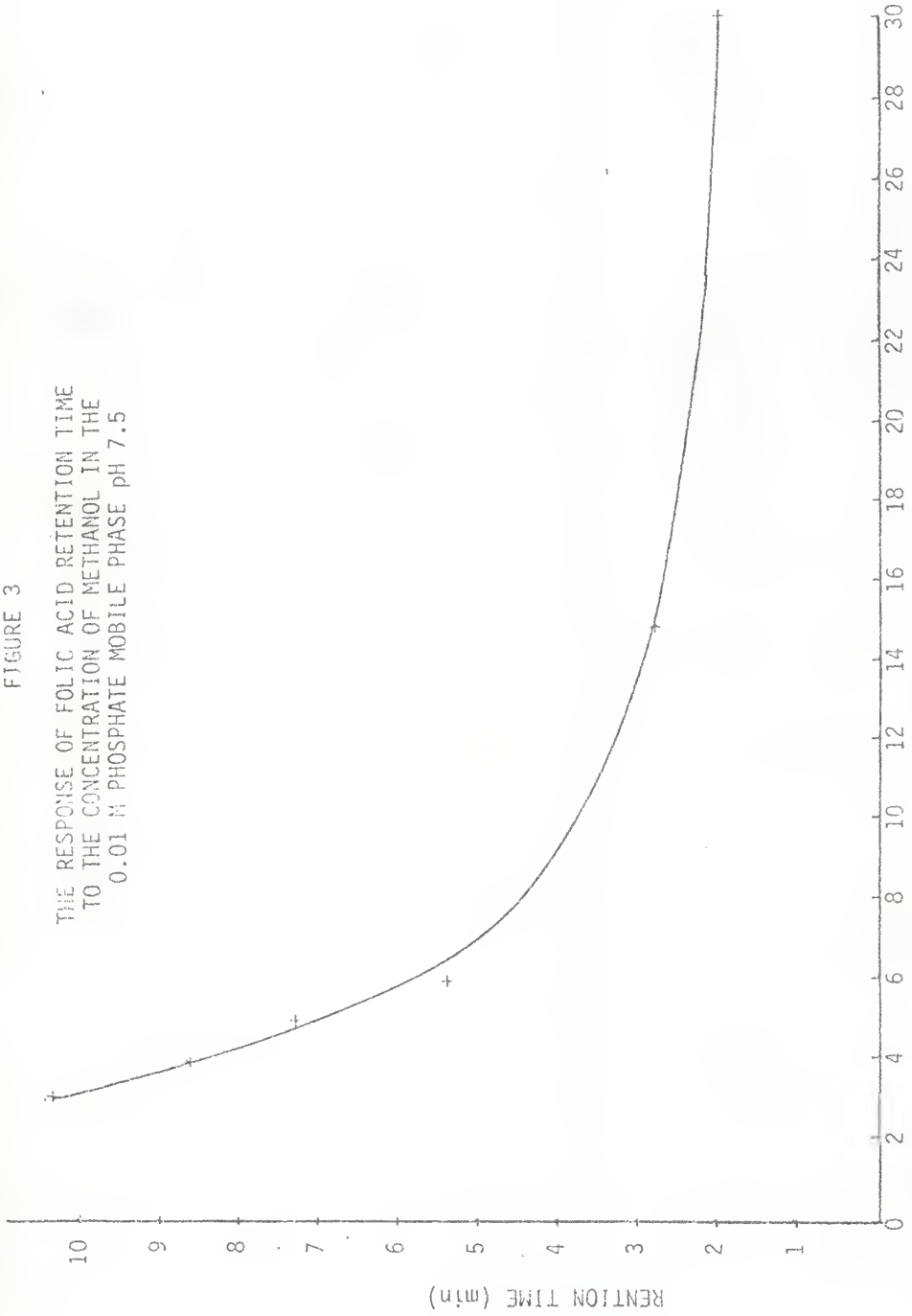
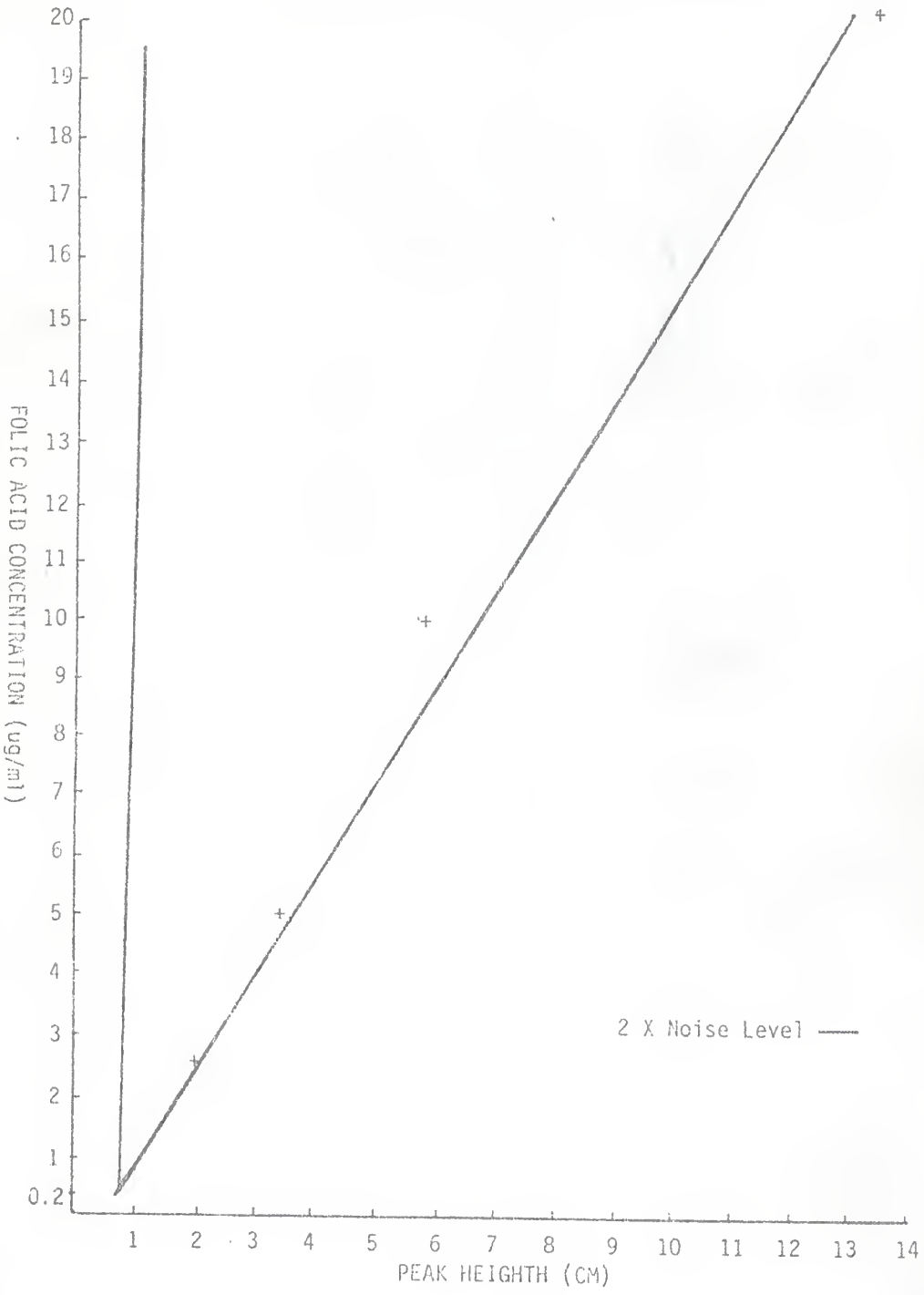


FIGURE 4

DETECTION LIMITS FOR FOLIC ACID AT 254nm



achievable using ultraviolet absorption is inadequate for the determination of folic concentrations in grain products.

It was then necessary to turn to fluorescence which offers greater sensitivity and selectivity for detection. Since folic acid itself does not fluoresce (25), several derivatizations were considered including labeling the C^2 amino group with 1-dimethylaminonaphthalene-5-sulfochloride (DANSYL chloride), and cleavage of the molecule to liberate the fluorescent pterin moiety. After attempts at dansylation were unsuccessful, and it became obvious that great interferences were being generated, it was abandoned.

Several methods for the cleavage of the folic acid molecule were attempted in order to determine which yielded the greatest amount of fluorescent analyte in a practical time frame. Acid cleavage, anaerobically and aerobically, generated significant amounts of fluorescent pterins only after 4 hours over steam, whereas oxidative cleavage at alkaline pH was immediate and nearly complete at room temperature (Figure 5). $KMnO_4$ was found to best facilitate this cleavage, being effective over a wide range of pH, however, at pH above neutrality some MnO_2 was formed.

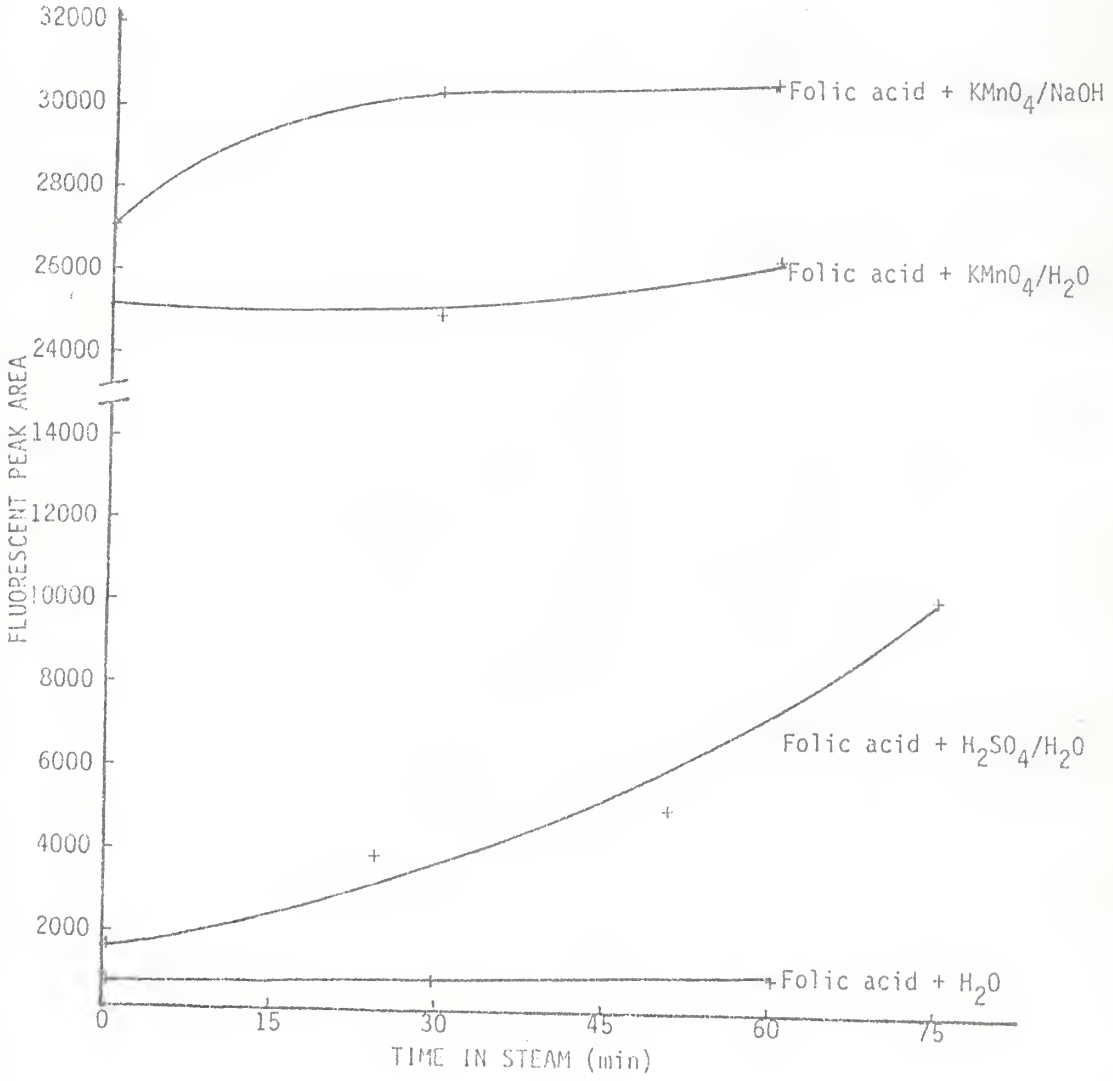
Fluorescent excitation and emission, and ultraviolet spectra for the oxidation product (Figures 6 and 7) agreed well with spectra for pterin-6-carboxylic acid found in the literature (22,48). Fluorescent detection limits were then determined for the oxidation product (Figure 8). It was found that sensitivity was increased by a factor of about 14 over detection at 280nm and 35 over that at 254nm.

Since folic acid is much more soluble in its ionic form at alkaline pH than near neutrality, an alkaline extraction process was chosen for its separation from the flour matrix. Many of the samples studied, however, predictably formed gels after exposure to temperatures above $60^{o}C$. It was,

FIGURE 5

COMPARISON OF OXIDATIVE CLEAVAGE CONDITIONS

Chromatographic conditions:
Concentration ---- 50ug/ml
Injection -------- 20ul
Detector attenuation 3
Mobile phase ----- 0.01 M KH_2PO_4, pH 6.0
Flow rate -------- 2ml/min



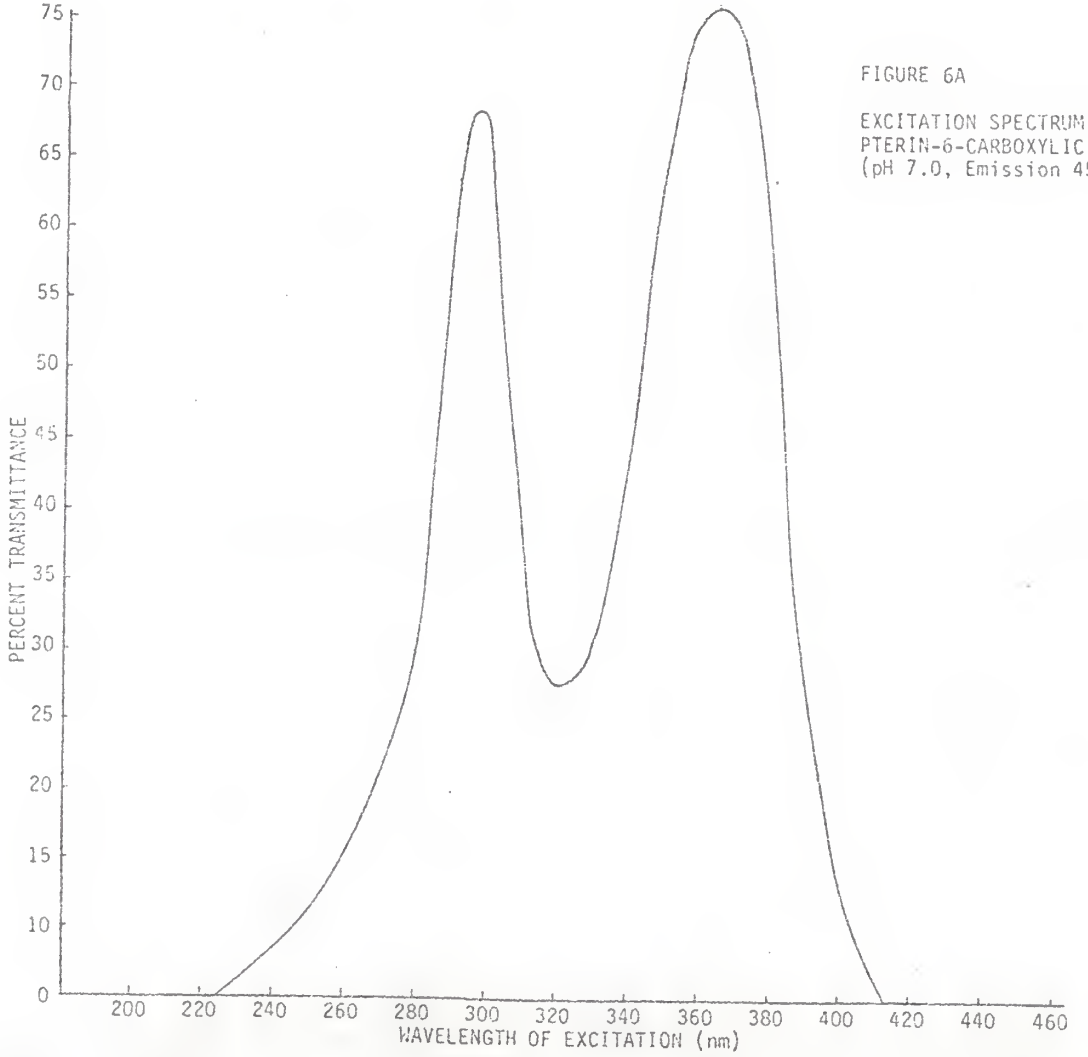


FIGURE 6A

EXCITATION SPECTRUM FOR
PTERIN-6-CARBOXYLIC ACID
(pH 7.0, Emission 450nm)

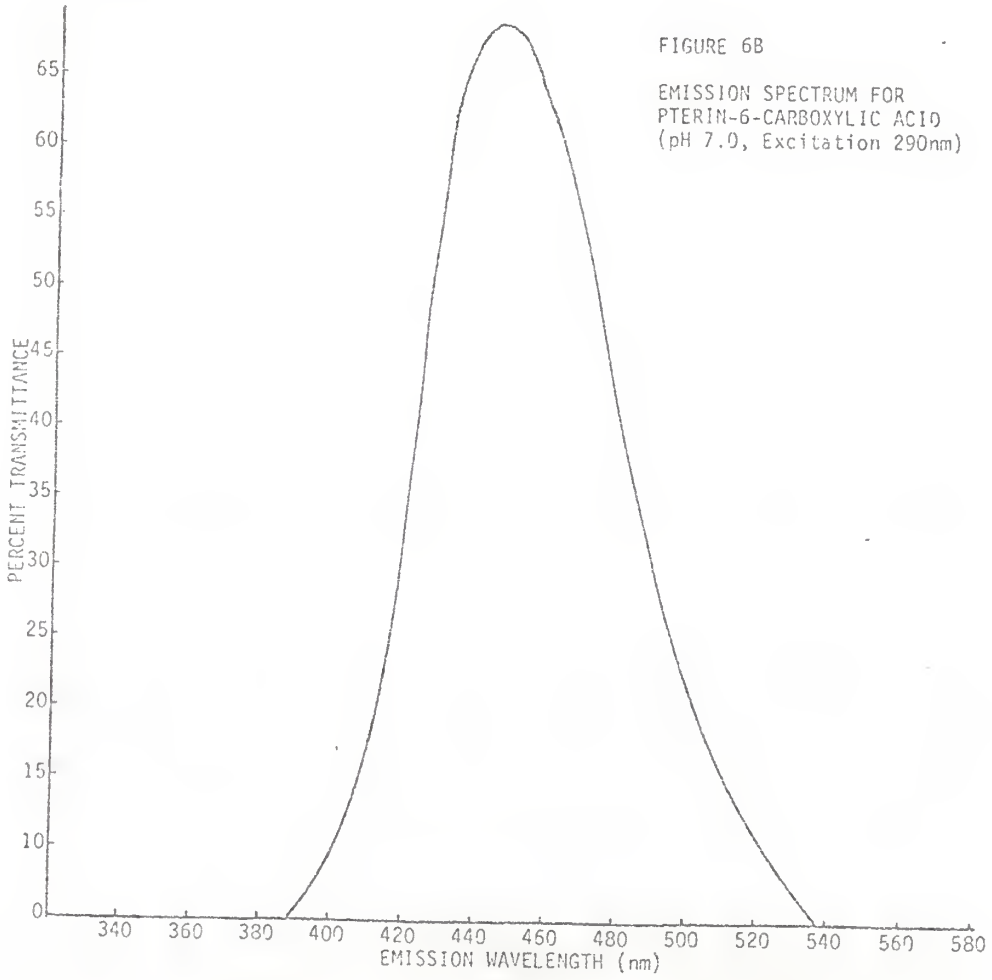


FIGURE 6B

EMISSION SPECTRUM FOR
PTERIN-6-CARBOXYLIC ACID
(pH 7.0, Excitation 290nm)

FIGURE 7

UV SPECTRUM FOR PTERIN-6-CARBOXYLIC ACID (pH 7)

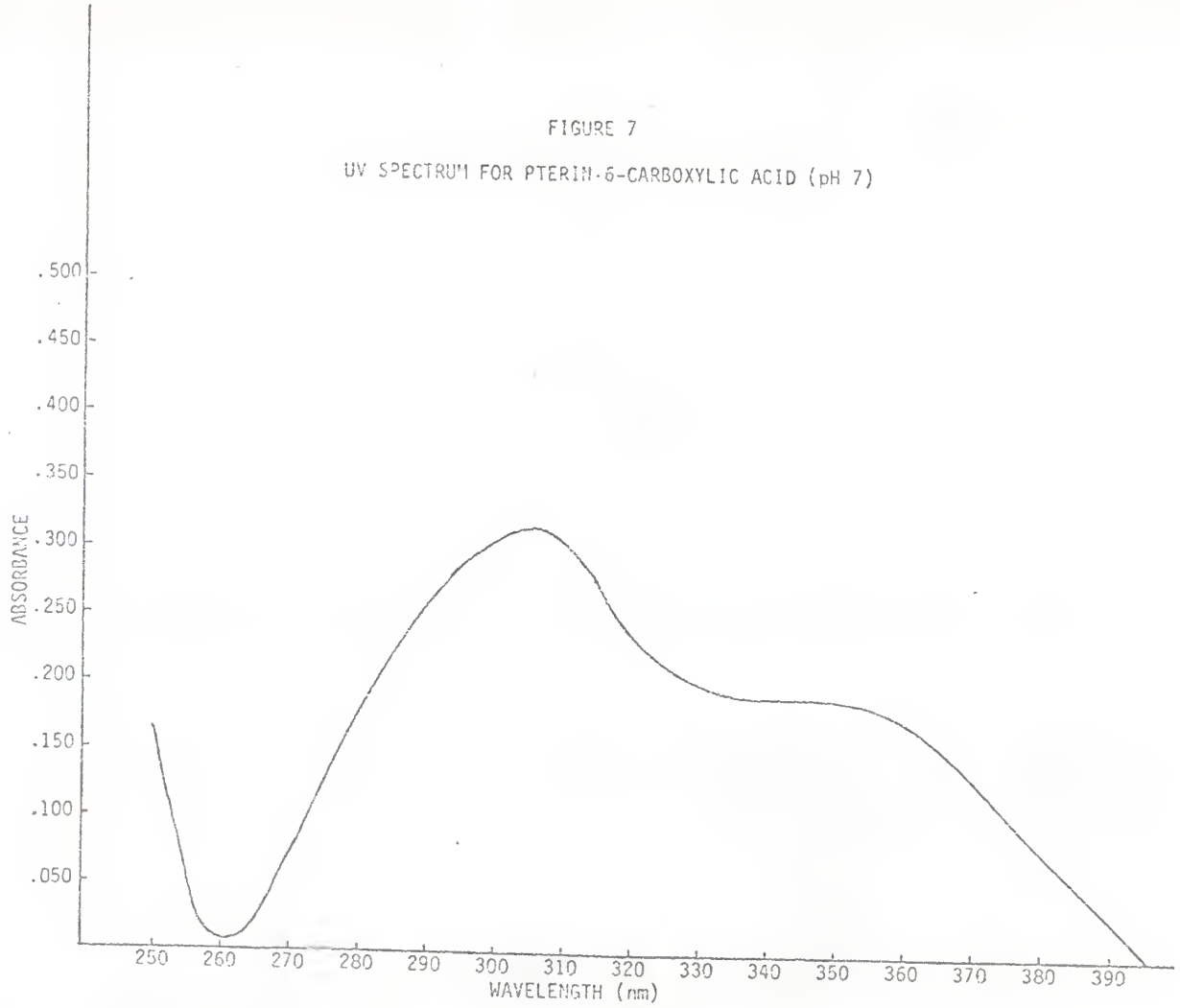
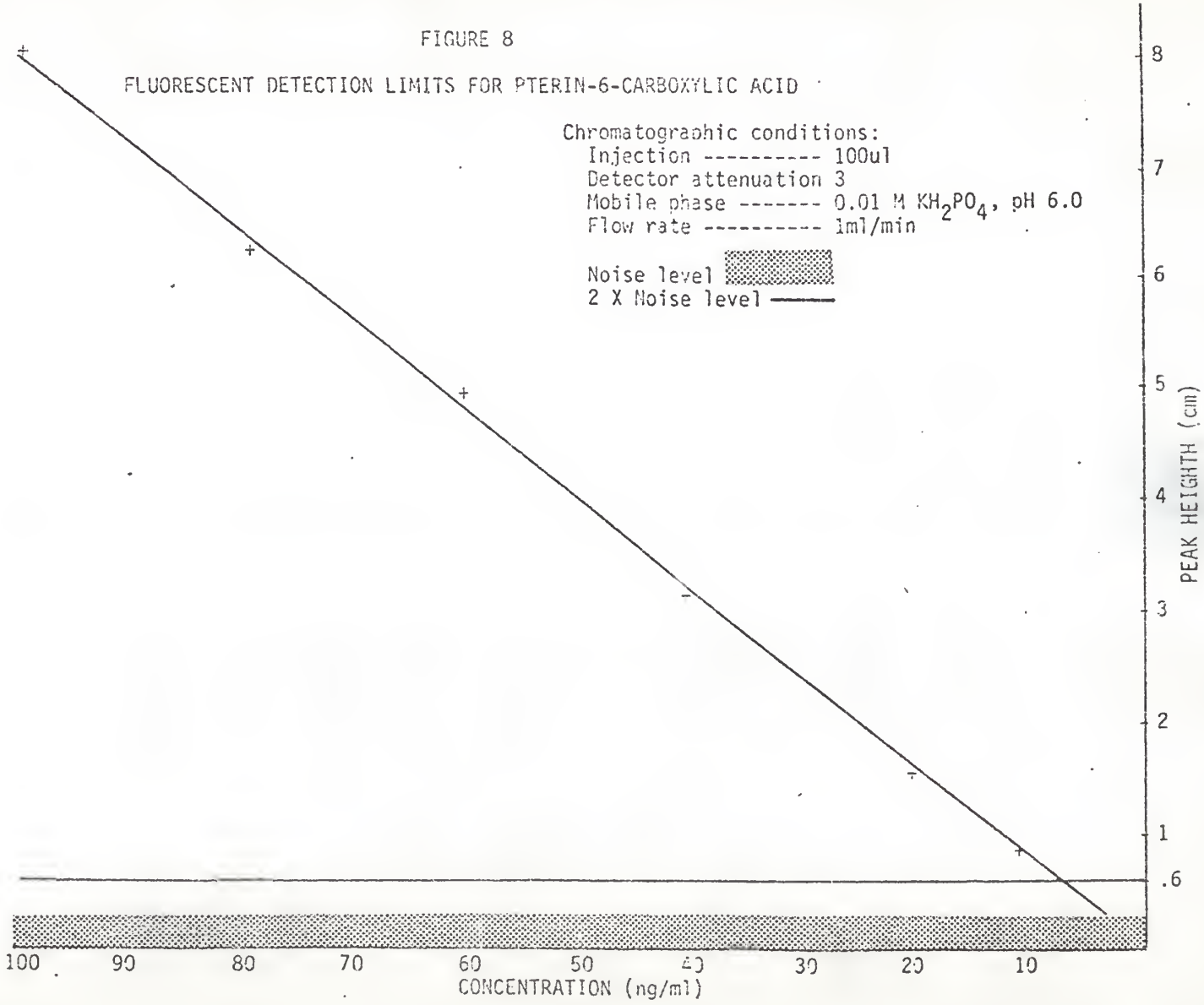


FIGURE 8

FLUORESCENT DETECTION LIMITS FOR PTERIN-6-CARBOXYLIC ACID



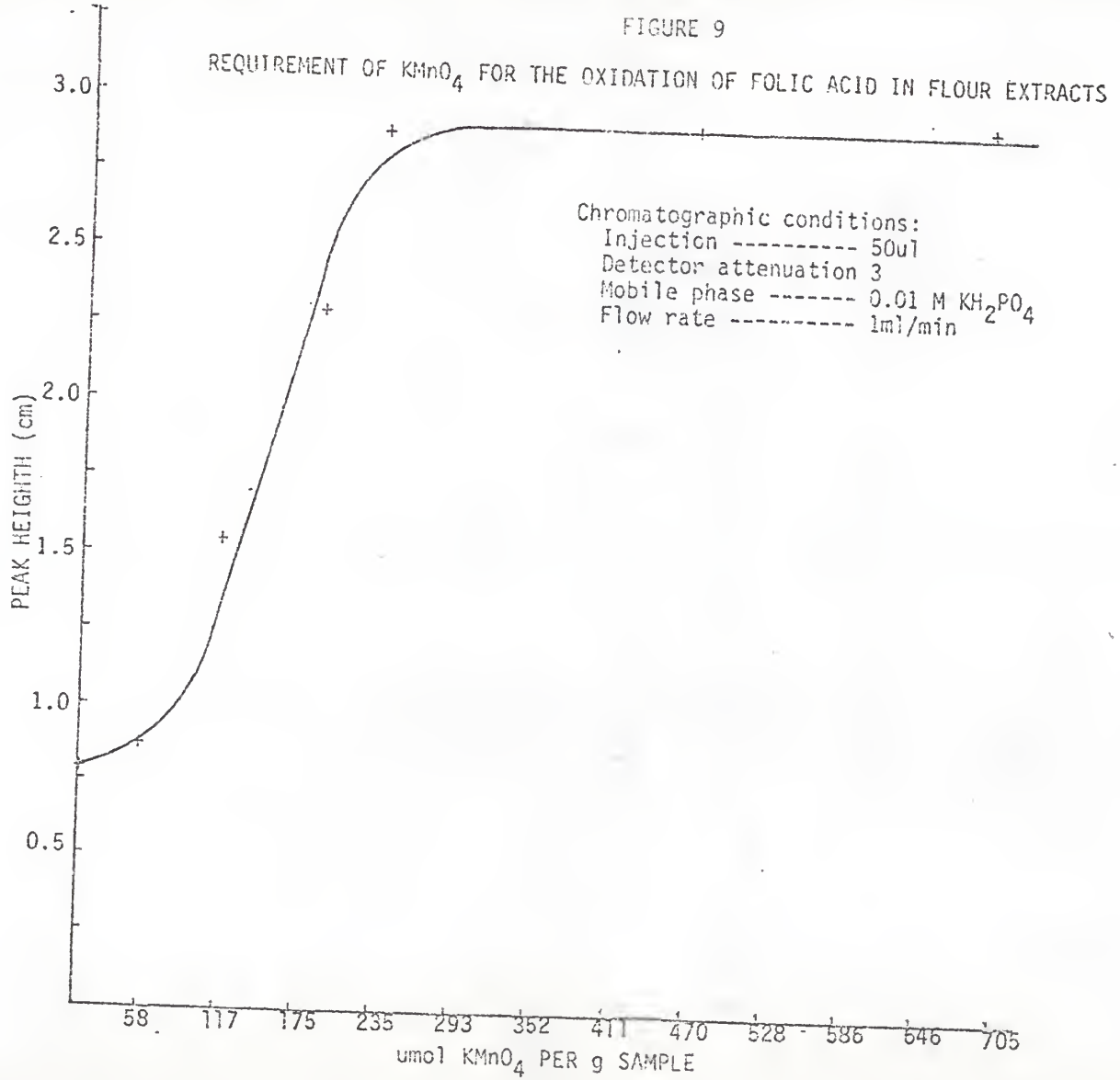
therefore, impossible to extract the samples in a manner similar to that used for the microbiological assay without devising an adequate method for liquifying the gels.

The levels of dilution used in the sample preparation were much smaller than those used in the microbial method and it was, therefore, impossible to homogenize the gels in excess water. Acid and enzymatic hydrolysis were tried, but neither proved to be of value for this assay. Acid hydrolysis, being limited to short duration because of the generation of competing folic acid degradation products, yielded a colloidal suspension after oxidation which could not be removed by centrifugation or filtration and gave a nephelometric response on the fluorescence detector when chromatographed. Enzymatic degradation using malt and fungal amylases, pepsin and pancreatin also caused dramatic increases in interferences, presumably because of the additional soluble protein added to the mixture. Denaturation of the protein, following enzymatic degradation of the starch gel, with water saturated phenol-chloroform (2:1) resulted in considerable precipitation, however, the redox potential of the extract solution was altered so severely that oxidation with less than 5ml of saturated $KMnO_4$ solution was impossible. For these reasons, extraction of the folic acid from the flour matrix was limited to a procedure including subgelatinization temperatures at high pH.

The extent of oxidation of folic acid in an alkaline flour extract is illustrated in Figure 9. A plateau in the level of generated fluorescent pterin-6-carboxylic acid was reached upon oxidation of the extract solution with 1.0ml of 0.47 M $KMnO_4$ and remained constant through the addition of 3.0ml. It was, therefore, determined that 2.0ml of the 0.47 M $KMnO_4$, corresponding to twice the amount necessary to oxidize the easily oxidizable compounds in the first midds hard wheat flour extract as well as the folic acid, would insure

FIGURE 9

REQUIREMENT OF $KMnO_4$ FOR THE OXIDATION OF FOLIC ACID IN FLOUR EXTRACTS



oxidative cleavage of folic acid when separated from a practical range of matrices.

The efficiency and reproducibility of extraction and oxidation on ten identical samples were then determined, the results being summarized in Table 7. The differences noted between the level added and the quantity of folic acid estimated may have been the result of several factors, the most obvious of which are quenching and inefficient extraction. Allfrey (34) reported quenching of pterin-6-carboxylic acid by several anions including acetate, borate, sulfate, chloride, and phosphate. However, at neutral to alkaline pH, these anions exist in an equilibrium favoring the dissociated state and because of the magnitude of the ionic character in the dissociated state, these anions should be rapidly separated from the pterin-6-carboxylic acid by chromatography in the reverse phase mode and elute near the solvent front.

Alternatively, the polar glucose polymers composing the starch granule provide excellent sites for interaction with the folic acid molecule. This is particularly evident when the many polar functionalities of folic acid and the tremendous starch surface area are considered.

Several grain flours and products were then assayed to determine the spectrum of applicability of this method. The results were calculated by a Hewlett-Packard data handling system calibrated with an external standard and adjusted to compensate for the 68.3% recovery efficiency as determined above. Parallel microbiological assays were run for comparison in duplicate on each sample as described above. The results are summarized in Figure 10. Baked and cooked products generally reflected lower recovery rates than flours. This may be attributable to entrapment of the folic acid in the partially

TABLE 7

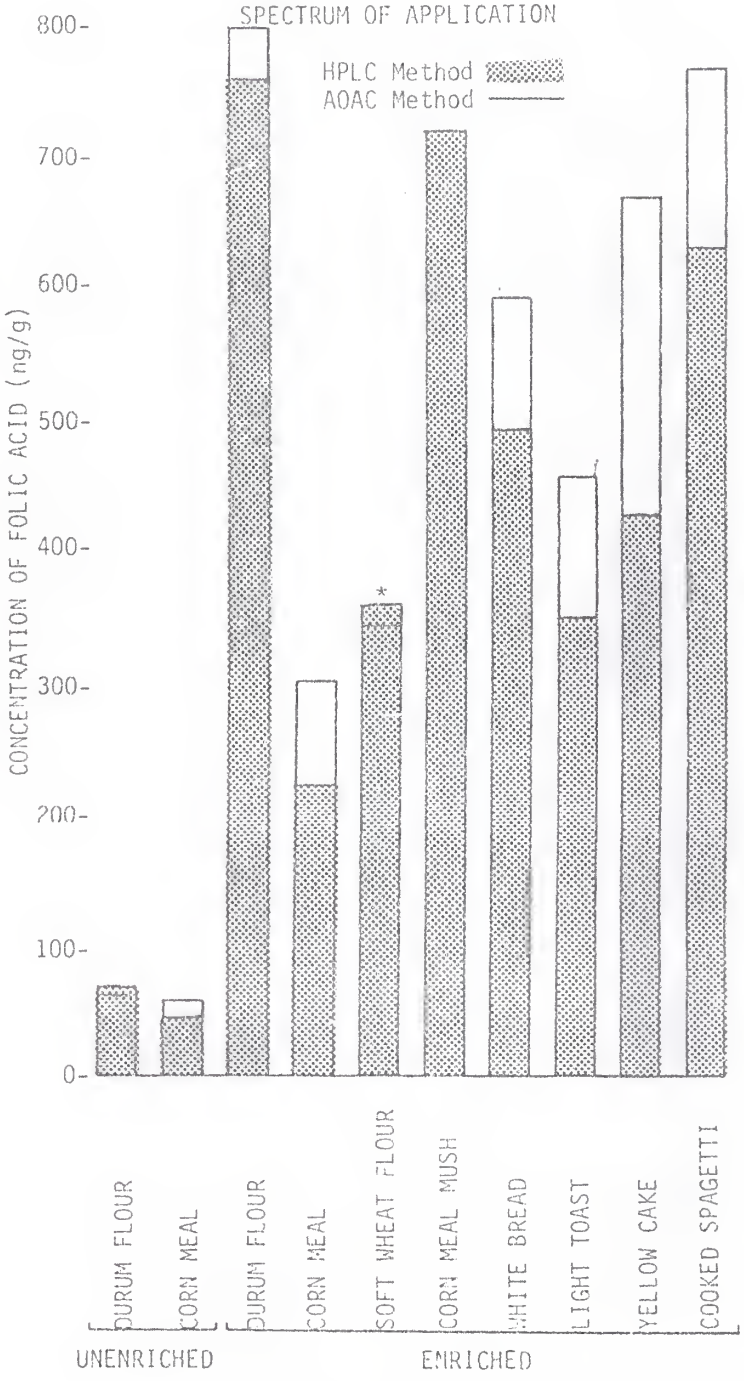
EFFICIENCY AND REPRODUCIBILITY OF EXTRACTION

Sample	Retention Time	Quantity (ng/g)
1	7.68	702
2	7.12	665
3	7.34	719
4	6.96	650
5	6.89	643
6	6.97	715
7	6.93	721
8	6.93	670
9	7.06	659
10	6.81	*
		$\bar{X}$= 683 $\pm$ 29ng/g

*Retention time was out of the calibration window.

Chromatographic conditions:
Injection ---------- 100ul
Detector attenuation 3
Mobile phase -------- 0.01 M KH_2PO_4, pH 6.0
Flow rate ---------- 1ml/min
Quantitation by external standard

FIGURE 10
SPECTRUM OF APPLICATION

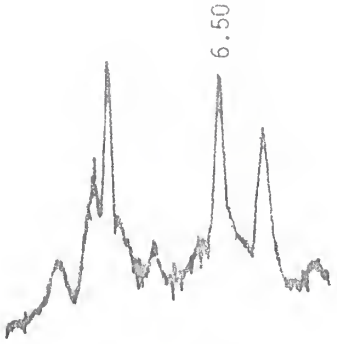


*Based on a single HPLC determination, all others were assayed in duplicate.
Actual values are given in Appendix I.

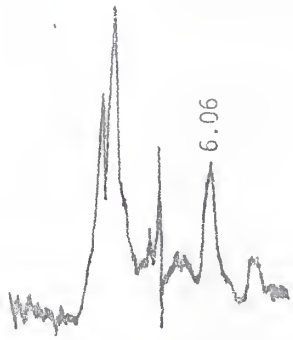
gelatinized starch matrices of these products. Recovery was assumed to be 100% with the AOAC analytical method because of the rigorous extraction procedures and its establishment as the standard method. It can also be seen that pterin-6-carboxylic acid (Figure 11) eluted as a discrete well resolved peak, thereby eliminating the problem of abberrant results arising from the coelution of interferring fluorescent compounds as reported by Allfrey (34).

FIGURE 11

SAMPLE CHROMATOGRAMS FROM THE SPECTRUM OF APPLICATION STUDY

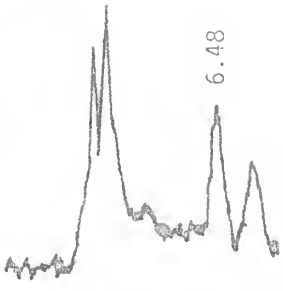


White Bread

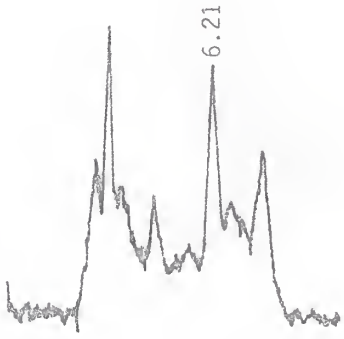


Light Toast

Chromatographic conditions:
- Injection ---------- 100ul
- Detector attenuation 3
- Mobile phase ------- 0.01 M KH_2PO_4, pH 6.0
- Flow rate ---------- 1ml/min
- Quantitation by external standard, corrected for 68.3% recovery



Cooked Spagetti



Yellow Cake

CHAPTER V

SUMMARY

With the advent of nutritional labeling and establishment of a minimum daily requirement for folic acid, a rapid method for its quantitation is essential, particularly in flour and bread as these are the recommended vehicles for fortification of the diet. Detection of folic acid by ultraviolet spectrophotometry has been shown to lack great enough sensitivity even at its wavelength of maximum absorption. With current "state-of-the-art" detection systems, then adequate sensitivity can be achieved through fluorescent determination.

Several degradative reactions were pursued in an attempt to establish the most rapid and complete method of cleaving the folic acid molecule into a free fluorescent pterin and p-aminobenzoylglutamic acid. It was found that derivatization to pterin-6-carboxylic acid at alkaline pH with $KMnO_4$ occurred almost instantaneously and yielded a fluorescent analyte for which detection limits were adequate for the analysis of folic acid in grain products.

It was demonstrated that folic acid could be extracted and oxidatively cleaved through the complex solution resulting from suspending flour samples in an alkaline mixture. However, operating at or near the detection limits dictated the use of subgelatinization temperatures for the extraction of folic acid. Results obtained by oxidizing extract solutions of various grain products, followed by high performance liquid chromatographic separation and fluorescent detection showed a high correlation to those determined by the AOAC standard method.

This chemical technique, then can potentially reduce the time required for a single assay from 24 hours for the microbiological method to as little

as 2 hours. Equally as important, this reduction in assay time is accomplished without a loss in throughput. The increasing need for a rapid method of folic acid analysis in industry then, will certainly dictate a shift to high performance liquid chromatographic determination. And, until technological advances of significant magnitude are made, fluorescence offers the only mode of detection with a sensitivity great enough to be of value to the assay of folic acid levels in cereal grains and their products.

BIBLIOGRAPHY

1. Wills, L. and H. S. Bilimoria. 1932. Studies in pernicious anemia of pregnancy, Part V: Production of macrocytic anemia in monkeys by deficient feeding. Indian Journal of Medical Research XX: 391.

2. Hogan, A. G. and M. Parrot. 1940. Anemia in chicks caused by a vitamin deficiency. J. Biol. Chem. 132: 507.

3. Binkley, S. B., O. D. Bird, E. S. Bloom, R. A. Brown, D. G. Calkins, C. J. Campbell, A. D. Emmet and J. J. Pfiffner. 1944. On the vitamin B_c conjugate in yeast. Science 100: 36.

4. Stokstad, E. L. R. and P. D. V. Manning. 1938. Evidence of a new growth factor required by chicks. J. Biol. Chem. 125: 687.

5. Snell, E. E. and W. H. Peterson. 1940. Growth factors for bacteria X: Additional factors required by certain lactic acid bacteria. J. Bact. 39: 273.

6. Hutchings, B. L., N. Bohonos, D. M. Hegsted, C. A. Elvehjem and W. H. Peterson. 1941. Relation of a growth factor required by Lactobacillus casei to the nutrition of the chick. J. Biol. Chem. 140: 681.

7. Stokstad, E. L. R. 1943. Some properties of a growth factor for Lactobacillus casei. J. Biol. Chem. 149: 573.

8. Mitchel, H. K., E. E. Snell and R. J. Williams. 1941. The concentration of folic acid. J. Amer. Chem. Soc. 63: 2284.

9. Keresztesy, J. C., E. L. Rickes and J. L. Stokes. 1943. A new growth factor for Streptococcus lactis. Science 97: 465.

10. Angier, R. B., J. H. Boothe, B. L. Hutchings, J. H. Mowat, J. Semb, E. L. R. Stokstad, Y. SubbaRow and C. W. Waller. 1946. The structure and synthesis of the liver L. casei factor. Science 103: 667.

11. Sauberlich, H. E. and C. A. Baumann. 1949. Further studies of the factor required by Leuconostoc citrovorum 8081. J. Biol. Chem. 181: 871.

12. Spies, T. D., C. F. Vilter, M. B. Koch and M. H. Caldwell. 1945. Observations of the anti-anemic properties of synthetic folic acid. Southern Medical Journal 38: 707.

13. Wagner, A. F. and K. Folkers. 1964. Vitamins and Coenzymes. Interscience Publishers, New York.

14. White, A., P. Handler, E. L. Smith, R. L. Hill and I. R. Lehman. 1978. Principles of biochemistry. McGraw-Hill Book Company, New York.

15. Toepfer, E. W., E. G. Zook and M. L. Orr. 1951. Folic acid content of foods. U.S.D.A. Handbook No. 29.

16. Recommended Dietary Allowances, 7th ed. 1968. National Academy of Science-National Research Council. Publication 1694, Washington, D.C.

17. Robinson, F. A. 1966. The vitamin co-factors of enzyme systems. Pergamon Press. Oxford, Eng.

18. Blakley, R. L. 1969. The biochemistry of folic acid and related pteridines. In Neuberger, A. and E. L. Tatum (ed.). Frontiers of Biology. North-Holland Publishing Company, Amsterdam, The Netherlands.

19. Stokstad, E. L. R., B. L. Hutchings, J. H. Mowat, J. H. Boothe, C. W. Waller, R. B. Angier, J. Semb and Y. SubbaRow. 1948. The degradation of the fermentation Lactobacillus casei factor I. J. Amer. Chem. Soc. 70: 5.

20. Hutchings, B. L., E. L. R. Stokstad, J. H. Mowat, J. H. Boothe, C. W. Waller, R. B. Angier, J. Semb and Y. SubbaRow. 1948. Degradation of the fermentation L. casei factor II. J. Amer. Chem. Soc. 70: 10.

21. Lowry, O. H., O. A. Bessey and E. J. Crawford. 1949. Photolytic and enzymatic transformations of pteroylglutamic acid. J. Biol. Chem. 180: 889.

22. Uyeda, K. and J. C. Rabinowitz. 1963. Fluorescence properties of tetrahydrofolate and related compounds. Anal. Bioch. 6: 100.

23. Duggan, D. E., R. L. Bowman, B. B. Brodie and S. Underfriend. 1957. A. spectrofluorometric study of compounds of biological interest. Arch. Biochem. Biophys. Acta 68: 1.

24. Wright, B. E., M. L. Anderson and E. C. Herman. 1958. The role of polyglutamylpteridine coenzymes in serine metabolism III: The enzymatic formation of dihydrofolic acid and dihydropterin. J. Biol. Chem. 230: 271.

25. Rouseff, R. 1979. The use of dual detectors for HPLC multivitamin analysis of citrus juices. In Liquid Chromatographic Analysis of Food and Beverages, Vol. I, Charalambous, G. (ed). Academic Press, New York.

26. Mitchel, H. K., E. E. Snell and R. J. Williams. 1941. The concentration of folic acid. J. Amer. Chem. Soc. 63: 2284.

27. Luckey, T. D., G. M. Briggs and C. A. Elvehjem. 1944. The use of Streptococcus lactis R for the measurement of folic acid. J. Biol. Chem. 152: 157.

28. Metz, J. 1963. Folates in megaloblastic anemia. Bull. World Health Org. 28: 517.

29. Tennant, G. B. 1977. Continuous-flow automation of the L. casei serum folate assay. J. Clin. Path. 3: 1168.

30. O'Dell, B. L. and G. Hogan. 1943. Additional observations on the chick antianemia vitamin. J. Biol. Chem. 149: 323.

31. Asenjo, C. F. 1948. Pteroylglutamic acid requirement of the rat and a characteristic lesion observed in the spleen of the deficient animal. J. Nutrition 36: 601.

32. Hutchings, B. L., E. L. R. Stokstad, J. H. Boothe, J. H. Mowat, C. W. Waller, R. B. Angier, J. Semb and Y. SubbaRow. 1947. A chemical method for the determination of pteroylglutamic acid and related compounds. J. Biol. Chem. 168: 705.

33. Bratton, C. A. and E. K. Marshall. 1939. A new coupling component for sulfanilamide determination. J. Biol. Chem. 128: 537.

34. Allfrey, V., L. J. Tepley, C. Geffen and C. G. King. 1949. Fluorometric method for the determination of pteroylglutamic acid. J. Biol. Chem. 178: 465.

35. Zakrzewski, S. F. and C. A. Nichol. 1953. The separation and determination of pterolyglutamic acid and related compounds. J. Biol. Chem. 205: 361.

36. Radhakrishnamurty, R. and P. S. Sarma. 1954. Paper chromatography of pteroylglutamic acid. Current Science (India) 23: 266.

37. Weiland, O. P., B. L. Hutchings and J. H. Williams. 1952. Studies on the natural occurrence of folic acid and citrovorum factor. Arch. Biochem. Biophys. 40: 205.

38. Usdin, E. 1959. Blood folic acid studies VI: Chromatographic resolution of folic acid active substances obtained from blood. J. Biol. Chem. 234: 2373.

39. Bertrino, J. R. 1965. Purification and properties of dihydrofolate reductase from Ehrlich Ascites Carcinoma cells. Biochem. 4: 839.

40. Branfman, A. R. and M. McComish. 1978. Rapid separation of folic acid derivatives by paired-ion HPLC. J. Chrom. 151: 87.

41. Wills, B. H., C. G. Shaw and W. R. Day. 1977. Analysis of water soluble vitamins by HPLC. J. Chrom. Sci. 15: 262.

42. Clifford, C. K. and A. J. Clifford. 1977. High pressure liquid chromatographic analysis of ffod for folates. J. A.O.A.C. 60: 1248.

43. Schuster, R. 1978. HPLC of pharmaceuticals. Hewlett-Packard Application Note AN 232-6.

44. Mader, W. J. and H. A. Frediani. 1948. Polarographic determination of folic acid. Anal. Chem. 20: 1199.

45. Huennekens, F. M. and M. J. Osborn. 1959. Folic acid coenzymes and one-carbon metabolism. Adv. Enz. 21: 369.

46. Rothenberg, S. P. 1965. A radio-enzymatic assay for folic acid. Nature 206: 1154.

47. Johns, D. G. and J. R. Bertrino. 1965. Folates and megaloblastic anemia: A review. Clin. Pharm. Therap. 3: 372.

48. Hutchings, B. L., E. L. R. Stokstad, N. Bohonos, N. H. Sloane and Y. SubbaRow. 1948. The isolation of the fermentation Lactobacillus casei factor. J. Amer. Chem. Soc. 70: 1.

49. Mowat, J. H., J. H. Booth, B. L. Hutchings, E. L. R. Stokstad, C. W. Waller, R. B. Angier, J. Semb, D. B. Cosulich and Y. SubbaRow. 1948. The structure of the liver L. casei factor. J. Amer. Chem. Soc. 70: 14.

APPENDIX I

COMPARISON OF HPLC AND CLASSICAL METHODS

SAMPLE	HPLC METHOD (NG/G FLOUR ± S.D.)	AOAC METHOD (NG/G FLOUR ± S.D.)
Durum flour unenriched	66 ± 17	60 ± 11
Corn meal unenriched	45 ± 17	56 ± 14
Enriched soft wheat flour	361*	343 ± 8
Enriched corn meal	224 ± 12	301 ± 11
Enriched flour light toast	350 ± 20	357 ± 17
Enriched flour bread	492 ± 4	590 ± 13
Enriched yellow cake	428 ± 22	668 ± 13
Corn meal mush	718 ± 13	719 ± 29
Durum flour stored 6 mo. $0^{o}C$	757*	796 ± 29
Cooked spaghetti	630 ± 17	761 ± 14

*Retention time fell outside the 5% window and was not calculated for the duplicate sample.

THE USE OF PTERIN-6-CARBOXYLIC ACID FOR THE INDIRECT DETERMINATION OF FOLIC ACID IN ENRICHED CEREAL GRAIN PRODUCTS BY HPLC WITH FLUORESCENT DETECTION

by

MICHAEL SHAFFER GILMORE

A.B. KALAMAZOO COLLEGE, 1977

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1979

ABSTRACT

With the advent of nutritional labeling and establishment of a minimum daily requirement for folic acid, a rapid method for its quantitation is essential, particularly in flour and bread as these are the recommended vehicles for fortification of the diet. Detection of folic acid by ultraviolet spectroscopy has been shown to lack great enough sensitivity, even at its wavelength of maximum absorption. With current "state-of-the-art" detection systems, then adequate sensitivity can be achieved only through fluorescent determination.

The oxidative cleavage of folic acid at alkaline pH was found to be the most rapid and complete method of derivatization to a fluorescent compound. This compouds, determined to be pterin-6-carboxylic acid by UV and fluorescent spectra, was found to have detection limits of 600pg with an injection volume of 100ul when separated from cereal grain matrices by reverse phase HPLC. This derivatization was shown to be reproducible with a relative standard deviation of $\pm$4.2%.

A broad spectrum of cereal grain products, both enriched and unenriched were assayed by this technique and the values obtained compared to those given by the AOAC standard microbiological method. A high correlation was demonstrated over the sample folic acid content range of 60-800ng/g.