DETERMINATION OF VITAMIN B-6, AVAILABLE LYSINE AND PYRIDOXYLLYSINE IN A NEW INSTANT BABY FOOD PRODUCT

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(ABSTRACT)

The purpose of this study was to compare the nutrient content of a new instant baby food product to jar baby food of similar product formulation. Instant and jar "Vegetable and Beef" and "Bananas" products processed in 1985 and 1987 were analyzed for available lysine, vitamin B-6 and pyridoxyllysine content.

The available lysine content of 100 grams of baby food was found to be higher in the instant products, but when adjusted for protein content, available lysine was higher in the jar products. This indicates that drum-drying used for the instant products is more detrimental in regard to lysine availability than retorting. The vitamin B-6 content of the instant products was found to be higher than that of the jar products. However, due to the addition of ingredients with little or no vitamin B-6 content to the jar products, no conclusion about processing effects on vitamin B-6 content

can be made. Products processed in 1985 tended to be lower in nutrient content than the products processed in 1987. Pyridoxyllysine, a compound thought to affect vitamin B-6 bioavailability, could not be detected in any of the baby foods, either by amino acid or HPLC analysis.

The instant products were found to be at least equal to the jar products with regard to available lysine and vitamin B-6 content. All products also appear to provide sufficient amounts of these nutrients to infants less than one year of age.

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List of Abbreviations

Ba = "Bananas" Formulation

°C = Degrees Centigrades

g = Gram

HCl = Hydrochloric acid

HPLC = High Performance (Pressure) Liquid Chromatography

I = Instant Formulation

J = Jar Formulation

kg = Kilogram

l = Liter

lys = Lysine

M = Molar

mg = Milligram

mg lys/g N = Milligram Lysine per Gram Nitrogen

mg lys/100 g food = Milligram Lysine per 100 Gram Food

min = Minutes

ml = Milliliter

N = Nitrogen

ng = Nanogram

nm = Nanometer

PIC = Paired-Ion Chromatography

PL = Pyridoxal

PLP = Pyridoxal-5-phosphate

PM	=	Pyridoxamine
PMP	=	Pyridoxamine-5-phosphate
PN	=	Pyridoxol (Pyridoxine)
PNP	=	Pyridoxol-5-phosphate
PPL	=	Phosphopyridoxyllysine
PSI	=	Pounds per Square Inch
RDA	=	Recommended Dietary Allowances
TCA	=	Trichloroacetic acid
TNBS	=	2,4,6-Trinitrobenzenesulphonic acid
TNP-L	=	2,4,6-Trinitrophenyl-lysine
ug	=	Microgram
um	=	Micrometer
v	=	Volume
VB	=	"Vegetable and Beef" Formulation
w	=	Weight

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CHAPTER 1

1. Introduction

The increased interest of the public in nutritional issues, and the great demand of dietitians and other professionals for food composition data, seem to require more detailed information about food composition. However, the quantification of the nutrients in all foodstuffs is a never-ending task similar to Sisyphus's[1]. Budgetary and personnel constraints limit the number of foods that can be analyzed, thus data for many foods will remain unavailable (Beecher and Vanderslice 1984). However, Stewart (1981) identified three areas of food analysis which are of particular interest. These areas represent nutrients associated with public health problems, nutrients for which data are inadequate, and nutrients for which analytical methods are good. The assessment of the nutrients that belong to all three categories is clearly the most appropriate way to approach the problem of food analysis (Stewart 1981).

This study is concerned with the analysis of various nutrients in baby food, and complies with the demands stated above. Baby foods were analyzed for their vitamin B-6, available lysine and pyridoxyllysine content. These nutrients

[1]**Sisyphus** n. Gk. Myth. A cruel king of Corinth condemned forever to roll a huge stone up a hill in Hades only to have it roll down again on nearing the top (The American Heritage Dictionary)

were chosen for analysis because of the high lysine requirements of infants (Snyderman et al. 1959), the vitamin B-6 deficiency that occurred in infant formulas in the 1950's (Snyderman et al.1953), and the decreased availability of the nutrients in the form of pyridoxyllysine (Gregory and Kirk 1978c). Both, jar baby foods preserved by canning, and new instant drum-dried baby foods were analyzed for their nutrient content. Jar and instant "Vegetable and Beef" and "Bananas" products processed in 1985 and 1987 were analyzed for their vitamin B-6, total and available lysine and pyridoxyllysine content. "Vegetable and Beef" and "Bananas" products were chosen due to expected differences in lysine and vitamin B-6 content.

CHAPTER 2

2. Review of Literature

2.1. Baby Foods

As more women enter the work force, there is an increased need for baby foods that are convenient in preparation and also meet the nutritional requirements of infants. Presently, most baby foods are canned in glass jars, and are ready-to-serve products. However, in recent years, a new instant product has found its niche in the market place. This product combines the convenience of preparation of jar products with requirements for travelling purposes. It is easily reconstituted, is lighter and requires less space than the jar products, and need not be refrigerated after opening. Whereas many baby foods have been analyzed for their nutrient content (Orr 1969), the newly developed instant product has not been subjected to nutrient analysis by the scientific community.

2.2. Preservation of Foods

For centuries, man has used various processing methods to preserve food. Heat processing is one of the most

important methods for extending the storage life of foodstuffs. Drying and canning, the two methods of interest to this study, belong to this category of food preservation.

Drying refers to the removal of water to preserve the food from spoilage by microorganisms (Potter 1986). Sun-drying is known to be the oldest method to dry and preserve food. It is still widely used throughout the world, but in some countries has been largely displaced by more sophisticated preservation methods like freeze- and spray-drying. (Bluestein and Labuza 1988).

Other methods for preserving food are based on Pasteur's discovery that life does not develop by itself in a matrix that has been heat-treated. During the first part of the 20th century, canning became one of the most important food preservation methods, and is still widely used. Many canned products can be consumed even after years of storage (Jackson and Shinn 1979).

However, the preservation of food by heat processing has a detrimental effect on nutrients since thermal degradation can and does occur (Lund 1988). Although not all nutrients are detrimentally affected by heating, for instance the denaturation of some proteins increases their digestibility, many of the essential nutrients such as vitamins are heat labile (Ball et al. 1963). The nutrients of interest in the

present study, vitamin B-6 and lysine, have been shown upon heating to break down or to form products that are not bioavailable (Erbersdobler 1986).

2.2.1. Canning of Foods

Canning of food means packing heat-sterilized food in hermetically sealed containers, usually made of glass or rigid metal (Jackson and Shinn 1979). Canned food products are considered "commercially sterile" because the processing conditions used do not sterilize the product in the true meaning, but usually leave spores of certain microorganisms unharmed (Lund 1988). The most important food spoilage microorganism is Clostridium botulinum, a heat resistant anaerobe that produces a potent exotoxin. Therefore the destruction of this microorganism and its spores has to be established in canned foods particularly in products with a pH above 4.6. Usually, the heat treatment for canned food is more severe than that required to destroy this microorganism.

One of two heating processes is used in canning food; the heating of the food in a heat exchanger before it is filled into the containers or the heating of the food after being placed into containers. When a heat exchanger is used,

live steam heats the food in a few seconds to a temperature in the range of 121-150°C, and the food is held at that temperature for a few seconds. After rapid cooling, the food is filled into the containers. Sterilizing the food in the container is a less efficient method, but has to be used for most foods due to their physical consistency. This process also uses steam as the heat source, and the rate of heating depends on the geometry and heat transfer characteristics of the container and the physical properties of the food. Most baby foods are commercially canned using this type of heat process (Jackson and Shinn 1979).

Some baby foods such as "Vegetable and Beef" are low-acid foods and require relatively high temperatures and long retort processing times, about 40-50 minutes at 121°C. Others, like "Bananas" belong to a group of baby foods that are acidified (see Appendix I) to a pH below 4.6, and can be given a short heat treatment of about 30 seconds at 121°C, followed by a hot fill and a holding time for sterilizing the container (Lopez 1981). However, the products under study are both retorted; the "Vegetable and Beef" product received a low-acid treatment, whereas the "Bananas" product received a high acid treatment (Marcy 1989).

Canning obviously can also destroy nutrients. Like the destruction of microorganisms, the extent of nutrient losses

in canned foods is dependent on time, temperature and rate of heat transfer. However, due to differences in the temperature coefficient of bacterial destruction and destruction of nutrients, a canning process that uses high temperature short time (HTST) treatment will result in greater nutrient retention than a low temperature long time treatment (Lund 1988, Ball et al. 1963). Up to 80% of thiamin, the most heat labile vitamin, can be destroyed during canning (Lund 1988). Similar losses have been reported for vitamin B-6 in certain foodstuffs (Orr 1969, Gregory and Hiner 1983). However, much of the data on the vitamin B-6 content of foods are considered unreliable (Jackson and Shinn 1979).

Another important factor in the canning process is the headspace remaining in the can or jar. The headspace gas is usually air and water vapor and for a few special foods it is replaced by an inert gas like nitrogen. The headspace volume has to comply with USDA regulations which require that no more than 10% of the total can volume can be left empty. The headspace volume can influence the sterilization process. Too small a headspace decreases the adequacy of the thermal process. Headspace pressure, commonly referred to as the "vacuum", helps to determine the quantity of oxygen in the container, prevents permanent distortion of the can ends, and gives a visual index to the condition of the contents by

maintaining a concave position of the closures (Jackson and Shinn 1979).

The nutrient content of canned foods is also dependent on the temperature and time of storage following processing. Under usual storage conditions of 26°C or lower, canned foods exhibit good nutrient stability (Jackson and Shinn 1979, Ball et al. 1963).

2.2.2. Drum-Drying of Foods

Virtually all drying methods are based on the principle of supplying heat to the food and removing water in the vapor state. The techniques of how the heat is supplied and the moisture removed distinguish the different drying methods.

Considering infinitely small time spans and infinitely small units of energy, supplying heat to food means either raising the temperature of the food or converting food moisture from the liquid state into the vapor state. In the beginning of the drying process, the so-called constant rate period, the bulk of the water is removed at a uniform rate and the temperature is constant at around 100°C. This period ends at a moisture level which is still above the moisture level where chemical reactions are at their maximum. Once this

period is over, the drying rate decreases and the temperature of the food increases. Drying methods with efficient nutrient retention should be fast in both stages because in the first stage undesirable conformational changes like shrinkage of the food occur, and in the second stage nutrient losses increase with increasing food temperature (Bluestein and Labuza 1988).

Many different methods are used commercially to dry foods. Selection of a drying method depends on product suitability and cost. Drum- or roller-drying is one of the methods used to dehydrate foods. It has been in use longer than freeze- or spray-drying, and usually results in greater nutrient losses (Erbersdobler 1986). Recently, efforts have been made to improve nutrient retention of foods during drum-drying (Erbersdobler 1986).

Drum-drying is commonly used to dry food slurries, which can be spread in a thin layer onto the surface of the rotating drum. Drum-dryers consist of a single or a pair of drums. Several different methods can be used to apply the food slurry onto the drums. The thickness of the food layer can be determined by the clearance between the drums, when a double drum-dryer is used (Potter 1986). The drums are heated with steam from the inside and have a temperature in the range of 120-170°C (Bluestein and Labuza 1988), with 150°C being the most often used temperature (Potter 1986). The drying time

is 20 seconds to three minutes, depending on the food material. Drying time is regulated by the speed of the rotating drums. The food is normally dry by the time it reaches the scraper blades after a single rotation (Potter 1986, Bluestein and Labuza 1988). Drum-drying is one of the cheapest drying methods, but since the product is in direct contact with the hot drums, it reaches a higher final temperature than when dried with other methods. This results in greater nutrient destruction (Bluestein and Labuza 1988), e.g. it has been shown that while spray-drying decreases lysine availability in dried milk by only 4%, drum-drying decreases it up to 16% (McDonald 1966).

2.3. Vitamin B-6

Vitamin B-6 was discovered and studied extensively in the 1930's. György (1934) described it as a factor which prevents rat dermatitis, and gave it its name, vitamin B-6. Since then it has been established that vitamin B-6 is not a single compound, but consists of six related compounds. The six forms are: pyridoxol (PN, also called pyridoxine), pyridoxal (PL), pyridoxamine (PM), pyridoxol-5-phosphate (PNP), pyridoxal-5-phosphate (PLP) and pyridoxamine-5-

phosphate (PMP) (Driskell 1984), of which PN, PLP and PMP are the forms mainly found in foods (Sauberlich 1985).

For about 20 years, little was known about the human requirements for vitamin B-6. This changed drastically after the events in 1951-53, when serious vitamin B-6 deficiencies occurred in 2-4 month old infants fed a liquid milk formula that contained less than 60 ug/l vitamin B-6. The low amount of vitamin B-6 in the milk formula was due to a newly introduced sterilization method. The infants suffered from severe convulsive seizures and some fatalities were reported (Coursin and Lancaster 1954, Malony and Parmelee 1954, Snyderman et al. 1953, Driskell 1984). This incident pointed out the need for more research to determine human vitamin B-6 requirements and vitamin B-6 content of foods.

2.3.1. Vitamin B-6 in Foods

Vitamin B-6 is found in foods mainly in the form of PN in plant foods, and PLP and PMP in animal products (Sauberlich 1985). Many foods have been analyzed for their vitamin B-6 content. An extensive review of the vitamin B-6 content of foods has been published by Orr (1969). On the average, animal foods are higher in vitamin B-6 content than plant

products. Whereas the vitamin B-6 content in baby foods made from meat products ranges from 0.2 to 0.8 mg per 100 g, vegetable products contain between 0.03 and 0.12 mg vitamin B-6 per 100 g, and fruit products range from 0.03 to 0.16 mg vitamin B-6 per 100g (Orr 1969).

In 1954, Hassinen et al. compared several commercially available infant formulas and processed milks in regard to their vitamin B-6 content. They found that all processed milk products and infant formulas were lower in vitamin B-6 content than fresh milk, with the exception of spray-dried products. The study also indicated that significant losses do not occur when the products are pasteurized or homogenized, but only when exposed to more radical heat treatments, like sterilization. In 1973, Miller et al. reported that the loss of vitamin B-6 due to retorting is only 2% in pinto beans, whereas the loss of vitamin B-6 in the cooking water was 35%. Drum-drying reduced the total vitamin B-6 content by 19% (Miller et al. 1973). These figures are lower than those reported by Everson et al. (1964), who reported a 10% loss of vitamin B-6 in beans due to canning and additional 20% loss due to storage. Harding et al. (1959) found much larger differences in vitamin B-6 content between fresh and stored army rations. On average, the stored rations were 45% lower in B-6 content than the fresh rations. Schroeder (1971)

reported the largest losses of vitamin B-6 on canning. In his report, the percentage of vitamin B-6 loss due to canning ranged from as low as 15% for dairy products to as high as 77% in legumes. More recently, Gregory and Hiner (1983) examined different factors affecting vitamin B-6 degradation in foods, and found that while major variables in vitamin B-6 degradation are processing time and temperature, food composition is a variable that must also be considered.

Another important factor that has to be considered when discussing the adequacy of vitamins in foods is bioavailability. A comparison of different food processing methods, namely freezing, canning and irradiation, indicated that thermal processing is the most detrimental in regard to vitamin B-6 bioavailability in foods. In addition, the loss of vitamin B-6 activity due to storage was also highest in thermally processed foods (Richardson et al. 1961). Even though this study assessed the vitamin B-6 bioavailable to rats, it gave no indication about the percentage of the total amount of vitamin B-6 that was bioavailable. To establish the percent bioavailability of vitamin B-6 in foods, two assays are necessary: a bioassay that determines the vitamin B-6 content that is available, and an assay or analysis that determines the total vitamin B-6 content.

Gregory and Kirk (1978a) showed that while severe thermal

processing in the form of roasting decreases the vitamin B-6 content of a dehydrated food model by 53-73%, the remaining B-6 vitamers were fully available. Similar results were observed in regard to storage time (Gregory and Kirk 1978b). However, when analyzing food systems instead of food models, Gregory (1980a) found that only 18-44% of the total vitamin B-6 content in a fortified rice breakfast cereal was bioavailable.

Whereas total vitamin B-6 is usually assessed by microbiological assays, and nowadays often by HPLC analysis, vitamin B-6 bioavailability is determined using rat bioassay, urinary excretion or plasma PLP concentrations. Conflicting data have been reported as to how these assays relate to one another. Tomarelli et al. (1955) found that the bioavailability of vitamin B-6 in heat treated milk is lower in rat bioassay than when determined by microbiological assays, whereas Davies et al. (1959), and Toepfer et al.(1963) reported that rat bioassay data were significantly higher than microbiological findings. In 1980, Gregory showed that microbiological assays, HPLC analysis and rat bioassay for vitamin B-6 are in close agreement for nonfat dry milk samples, but considerably different for cereal samples (Gregory 1980a). Other researchers found greater amounts of vitamin B-6 in foods when analyzed by HPLC than with micro-

biological assays (Lim 1981, Vanderslice et al. 1980). More recently, Nguyen and Gregory (1983) conducted a study that assessed the bioavailability of the B-6 vitamers in different foods at different stages of processing. They found that the food type and composition plays an important role in regard to vitamin B-6 bioavailability. For instance, vitamin B-6 is more available in raw beef than in cooked beef, whereas in spinach, it is less available in raw than in cooked spinach. They found highly significant interactions between vitamin B-6 bioavailability and diet composition and concluded that the relationship of food composition and vitamin B-6 bioavailability is rather complex. On average, vitamin B-6 bioavailability in a regular American diet is reported to be between 61% and 81% (Tarr et al. 1981).

The determination of vitamin B-6 in foods is difficult in part because vitamin B-6 exists in six biologically active forms in foods (Gregory et al. 1986). Of the many methods used to quantify vitamin B-6 in foods, High Performance (Pressure) Liquid Chromatography (HPLC) seems to be the most promising because of its capability to not only separate the six different B-6 vitamers, but also because it is possible to detect B-6 derivatives and degradation products that render vitamin B-6 unavailable to the human body. The possibility of quantifying different B-6 vitamers and B-6 analogs using

HPLC is a great advantage over microbiological assays which are much more restricted in this respect. However, almost all reported vitamin B-6 values in foods are based on microbiological assays. Rat bioassays which are very useful for determining the bioavailability of vitamin B-6 in foods are rather expensive, and time consuming, and have other restrictions. If the bioavailability of the different B-6 derivatives and analogs is known, the quantification with HPLC can give a good estimate of the total B-6 availability.

When analyzing food for available vitamin B-6, the extraction method of the vitamers and their analogs is of particular interest. Many derivatives and analogs of vitamin B-6 are known to occur in foods, of which pyridoxic acid and more recently pyridoxine-ß-glycoside are major forms of interest (Kabir et al. 1983, Ink et al. 1986, Gregory and Ink 1987, Trumbo et al. 1988), whereas pyridoxyllysine is one of the vitamin B-6 derivatives which has received less attention than others. HPLC is an excellent method for separating these compounds. However, good sample clean up is important for HPLC analysis, which at this time is still a challenging task when complex food systems are used.

2.3.2. Nutritional Significance of Vitamin B-6

Vitamin B-6 is an essential nutrient to the human body, which serves as a co-factor in various enzymatic reactions. PLP is the form primarily involved in the metabolism as a co-enzyme. The most important enzymatic reactions in which PLP participates are transaminations and deaminations in amino acid anabolism and catabolism, decarboxylation in the synthesis of gamma-amino-butyric acid, serotonin, epinephrine and norepinephrine, in the synthesis of porphyrins, and the conversion of tryptophan to niacin (Driskell 1984). The major B-6 vitamers found in foods are PN, PLP and PMP, of which PLP and PMP are dephosphorylated by phosphatases in the intestine prior to absorption (Gregory 1980c). PN and PM are readily transformed into the aldehyde form PL by pyridoxine-dehydrogenas (EC 1.1.1.65) and pyridoxamine-phosphate-oxidase (EC 1.4.3.5), respectively. The unphosphorylated forms are converted into the phosphorylated forms via the ATP-dependent pyridoxalkinase (EC 2.7.1.35) (Driskell 1984).

The Recommended Dietary Allowance for vitamin B-6 for a 0-0.5 year old infant has been set at 0.3 mg per day (Food and Nutrition Board 1980).

2.4. Lysine

Lysine is one of nine essential amino acids for the human adult. Its side chain consists of a hydrocarbon chain with a terminal epsilon amino group. This epsilon amino group can react chemically with various food constituents during food processing and storage, rendering lysine complexes biologically unavailable. The reaction sequence of Maillard Browning is probably the best studied naturally occurring reaction of lysine in foods (Bender 1972).

2.4.1. Lysine in Foods

Since lysine is the limiting amino acid in many plant proteins, its availability is important to protein quality. It is well known that heating decreases the bioavailability of lysine in foods. Lysine is considered to be the amino acid most susceptible to heat degradation (Hurrel and Finot 1985). The loss of lysine due to thermal destruction and decrease in lysine bioavailability due to chemical reactions gradually increases with increasing temperature (Palamidis and Markakis 1980, Björck et al. 1983, Dexter et al. 1984, McAuley et al. 1987), and with the amount of reducing sugars present in the

food (Adewusi and Oke 1984). Pedrosa et al. (1981) found that heat treatment does not affect the nutritive value of baby food in regard to lysine, as long as the carbohydrate:protein ratio is not high.

Storage has also been found to affect the available lysine content of foods. Horvatic and Grüner (1984) found a decrease in lysine content in seven different infant formulas which averaged 4.2% loss after 6 months and 6.9% after 12 months. Larsen et al. (1986) found a decrease in available lysine from 96% to 88% within two weeks of storage for nitrite or sorbate treated frankfurters.

In general, N-substituted lysine can be classified into three groups, according to their biological availability: biologically unavailable, partly available and fully available derivatives (Finot et al. 1980/81). Studies with lysine derivatives like methylated or acetylated lysine have shown that the bioavailability of lysine decreases with increasing methylation and that acetylation decreases the availability even further (Boggs 1978). The relative growth response of mice fed lysine derivatives was between 6.6 % and 12.3% compared to mice fed L-lysine (Friedman and Gumbmann 1981).

The amount of lysine available in foods is mainly dependent on the extent of the Maillard Browning reaction (Almas and Bender 1980). This reaction also makes the <u>in</u>

vitro determination of available lysine difficult, due to early reaction products such as epsilon-deoxy-fructosyl-lysine which are biologically unavailable but react like biologically available lysine in *in vitro* methods (Plakas et al. 1988). These early reaction products can decrease the lysine availability in foods by up to 76% (Steinig and Montag 1982). Most researchers determined the available lysine content of food models or foods containing protein as the main macronutrient. Reported values range from 385 mg lys/g N for egg albumin to 510 mg lys/g N for casein (James and Ryley 1986). For meat and meat products, values between 326 mg lys/g N for garlic sauce, and 613 mg lys/g N for beef in natural juice have been reported (Dvorak and Vognarova 1965). Total lysine has been determined for many foodstuffs, and extensive compilations of data are available (Souci et al. 1986, USDA 1976-1984).

2.4.2. Nutritional Significance of Lysine

Children and infants require higher amounts of lysine than adults due to their fast growth (Finot and Hurrel 1985). The Food and Nutrition Board (1980) recommends 99 mg/kg body weight lysine in the diet for a 4-6 month old infant, and

44 mg/kg body weight for a child of 10-12 years of age. Snyderman et al. (1959) established that the lysine requirement for infants is 105 mg/kg body weight, which is higher than that for children (1.6 g/day or 60 mg/kg body weight) (Nakagawa et al. 1961) or for adult males (0.8 g/day) (Rose et al. 1955) and adult females (0.63 g/day) (Jones et al. 1955).

Dietary deficiency of lysine has been shown to cause decreased feed intake and induces decreased weight gain in weaning rats (Bolze et al. 1985). Weight gains of mice are proportionally (Finot et al. 1978) or semilogarithmically (Friedman and Gumbmann 1981) related to the amount of lysine in the diet. Lysine-deficient protein intake also alters biochemical parameters. For instance, the synthesis and turnover rate of cholesterol is reduced in rats fed a lysine-deficient diet (Gosh and Misra 1987). Also, a diet that is not absolutely but relatively deficient in lysine when compared to other amino acids does not support weight gain of weanling rats as a balanced diet does, if lysine is below 0.72% of the diet. This observation is known as amino acid imbalance and can be found as well with other essential amino acids (Cieslak and Benevenga 1984). While some experiments seem to indicate that amino acid imbalance depresses voluntary food intake (Leung et al. 1968), a more recent study found

that rats fed suboptimal levels of lysine will consume more energy per unit of body weight than rats fed diets with adequate lysine levels (Cieslak and Benevenga 1984) which is similar to the findings reported for rats fed a lysine-deficient diet (Brooks et al. 1972). This increased energy consumption appears to increase body fat deposition in the rat carcass (Cieslak and Benevenga 1984).

2.5. Pyridoxyllysine

Pyridoxyllysine (Figure 1) was discovered along with other pyridoxylamino acids in the late 1940's (Heyl et al. 1948). It has a molecular weight of 297.2, and is of original and current interest to many researchers because of its significance in the binding of pyridoxal and pyridoxalphosphate to the epsilon amino group of lysine in apoenzymes, and its characteristic absorbance spectrum (Fisher et al. 1958, Dempsey and Snell 1963, Taylor and Jenkins 1966, Anderson et al. 1966, Kazarinoff and McCormick 1975, Dominici et al. 1986 and Choi et al. 1987). Pyridoxyllysine was not thought to play a significant role in foods or in the bioavailability of vitamin B-6 until Gregory encountered it while studying the interactions of vitamin B-6 with proteins in model food

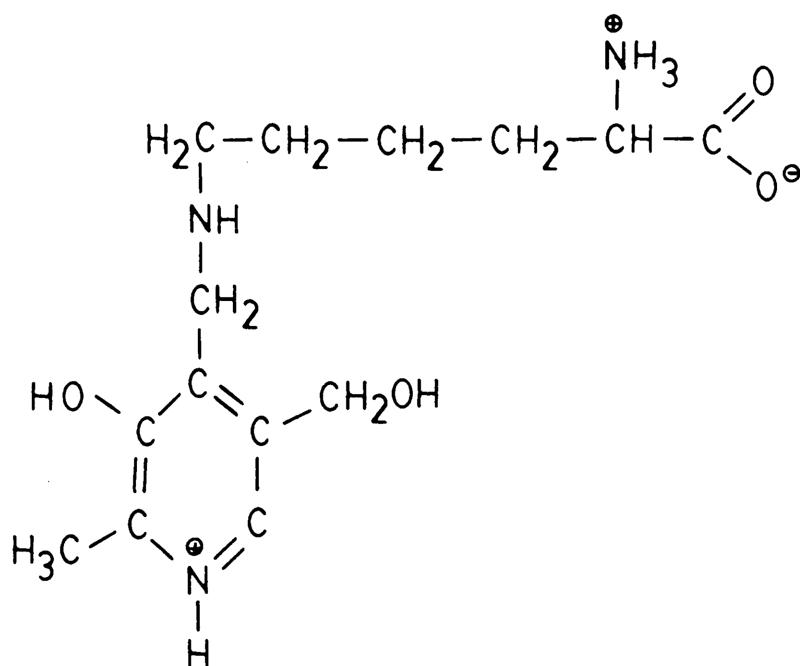

Figure 1: Epsilon-N-Pyridoxyllysine

(adapted from Reynolds 1988 and Gregory 1989)

systems (Gregory 1978).

The reaction of vitamin B-6 with alpha-amino groups of amino acids in the form of Schiff base linkages as a coenzyme in transaminases, deaminases or decarboxylases has been studied extensively (Snell et al. 1963, Evangelopoulos 1983). Much less attention has been given to the reaction of PL with epsilon-amino groups of lysyl residues or with free sulfhydryl groups of cysteinyl residues of proteins. These interactions have been suggested to be of major importance to the vitamin B-6 bioavailability. The compound pyridoxyl disulfide which is formed by the reaction of PL with cysteinyl residues has been shown to have no vitamin B-6 activity in rats. However, only 3% of PL was found to be involved in this reaction (Srncova and Davidek 1972, Gregory and Kirk 1977).

Therefore, Kirk and co-workers suggested that there must be another compound formed during heat-processing that accounts for major losses in vitamin B-6 bioavailability. In 1977, Gregory and Kirk proposed the formation of pyridoxyl-amino compounds in foods, where the Schiff-base linkage is reduced to a secondary pyridoxylamine which is likely when reducing agents such as ascorbic acid are present. Since foods normally contain but small amounts of free amino acids, most of the available amino groups are epsilon-amino groups and the few N-terminal groups of proteins. Gregory and Kirk

(1977) found that the pyridoxylamino complexes account for 60% of all protein-bound PLP in a liquid model food system which was 10% of the total added PLP. In this study, the model food system contained 50 ug/ml PL and 67 ug/ml PLP (Gregory and Kirk 1977). They also demonstrated that a greater loss in free amino groups of a dry food model occurred when any of the B-6 vitamers were added (Gregory and Kirk 1978b). In one study, freeze-dried model food systems were stored in two different types of cans and the formation of browning pigments and the degradation of vitamin B-6 was found to be less in cans with a larger headspace. This suggests a possible link between vitamin B-6 degradation and nonenzymatic browning. After processing, 21% of all PLP was found to be bound in the form of a Schiff-base link, as substituted aldamines or pyridoxyl-amino complexes (Gregory and Kirk 1978c); while after 128 days of storage 88% of PLP was bound, and pyridoxyl-lysine formation accounted for 50% of the vitamer degradation (Gregory and Kirk 1978b). However, in these studies, pyridoxyllysine formation was only observed in the PL-fortified food models presumably because the aldehyde-group of PL or PLP is necessary for the reaction to take place. The assessment of the biological availability of the remaining B-6 vitamers indicated that they retained full activity (Gregory and Kirk 1978b). In this study, the model food

system was fortified with 25% the USRDA of vitamin B-6 (0.5 mg) per ounce. A more recent study confirmed that pyridoxyllysine is a major mode of binding PL and PLP to food proteins during processing. This study was conducted to investigate the irreversible binding of PL and PLP to proteins at concentrations comparable to endogenous levels in foods (8.5 uM PLP and 21.2 uM PL+PN). The processing conditions were kept similar to those of a regular retort process, and the model food system was a high protein system (casein, evaporated milk, chicken liver, and chicken muscle) (Gregory et al. 1986).

Even though pyridoxylamino acids and pyridoxylamines in general have been shown to exhibit a very low microbiological vitamin B-6 activity, in rat bioassay their activities ranged from 50-100% compared to free PN (Snell and Rabinowitz 1948, Rabinowitz and Snell 1953, Heyl et al. 1952). Since these degradation products of vitamin B-6 are structural analogs of the vitamin, this finding is not surprising (Gregory 1980c). On the other hand, pyridoxylamino acids have been found to inhibit the activity of aminotransferases (Severin et al. 1969).

The vitamin B-6 activity, or antivitamin property of epsilon-pyridoxyllysine in particular, however, have not been investigated (Gregory and Kirk 1978c). Gregory and Kirk (1978c) found that pyridoxyllysine exhibits 50-60% of the

vitamin B-6 activity of free PN and can satisfy the vitamin B-6 requirements with respect to growth, feed efficiency and liver PLP concentration when fed in sufficiently high levels (Gregory 1979, 1980b). Since pyridoxyllysine is stable to acid hydrolysis (6 N HCl, 24 hrs, 110°C), these results suggested a release of vitamin B-6 by an enzymatic process (Gregory and Kirk 1978c). However, in this first study (Gregory and Kirk 1978c) pyridoxyllysine also inhibited the utilization of over half of the PN added. Later it was shown that in addition, when fed at low levels without other vitamin B-6 supplementation, pyridoxyllysine induced vitamin B-6 deficiency symptoms at an even faster rate than when a totally vitamin B-6-deficient diet is fed. However, this deficiency could be readily compensated by low amounts of PN (0.5 ug-1.0 ug/g diet) added to the diet. At high levels of pyridoxyllysine, no deficiency symptoms occurred, which indicates an inverse relationship between the pyridoxyllysine content of foods and any antivitamin activity (Gregory 1979, 1980c). It could be demonstrated that pyridoxyllysine depresses the erythrocyte aspartate amino-transferase (AspAT) (EC 2.6.1.1) activity, through inhibiting either the cellular formation of the apoenzyme, or the coenzymatic function of PLP (Gregory 1979, 1980b, c). The antivitamin activity of pyridoxyllysine has also been attributed at least partially to competitive

inhibition of pyridoxalkinase (EC 2.7.1.35) through formation of phosphopyridoxyllysine (PPL). While pyridoxyllysine does not show any substrate activity for pyridoxamine-5-phosphate-oxidase (EC 1.4.3.5), PPL can be the substrate for this enzyme, which is considered to be the mechanism by which pyridoxyllysine does exhibit partial vitamin B-6 activity (Gregory 1980b). How well PPL functions as a substrate for pyridoxamine-5-phosphate-oxidase is, however, unclear, and appears to depend on the orientation and mode of binding of lysine to PLP (Kazarinoff and McCormick 1975, Gregory 1980b).

Even though the bioavailability of vitamin B-6 in pyridoxyllysine is relatively high, it also exhibits antivitamin activity at low levels. Thus, it seems that small concentrations of pyridoxyllysine could be deleterious to vitamin B-6 status when the diet is otherwise B-6-deficient (Gregory 1980c).

CHAPTER 3

3. Objectives of the Study

As mentioned in the introduction, the best approach to nutrient analysis in foods is to question if there is a need for the analysis, either in the form of a public health problem associated with the nutrient, or in the form of inadequate data for the particular nutrient. Pardue (1984) summarized the problem in the form of the following statement: "The principal role of an analysis is to aid in the resolution of some type of problem". With this statement in mind, we have to ask how well our study complies with this demand.

The Food and Nutrition Board stated in their latest issue of the Recommended Dietary Allowances that data on vitamin B-6 content of foods are insufficient, information on the bioavailability of the vitamin is lacking, and the methodology for the quantitation of vitamin B-6 is inadequate (Food and Nutrition Board 1980). It is also known that although vitamin B-6 status of most of the U.S. population appears adequate, there are some groups whose intake is considered marginal, or who seem to have higher requirements for this vitamin (Driskell 1984). Hence, our study appears to meet the criteria cited by Pardue. The baby food of interest is a formulation that is not fortified with pyridoxinehydro-

chloride, a stable B-6 vitamer commonly used for fortification of foods. As pointed out in the literature review, PL and PLP, two of the four major B-6 vitamers found in foods, may participate in formation of pyridoxyllysine during thermal processing (Gregory and Kirk 1978c). Since both types of baby food chosen for the study (jar and instant) undergo thermal processing, we should be able to detect pyridoxyllysine in both products. In addition, pyridoxyllysine has been determined only in model food systems, and the extent of its formation in commercial food products is not known (Gregory 1980b, Gregory et al. 1986).

The objectives of this study are:

1. To determine if there are significant differences in vitamin B-6 and available lysine content of similar jar and instant baby foods.
2. To determine if there are significant differences in vitamin B-6 and available lysine content of baby foods due to product formulation or storage time.
3. To determine if there is any association between the pyridoxyllysine content of jar and instant baby foods and storage losses of vitamin B-6 and available lysine.

CHAPTER 4

4. Materials and Methods

4.1. Materials

The content of total and available lysine, vitamin B-6 and pyridoxyllysine was determined in different baby foods. The intention of this study was to evaluate the effect of drum-drying on the nutrient content of these baby foods, and to compare nutrient retention in drum-dried products to that of conventionally canned products. The particular products used in this study have been sterilized using a steam injection heat exchanger and flash cooler before being put on the drum (Marcy 1989).

Two different kinds of drum-dried instant baby foods, namely "Vegetable and Beef", and "Bananas" and their matching jar products were kindly donated to us by H.J. Heinz Co., Pittsburg, PA 15212 in Winter 1985/86. Both products were processed at about the same time, and were stored in the dark at room temperature. More recently processed products of similar formulation were purchased in Spring 1988. A list of baby food ingredients is given in Appendix I.

The chemicals used in this study were purchased from several different vendors. All chemicals were at least

reagent-grade chemicals, unless otherwise stated. Vendors of chemicals and equipment are listed in Appendix II, and the preparation of chemical solutions is given in Appendix III.

4.2. Experimental Design

One of the principal objectives of this study was to determine if there are significant differences in Vitamin B-6, available lysine and pyridoxyllysine content in baby foods due to processing method. In addition, since we were able to obtain baby food produced in two different years, this enabled us to investigate the effects of storage on nutrient content. Since the lysine content of baby food also depends on the product formulation (i.e.: amount of protein), the lysine data were adjusted to a standardized amount of protein. It was then possible to consider whether changes in the content of a particular nutrient depend on product formulation, or if all product formulations show similar changes, regardless of the original nutrient content.

However, since the nutrient content of raw materials used in the baby foods was not determined, it was impossible to directly assess nutrient losses due to food processing. This causes a problem in regard to determining which one of the two

processes, canning or drum-drying, results in greater losses of specific nutrients, particularly because we have to assume that the different products were made from different raw materials. However, if the assumption is made that the nutrient content of the different raw materials and product formulations is the same, it is then possible to compare the effects of the different processing treatments on the products.

Preliminary analyses indicated that there are no significant differences in available lysine or vitamin B-6 content for different jars or lots of baby food. This suggests that raw materials are not significantly different in nutrient content for the same product. It should also be mentioned that the raw materials are delivered to the company in bulk quantities (bananas are aseptically processed and packaged in 50 gallons drums) which results in a more uniform product in regard to quality and nutrient content (Marcy 1989). It should be emphasized, however, that different raw materials may be used for different products.

For the actual analysis of the baby foods, every product was sampled four times with duplicate subsamples taken for the available lysine analysis, whereas one sample with duplicate subsamples was considered to be sufficient for the total lysine analysis. Foods were sampled three times with tri-

plicate subsamples for the vitamin B-6 analysis and two times for the pyridoxyllysine analysis. This sampling procedure is described in Table 1. Figure 2 shows the statistical model for the analyses.

Baby food products were handled and reconstituted as described on the label, to insure that products were as similar in composition as possible to actual foods consumed by a child.

4.3. Methods

4.3.1. Proximate Analysis

The methods used for the proximate analysis are modifications of the methods of analysis recommended by the Association of Official Analytical Chemists.

4.3.1.1. Determination of Crude Protein

The method used to determine the crude protein content of the products is a modification of AOAC Method 7.015, better known as the Kjeldahl Method (AOAC 1984). This method employs

Table 1

Sampling Procedure Used in This Study

		Instant Product		Jar Product	
		Veg & Beef	Banana	Veg & Beef	Banana
1985	Av.Lys	4	4	4	4
	Lysine	1	1	1	1
	Vit B6	3	3	3	3
	PyxLys	2	2	2	2
1987	Av.Lys	4	4	4	4
	Lysine	1	1	1	1
	Vit B6	3	3	3	3
	Pyxlys	2	2	2	2

Difference in nutrients = Processing Method
+ Storage Effect
+ Product Formulation
+ Processing Method * Storage Effect
+ Proc. Method * Product Formul.
+ Storage Effect * Product Formul.
+ Stor.Eff.* Prod.Form. * Proc.Meth.
+ Error Term

Figure 2: Statistical Model

the sulfuric acid digestion of the product followed by the release of nitrogen in the form of ammonium, which is distilled into 4% boric acid. The amount of ammonium borate that is formed is determined by titration against a standardized hydrochloric acid solution. In order to estimate total protein, the nitrogen content of the samples is multiplied by 6.25 (Protein on average contains 16% nitrogen).

4.3.1.2. Determination of Crude Fat

The crude fat content of samples was determined by AOAC method 7.060 (AOAC 1984), also known as the ether extract method. The procedure is based on the determination of the weight loss of a known amount of sample due to the extraction of ether-soluble compounds for 36 hours using anhydrous petroleum ether.

4.3.1.3. Determination of Moisture

The AOAC method 7.007 (AOAC 1984) was used to determine the moisture content by drying samples placed in aluminum dishes to constant a weight, in a Brabender oven at 135°C.

4.3.1.4. Determination of Ash

A modified AOAC method 7.009 (AOAC 1984) was used to determine the ash content of the samples. Ash was determined by placing a weighed sample in a porcelain crucible, combusting the sample in a muffle furnace at 600°C for 12 hours and after cooling reweighing the sample. The weight of the residue corresponds with the ash content of the sample.

4.3.1.5. Determination of Neutral Detergent Fiber

There is no recommended AOAC method for the determination of the dietary fiber content of foods. Therefore, a method proposed by Robertson and Van Soest (1977), was chosen, because it is commonly used in our department. The method is based on the digestion of the sample with amylase in a neutral-detergent solution. The residue after digestion is then ashed and the loss in weight on ashing is an estimate of dietary fiber.

4.3.1.6. Determination of Crude Carbohydrate

The crude carbohydrate content of the samples was determined by difference. The crude carbohydrate content is equal to 100 minus the percentage of crude protein, crude fat, moisture and ash.

4.3.2. Determination of Available Lysine

The method used for the determination of available lysine was a trinitrobenzenesulfonic acid (TNBS) method, adapted from Hall et al. (1973). Due to a relatively high carbohydrate concentration in the products, some modifications of this method were required (James and Ryley 1986, Hall and Henderson 1979, Hall et al. 1975). The determination of available lysine employs the reaction of the free epsilon-amino group of lysine with trinitrobenzenesulphonic acid (TNBS ≡ Picryl-sulphonic acid), forming trinitrophenyl-lysine (TNP-L) (Figure 3), which can be measured spectrophotometrically at 415 nm.

i) Depending on the type of sample, 5 (VB Instant), 10 (VB Jar), 20 (Ba Instant) or 30 (Ba Jar)g of homogenized sample were placed into a 50 ml flask containing 4 ml acetone. The

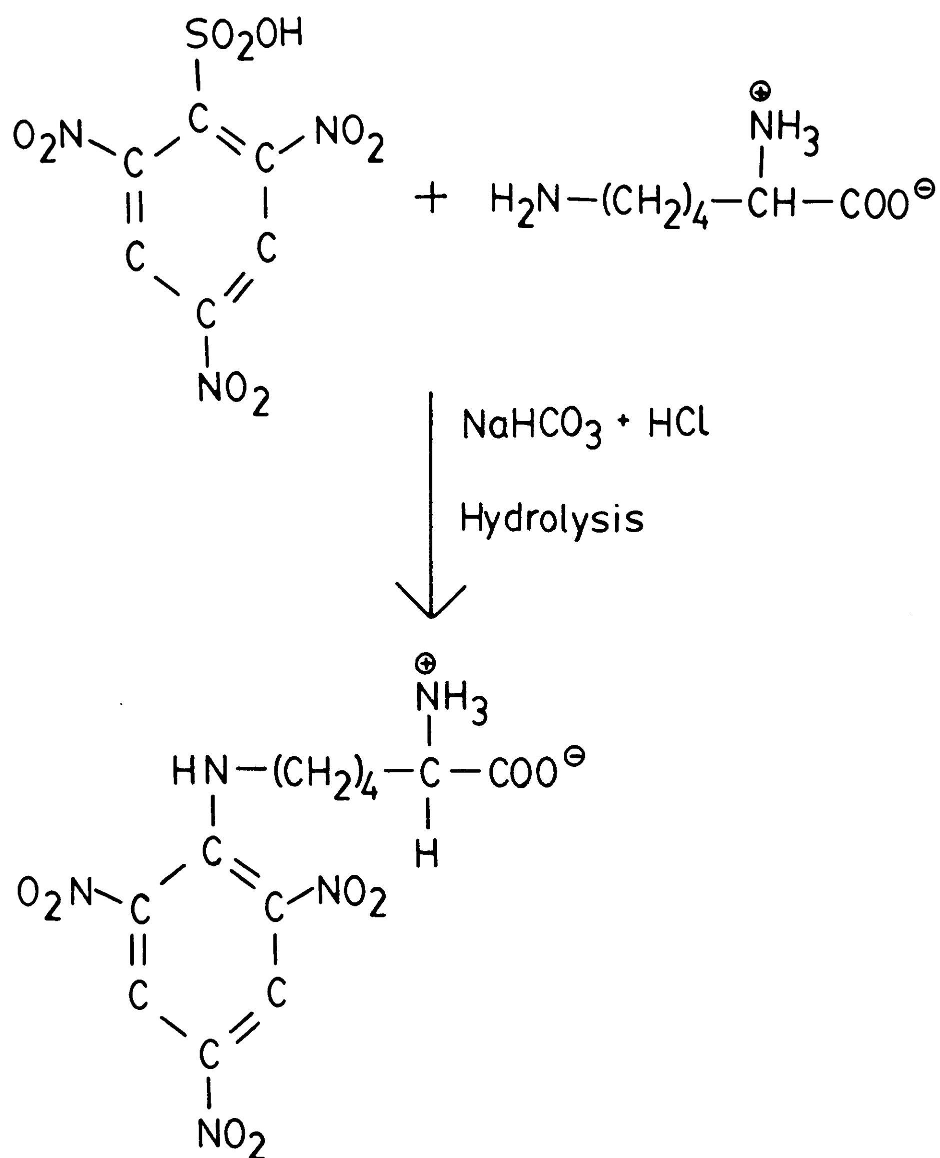

Figure 3: Reaction of TNBS With Lysine to Form TNP-L (adapted from Hall et al. 1973)

flask was filled to volume with agar solution (0.1% w/v), which was preserved with octane-2-ol and shaken vigorously for 1 minute (sample suspension).

ii) Then 0.5 ml of the vegetable and beef sample suspension or 1.0 ml of the banana sample suspension was transferred in duplicate into separate tubes and 0.5 ml 1 M $NaHCO_3$ was added to both suspensions. To one tube (sample) 1 ml of TNBS solution (1% w/v) was added and mixed. To the other tube (sample blank) first 3.0 ml concentrated HCl and then 1 ml of TNBS solution was added and mixed. All test tubes were stoppered and incubated at 30°C for 30 minutes.

iii) Then 3 ml of concentrated HCl was carefully added with a burette to each sample tube. All tubes were then covered and placed into a vigorously boiling water bath for 1 hour. After boiling, the tubes were cooled, and filled to a volume of 10 ml with water.

iv) The solutions were then filtered through Whatman #40 filter paper to remove solids and 3.0 ml from each sample test tube was pipetted in duplicate into separate test tubes. Three ml of the sample blank were also pipetted into another test tube. Water was added to all test tubes to give an approximate volume of 8.0 ml, and 5.0 ml ethyl ether was added to remove free TNBS and other TNP amino acids formed since they are soluble in ether. The test tubes were vigorously shaken for

10-15 seconds, then the stoppers were removed and rinsed into the tubes with water. Most of the ether was removed with a Pasteur pipette attached to a water pump. The ether extraction was repeated. Any remaining ether was removed by placing the tubes in a 40°C water bath.

v) The banana formulations were bleached with hydrogen-peroxide (3% v/v) and potassium permanganate (4% w/v) solutions. The volume of all test tubes was made up to 10 ml with water and mixed by inversion. The absorbance of the resulting solution was measured at 415 nm using a Bausch & Lomb Spectronic 2000 spectrophotometer. The absorbance of the sample blanks was subtracted from the absorbance of the samples and the corresponding available lysine values were found using a standard curve (Appendix IV).

Standards

Epsilon-TNP-L-lysine monohydrochloride monohydrate has a molecular weight of 411.77 and consists of 35.5% lysine (MW lysine: 146). 14.1 mg TNP-lysine (≡ 5 mg lysine) was placed into a 25 ml volumetric flask and made up to volume with water. Then 0.00 (blank), 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, and 0.9 ml of this solution were placed into 10 ml test tubes and

made up to 1.0 ml volume with water and treated identically to the samples in ii). These standards correspond to final lysine concentrations of 6, 12, 18, 24, 30, 42 and 54 ug/10ml respectively.

Calculation of Available Lysine

The values of available lysine read from the standard curve are in ug lysine per 10 ml solution ((x) ug lys). The following equations were used to convert these numbers into values presented in Appendix IX.

$$\text{(y) mg lys/g food} = \frac{\text{(x) ug lys} * \text{Dilution Factor} * \text{1 mg}}{\text{Sample weight g} * \text{1000 ug}}$$

$$\text{Dilution factor} = \frac{\text{50 ml} * \text{10 ml}}{\text{0.5 ml} * \text{3 ml}} = \frac{500}{1.5} = 333.33$$

Values for (y) mg lys/g food were converted to (z) mg lys/g nitrogen using the following equation:

$$\text{(z) mg lys/g N} = \frac{\text{(y) mg lys} * \text{6.25 g N}}{\text{\% Protein}} * 100\%$$

where % protein equals the percentage of crude protein in the specific baby food.

Several different *in vitro* methods for determining available lysine in foods have been developed, each having advantages and disadvantages. The four best known methods are the FDNB (Flourodinitrobenzene) method, the TNBS method, the DNBS (Dinitrobenzenesulfonic acid) method and Udy-dye method. All methods are based on the reaction of lysine's free epsilon amino group with a particular chemical reagent.

Since certain problems were experienced with the TNBS method used in this study, a short discussion of those shortcomings and attempts to solve them is considered necessary.

The TNBS method was developed by Kakade and Liener (1969) for the determination of available lysine in proteins and was modified by Hall et al. in 1973. However the method was not considered applicable to carbohydrate-rich foods because of the "caramelization" of carbohydrates by hydrochloric acid at 100°C. The interference was twofold: 1. it added color that absorbs at 415 nm to the solution and 2. the carbon particles formed (also referred to as "humins" or "charring") could adsorb some of the TNP-lysine (Hall et al. 1975). However, James and Ryley (1986) found that the adsorption of TNP-lysine by the carbon particles does not contribute to the problems

of determining available lysine in carbohydrate-rich food with the TNBS-method. The interference of the "humins" and "caramelization" has been overcome by several means:
1. shortening the reaction time of TNBS with lysine from 75 min to 30 min (Hall et al. 1975), 2. "bleaching" the samples with hydrogen peroxide and $KMnO_4$ solutions (James and Ryley 1986, Hall and Henderson 1979), 3. filtering the samples after hydrolysis (Posati et al. 1972), 4. reducing the hydrolysis time from two hours to one hour (James and Ryley 1986), and 5. decreasing the reaction temperature from 40°C to 30°C (Hall et al. 1975).

4.3.3. Determination of Vitamin B-6

A microbiological method was used to determine the total amount of vitamin B-6, in the baby food samples. The method is a modification of AOAC procedure 43.229 (AOAC 1984, Toepfer and Polansky 1970, Haskell and Snell 1970).

Assay Inoculum

A Saccharomyces uvarum (ATCC 9080, formerly known as

Saccharomyces carlsbergensis) culture was streaked fresh weekly onto Difco bacto YM agar slants, and slants were incubated overnight in a shaking waterbath at 30°C. Ten ml of pyridoxine Y media for each inoculum were transferred to two screw top centrifuge tubes and steamed for ten minutes. Saccharomyces uvarum cells were aseptically transferred from the slant to the cooled media. These cells were then incubated at 30°C in a shaking water bath for 20 hours. Then the cells were centrifuged, the supernatant decanted, and the precipitate washed three times with 10 ml sterile saline solution. The final suspension was used as the assay inoculum.

Sample Preparation

The frozen samples were defrosted at 5°C overnight. One hundred seventy ml of 0.44 M HCl were added to approximately 2 g of each sample, and the mixture was autoclaved for 2 hours at 121°C and 15 psi. Samples were cooled, adjusted to pH 4.5 with 6 M KOH, and glacial acetic acid, then brought to 250 ml with distilled deionized water, and filtered through two sheets of Whatman #42 filter paper. The filtrate was designated as the extracted sample.

Sample Assay

All analyses of standards and samples were run in triplicate. Sample extracts, 0.1 ml for BaI, 0.5 ml for BaJ and VBI and 0.8 ml for VBJ, as well as working standards (0, 0.5, 1, 2, 4 ng PN HCl) were pipetted into test tubes. Then 5 ml of Pyridoxine Y media was added to the sample extract, and the volume brought to 10 ml with distilled deionized water. The tubes were then steamed for 10 minutes, cooled, and aseptically inoculated with two drops of assay inoculum. The tubes were then incubated for 20 hours at 30°C in a shaking waterbath. At the end of the incubation period, the contents of the tubes were steamed for five minutes in order to stop growth of the test organisms; the contents were then allowed to cool. The content of each tube was vortexed and decanted into a spectrophotometer tube. A Bausch & Lomb Spectronic 20 was used to read transmittance at 550 nm.

Calculation of Vitamin B-6

The vitamin B-6 content of each extracted sample was estimated from a standard curve (Appendix V), and inserted in the following equation in order to calculate ug B-6 per 100

g of food:

$$\text{ug B-6/100g food} = \frac{\text{ng/tube} * \text{250 ml} * \text{100 g} * \text{1 ug}}{\text{aliquot ml} * \text{sample weight g} * \text{1000 ng}}$$

4.3.4. Determination of Pyridoxyllysine

Several variations of a method described by Gregory et al. (1986) for extracting pyridoxyllysine from model food systems were tried. The HPLC method used for the separation and detection of pyridoxyllysine is a method developed in our department for the separation of the six B-6 vitamers, 4-pyridoxic acid and the internal standard 4-deoxypyridoxine (Chrisley et al. 1988). The HPLC system consisted of a Model 730 Data Module, a Model 720 System Controller, a Model U6K Universal Injector, a Column Temperature Control System, two Model 45 Solvent Delivery Systems, and a Model 420 E/AC Fluorescence Detector (Mercury lamp). The HPLC conditions for injection of samples and standards are shown in Figure 4.

Whereas the HPLC conditions for the pyridoxyllysine detection were kept constant, various changes were made in the extraction method. The following changes (in chronological order) were made in the extraction method:

Column: Micro-Bondapak Octadecylsilane (30 cm * 3.9 mm I.D., 10 um porous packing (plus guard-column)

Injection Size: 25 ul

Mobile Phase: Solvent A: Methanol : Water (80:20 v/v)

Solvent B: Heptane sulfonic acid (PIC B7) and octane sulfonic acid (PIC B8) and 1% acetic acid

Flow Rate: 1 ml/min at ambient temperature (37°C)

Gradient Elution:

Time (min)	Solvent A (%)	Solvent B (%)
0	0	100
5	25	75
8	75	25
12	40	60
16	0	100
20	0	100

Detection: Fluorescence at 400 nm (300 nm excitation)

Figure 4: HPLC System Used for Pyridoxyllysine Detection

It was our original intention to incorporate the pyridoxyllysine extraction into the vitamin B-6 extraction procedure described by Chrisley et al. (1988). In this method, the B-6 vitamers are released from the food, and protein coagulated using 5% TCA. The mixture is centrifuged, and the supernatant is decanted. The supernatant was to be used for the B-6 analysis, and the coagulated protein hydrolysed to release pyridoxyllysine. After hydrolysis the hydrolysate was to be purified by evaporation and ion-exchange chromatography according to Gregory et al. (1986). The major problem with this method was that it did not give reproducible results, either for vitamin B-6 or for pyridoxyllysine. The method had been developed for plasma samples and does not seem to be applicable without further modifications to complex food systems. Instead of obtaining two nicely separated layers after centrifugation as with plasma samples, three layers formed, of which the middle layer was a whitish, suspended layer. Qualitative analyses indicated that this middle layer consisted mainly of complex carbohydrates (iodine reaction) and some protein (biuret reaction). Increasing the TCA concentration used to precipitate the protein did not improve the properties of the middle layer in regard to separation. It was not possible to remove the supernatant without disturbing this layer or completely remove the layer with the

supernatant, which is in fact not desirable since trace amounts of protein in this layer could contain bound pyridoxyllysine.

The extraction method of Gregory et al. (1986) with slight modifications was then tried. This method is described as follows:

i) One g of sample was diluted with 3 ml HPLC-grade water and dialyzed against 500 ml of 0.1 M potassium phosphate buffer, pH 7.0, for 16 hours. The buffer was changed once after 8 hours. The contents of the dialysis bags were then subjected to acid hydrolysis in 6 M HCl at 105°C for 48 hours in total darkness. After hydrolysis, the samples were filtered through Whatman #44 filter paper.

ii) Filtrates were evaporated to dryness at 50°C using a rotary evaporator, washed with HPLC-grade water and evaporated again. After evaporation, filtrates were again washed with HPLC-grade water and evaporated. Samples were then reconstituted in 5 ml HPLC-grade water and applied to a Bio-Rex 70 column (0.5*10 cm) which was previously equilibrated with 150 ml 1 M ammonium formate, pH 4.0, and washed with 20 ml HPLC-grade water. The column was washed again with water and the pyridoxyllysine was eluted with 1 M acetic acid (HPLC-grade).

iii) The eluate was dried with a rotary evaporator and taken up in 2 ml of solvent B (PIC B7 & B8: 0.005 M heptane sulfonic

acid and octane sulfonic acid and 1% acetic acid). The resulting solution was adjusted to pH 2.9 with HPLC-grade glacial acetic acid, and filtered through 0.2 um Acrodisc and C_{18} Sep-pak, and injected into the HPLC.

This method gave reproducible chromatograms, but Maillard browning products, which formed due to the high amount of carbohydrates in the baby food samples, interfered with the fluorescence detection of any pyridoxyllysine.

We attempted to solve this problem by using the amylase and amyloglucosidase enzymes to hydrolyze the complex carbohydrates into small soluble saccharides which could then be removed by dialysis. It was hoped that this would dramatically decrease problems with fluorescence detection of pyridoxyllysine due to Maillard browning products. While the Maillard browning problem was in fact alleviated, we believe that the enzymes might have been contaminated with proteases, since pyridoxyllysine was not detected in any of these samples and was presumably lost in the dialysate. An attempt to stop enzyme activity after 3 hours with TCA in order to decrease the possibility of significant proteolysis did not result in improved chromatograms or in the detection of pyridoxyl-lysine. The use of enzymes with higher purity resulted in interference with the fluorescence detection due to high fluorescence of the enzymes.

Other trials that were attempted included hydrolyzing the samples without dialysis or enzyme treatment and including or excluding the use of an ion-exchange column. Sample sizes were varied between 1 g and 20 g. However an increase in sample size aggravated the interference from Maillard browning products. In every case, it was possible to separate the pyridoxyllysine standards and spikes in the samples from the B-6 vitamers and/or interfering peaks. In the case of large sample sizes (20 g) the spikes were only visible as small shoulders in the decay curve of the interfering peaks. Pyridoxyllysine was not detected in any of the baby food samples by any of these methods. For the sake of completeness, it should be mentioned that a basic protein hydrolysis was also tried, but photospectrometric and fluorospectrometric qualitative analyses showed that pyridoxyllysine was unstable under such conditions.

Pyridoxyllysine, an amino acid derivative, was also analyzed using an amino acid analyzer (see Chapter: 4.3.5. Determination of Total Lysine, for details). It was possible to detect and separate the pyridoxyllysine standard from the other amino acids (Appendix VI), but no pyridoxyllysine was found in the baby food.

Pyridoxyllysine Standards

Pyridoxyllysine was prepared according to Dempsey and Christensen (1962) and Dempsey and Snell (1963), with some modifications (Gregory 1980b, Severin et al 1969). The method is based on the condensation of acetyllysine with pyridoxal hydrochloride, forming alpha-acetyl-epsilon-pyridoxyllysine. The acid hydrolysis of this compound cleaves the acetylgroup off and yields epsilon-pyridoxyllysine.

i) 376 mg (2 mM) of N-alpha-acetyl-L-lysine and 157 mg of potassium hydroxide were dissolved in 10 ml methanol. 334 mg (2 mM) pyridoxalhydrochloride was added and the solution stirred for 15 min at room temperature. The solution was filtered through Whatman #40 filter paper to remove all solids. Five-fold molar excess (=378 mg) of solid sodiumborohydride was slowly added at 5°C and allowed to react for one hour. The solution was then acidified to pH 4.4 with glacial acetic acid and evaporated to dryness under vacuum at 50°C.

ii) Salts and unreacted material were removed by column chromatography, using a Sephadex G-10 column (2.6*60 cm). Fractions containing acetylpyridoxyllysine were pooled and evaporated under vacuum to yield pale-yellow crystals. The

crystals were dissolved in 6 M HCl and autoclaved for 40 min at 121°C, then neutralized with NH_4OH. This solution was taken to total dryness under reduced pressure at 50°C, and then dissolved in water. It was again dried under reduced pressure at 50°C and dissolved in 50 ml water.

iii) The resulting solution was applied to a BioRex 70 column (2.6*30 cm) which was previously equilibrated with 1.0 M ammonium-formate and washed with 250 ml water. The column was washed with 200 ml water and the pyridoxyllysine was eluted with 1.0 M acetic acid. Collected fractions eluted from the column were checked for epsilon-pyridoxyllysine by absorbance at 323 nm and eluted fractions showing this reaction were combined. The eluate was dried under reduced pressure at 50°C and the oil residue was dissolved in ethanol and dried again. The residue was again dissolved in ethanol and stood for 12 hours at room temperature. Pure epsilon-pyridoxyllysine crystallized out and was dried under reduced pressure at 50°C yielding a viscous oil. The purity of pyridoxyllysine was verified by mass-spectrometry.

The UV-visible absorbance spectrum of the synthesized compound is shown in Figure 5 and its mass spectrum in Figure 7 with reference spectra from the literature shown in Figure 6 and 8. A pyridoxyllysine standard curve used in the HPLC analysis is shown in Appendix VII.

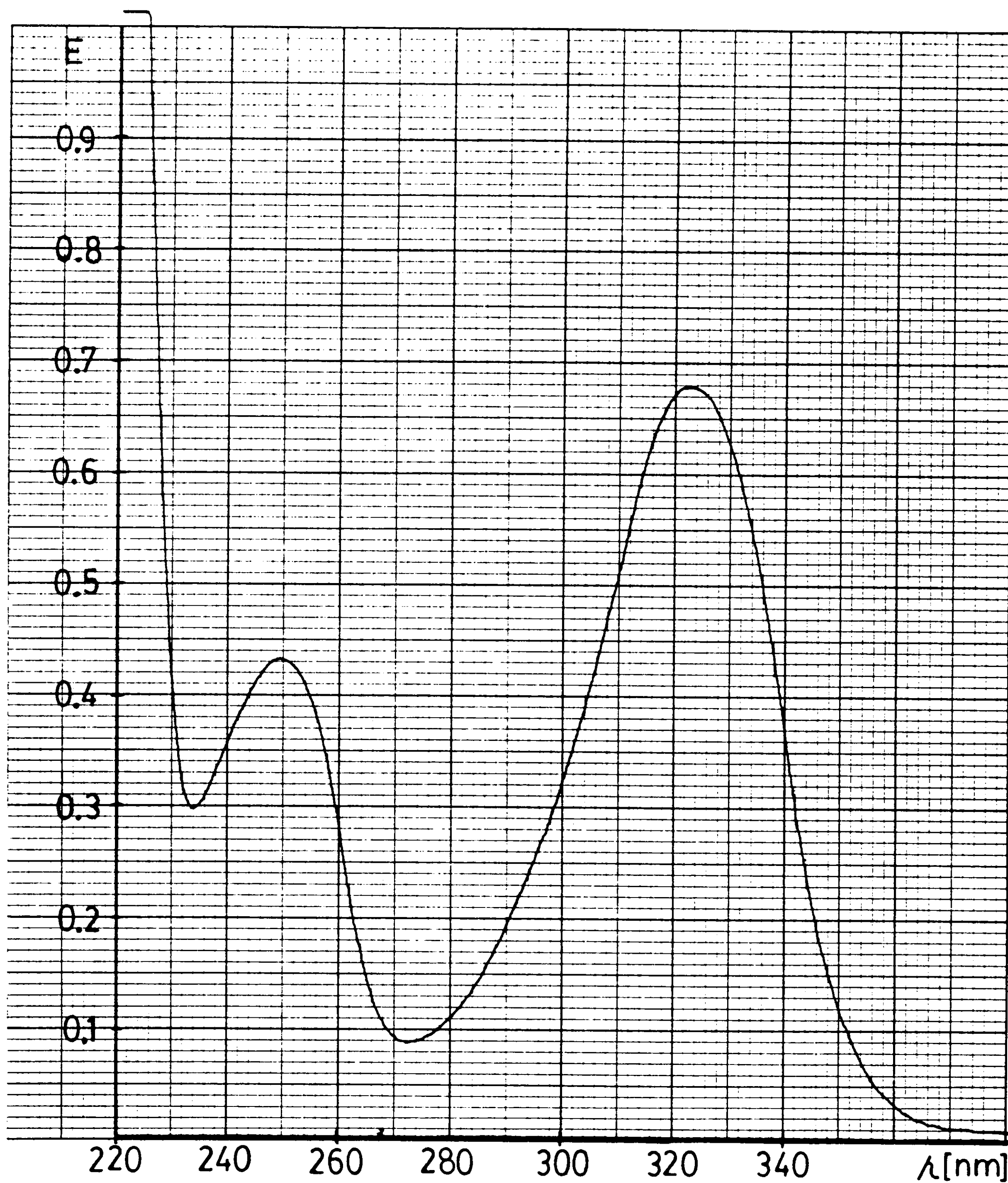

Figure 5: Absorbance Spectrum of Synthesized Epsilon-N-Pyridoxyllysine (35 ug/ml in water)

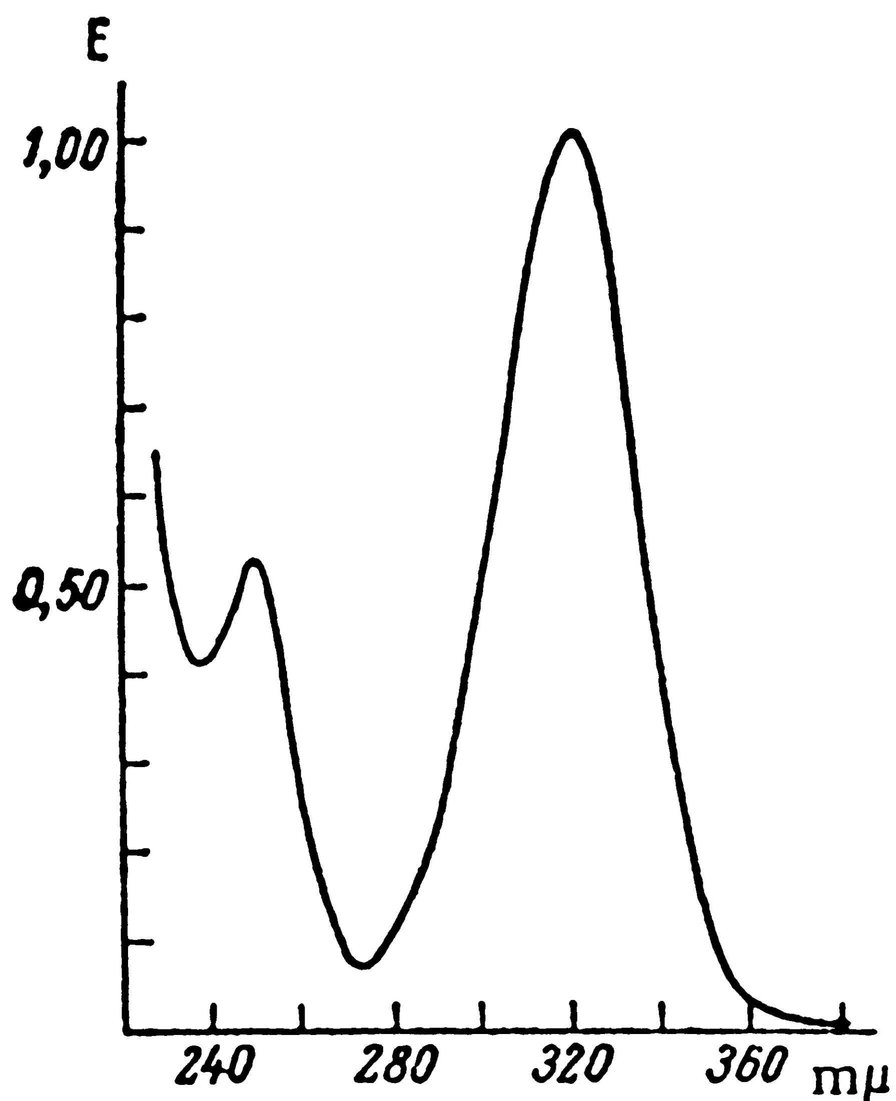

Figure 6: Reference Absorbance Spectrum of Epsilon-N-Pyridoxyllysine (50 ug/ml in water)(Polyanovskii, 1963)

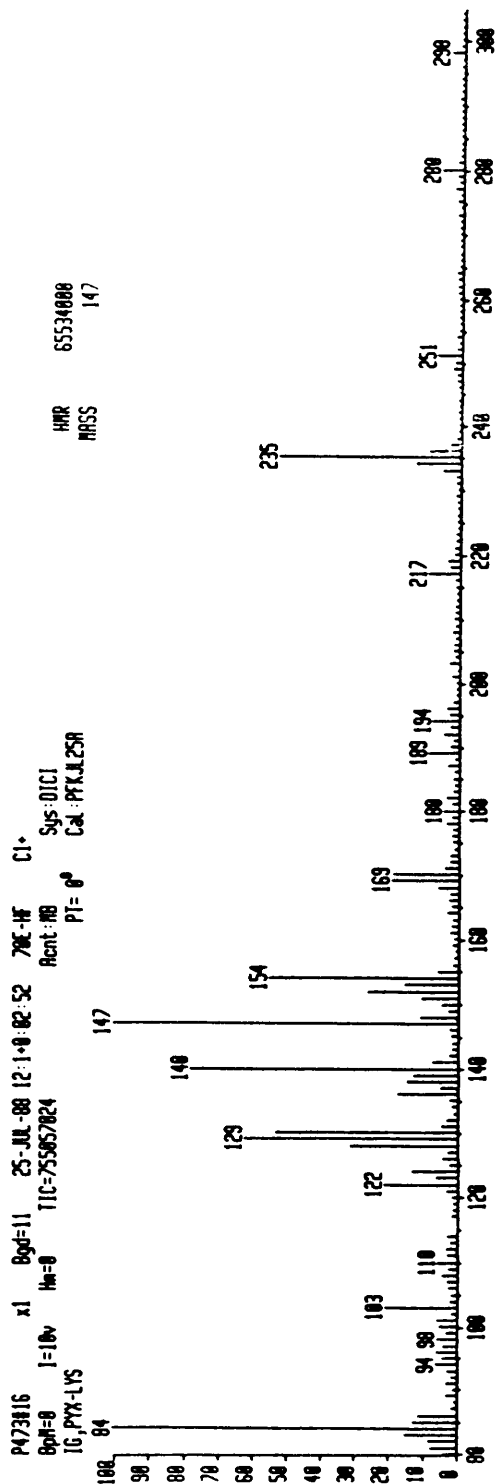

Figure 7: Mass-spectrum of Synthesized Epsilon-N-Pyridoxyllysine

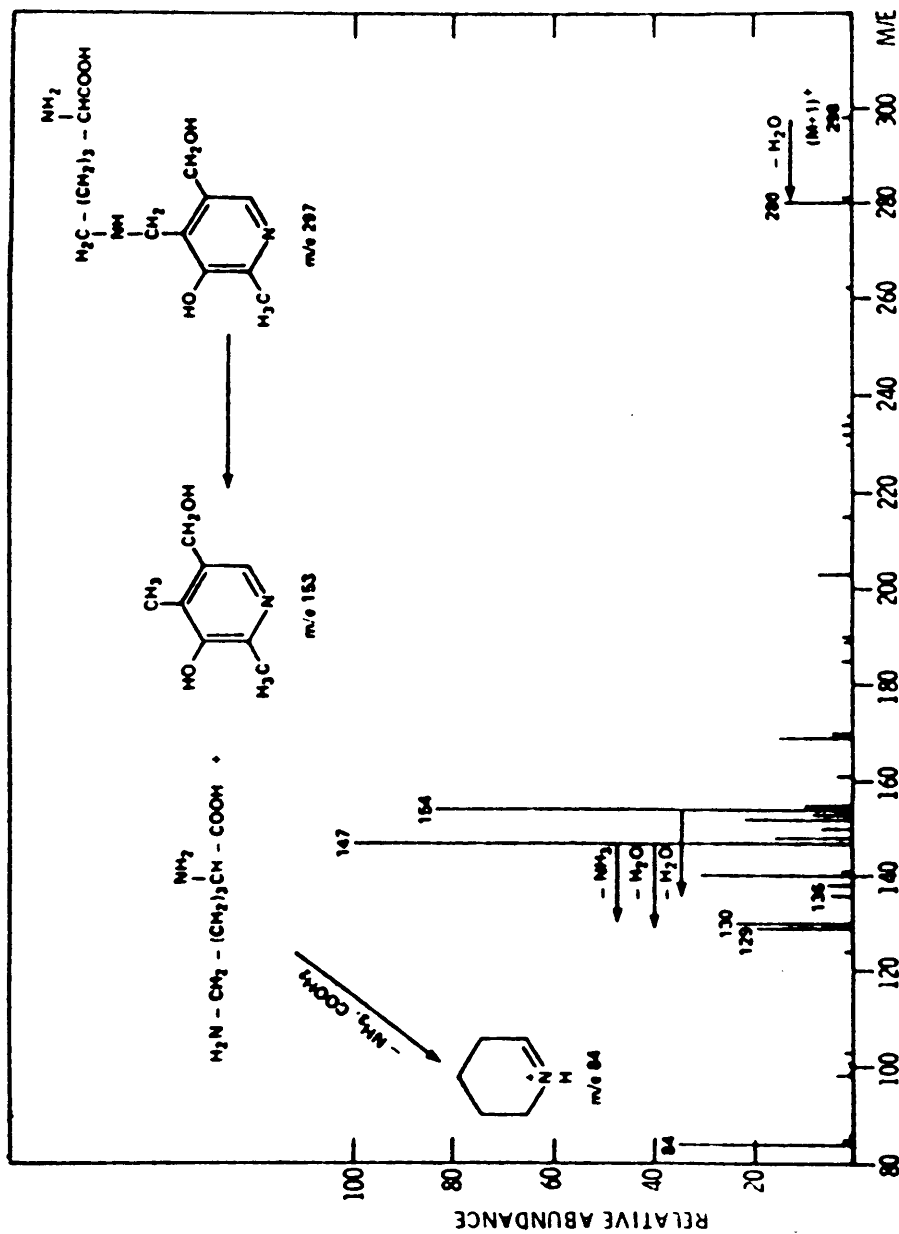

Figure 8: Reference Mass-Spectrum of Epsilon-N-Pyridoxyllysine (Miles et al. 1972)

4.3.5. Determination of Total Lysine

Total lysine was determined using an amino acid analyzer. In this procedure samples are first hydrolyzed in 6 M HCL, and the resulting amino acids are derivatised with phenylisothiocyanate to form phenylthiocarbamyl amino acids. These amino acid derivatives are then separated by HPLC before detecting them spectrophotometrically at 254 nm.

i) 0.1 g of samples was weighed into 20 ml glass ampules. 7.5 ml of 6 M HCL was added to the ampules. The vials were vortexed and another 7.5 ml of 6 M HCL was added. The ampules were then sealed and placed in an autoclave. The samples were autoclaved at 132°C for 6 hours, and then cooled to room temperature.

ii) 5 ml of 5 uM/ml alpha-amino butyric acid (internal standard) was added to 25 ml volumetric flasks. The contents of the ampules were poured into the flasks, mixed and the volume was brought up to 25 ml with water and mixed again. A 1 ml aliquot was removed using a tuberculin syringe and passed through a 0.45 micron filter into a small screw top glass vial. The vial was purged with nitrogen and frozen at -20°C. At the time of analysis the sample was thawed, and a 10 ul aliquot was placed into sample tubes and dried to 70 m Torr

on the vacuum station.

iii) Ten ul of Redry solution was added to each standard and sample tube and vortexed. The tubes were placed in a reaction vial and dried to 70 m Torr. Then 20 ul of derivatization reagent was added to each tube. The tubes were vortexed and placed into the reaction vial. The reaction vial was placed in the vacuum station and the vacuum port was opened after a derivatization time of 20 minutes. The samples were dried to 70 m Torr.

iv) 200 ul of diluent was added to each sample tube and vortexed. The sample tubes were then centrifuged in a micro-centrifuge for 30 seconds. The supernatant was removed to limited volume inserts and placed in the appropriate WISP vials. The vials were closed with a teflon septa and screw cap, and were placed into the automatic sampler.

The HPLC system used for derivatised amino acids consisted of a WISP Model 712, a Column Temperature Control System, two Model 510 solvent delivery systems, a PICO*TAG column (3.9 mm*15 cm), a Model 441 Absorbance Detector, and a System Interface Module that connected the system to a NEC APC IV personal computer. The injection size was 10 ul. The elution conditions are shown in Table 2. The lysine concentration in the samples was calculated by the computer software "Maxima

Table 2

Elution Conditions for Total Lysine Analysis

Time	Flow Rate (ml)	Eluent A (%)	Eluent B (%)
0.0	1.0	100	0
20.0	1.0	56	44
20.5	1.0	0	100
21.0	1.5	0	100
25.0	1.5	0	100
25.5	1.5	100	0
38.0	1.5	100	0
38.5	1.0	100	0

820" used by the system, and recorded as g lysine per 100 g sample. The total lysine content was then calculated as mg lysine per g nitrogen.

Standards

An amino acid standard solution, containing 2.5 uM/ml of each amino acid (except cysteine which contained 1.5 um/ml) from Pierce was used in the analysis. The lysine concentration in the standard was 0.008012 mg/ml. Alpha-amino-n-butyric acid was used as an internal standard. Equal parts (0.2 ml) of the solutions were mixed. 10 ul of the mixture was placed into a vacuum vial and dried in the vacuum station to 70 m Torr.

4.4. Statistical Analysis

Significant differences between means were determined by using the statistical method "Analysis of Variance" and "Duncan's Multiple Range Test" which are part of the statistical package SAS (Statistical Analysis System) employed by the Computing Center of the Virginia Polytechnic Institute and State University.

CHAPTER 5

5. Results and Discussion

5.1. Proximate Analysis

The results of the proximate analysis for the individual samples are presented in Appendix VIII. The mean percentages for protein content, fat content, moisture content, ash content, fiber content and carbohydrate content of the selected baby foods are presented in Table 3. The coefficients of variation for each of the analyses are as follows:

Moisture Determination	0.0006
Ash Determination	0.0599
Fat Determination	0.0744
Protein Determination	0.0888

No coefficient of variation has been established for the Neutral Detergent Fiber analysis.

Only the protein content of the selected baby foods was subjected to statistical analysis, since it is the only proximate constituent of the foods clearly related to the three nutrients under study. The results of the statistical analysis are presented in Table 4. While the protein content

Table 3

Mean Percentages of Moisture[1], Ash[2], Fat[3], Protein[4], Fiber[5] and Carbohydrate[6] Content of Selected Baby Foods

Product	Moisture	Ash	Fat	Protein	Fiber	Carbohydrate
VB J	88.0	0.29	0.67	2.6	2.5	8.44
VB I	88.8	0.29	1.95	4.0	4.3	4.96
Ba J	82.8	0.19	0.43	0.32	3.8	16.26
Ba I	84.9	0.70	0.20	0.65	3.1	13.55

[1] Based on the analysis of four samples

[2] Based on the analysis of four samples

[3] Based on the analysis of eight samples

[4] Based on the analysis of four samples

[5] Based on the analysis of two samples

[6] Determined by difference

Table 4

Results of Duncan's Multiple Range Test on the

Protein Content of Selected Baby Foods

Product	Protein (%)	Std.Dev.	Duncan's Grouping[1]
VB I 85	4.10	0.04	A
VB I 87	3.85	0.15	B
VB J 85	2.66	0.04	C
VB J 87	2.56	0.08	C
Ba I 87	0.69	0.04	D
Ba I 85	0.61	0.02	D
Ba J 87	0.32	0.01	E
Ba J 85	0.31	0.02	E

[1]Means with the same letter are not significantly different at the alpha = 0.05 level

depends clearly on the product formulation; it is significantly ($p<0.05$) higher for the "Vegetable and Beef" than for the"Bananas" formulation, the processing year did not influence the protein content, with the exception of the instant "Vegetable and Beef" product. The protein content is also significantly ($p<0.05$) higher in the instant products for all product formulations and processing years.

While it was somewhat expected that the protein content of the product formulations, "Vegetable and Beef" and "Bananas" would be different, it is more surprising that the protein content is significantly different for the same formulations processed by two methods. This difference in protein content is, however, not due to varying processing methods, because even though proteins are affected by heat in regard to their nutritive value, the total amount of protein does not change upon thermal processing (Erbersdobler 1986). It is obviously due to the different formulation of the products, and can be considered a "dilution" effect. As it can be seen in Appendix I, the instant "Bananas" product consists solely of dried bananas and some vitamin C, whereas the jar product has many more ingredients which contain no protein, such as starches and corn syrup. For the "Vegetable and Beef" product this is not as obvious. Whereas the jar product contains added corn starch and soy flour, the instant

product has rice- and oat flour, but also some added soy protein. Considering the placement of these ingredients on the label, however, the jar product seems to contain more added carbohydrates than the instant product, which may account for the significantly different protein content.

5.2. Available and Total Lysine Analysis

The coefficient of variation for the available lysine analysis is 0.054 and was established by analyzing five identical samples. Available lysine was determined on four samples which were subsampled in duplicate for each treatment combination. The results of the available lysine analysis for the individual samples, expressed first in mg lys / 100 g food, and then adjusted for protein content and expressed as mg lys / g N, are presented in Appendix IX. A standard curve used in the available lysine analysis is shown in Appendix IV. The results of the statistical analysis of these data using the Analysis of Variance (ANOVA) from the Statistical Analysis System (SAS) are presented in Table 5. The R-Square values for the models, describing the differences in available lysine, are 0.995 and 0.922 respectively. This indicates that the models give a good explanation for the differences found.

Table 5

P-Values for the Model Describing the Differences in Available Lysine, Expressed as mg Lysine per 100g Food and mg Lysine per g Nitrogen

Source of Variation	P-Values for mg lys/100 g Food	P-Values for mg lys/g Nitrogen
Product Formulation	0.0001	0.0001
Processing Method	0.0001	0.0001
Storage Effect	0.1468	0.0011
Prod.Form.*Proc.Meth.	0.0001	0.0028
Prod.Form.*Stor.Effect	0.6940	0.0245
Proc.Meth.*Stor.Effect	0.4168	0.0142
Prod.F.*Proc.M.*Stor.E.	0.7570	0.1025

Due to the significant interactions of the main effects it is not possible to draw conclusions about the main effects from the analysis. Therefore, it was necessary to analyze the data in more detail. This was done in two ways: First, the interactions were visualized by drawing graphs of the interactions. These are presented in Appendix X and XI. Secondly, two main effects were blocked and one fourth of the data set was used to analyse for the third main effect. This was done for all levels of all main effects. The results of these analyses are presented in Table 6 and 7.

The results of the available lysine content in mg per 100 g food will be discussed first. Graph 1 and 2 in Appendix X show that the significant interaction between the product formulation and the processing method is due to the fact that the difference in available lysine content between instant "Vegetable and Beef" and jar "Vegetable and Beef" is much larger than it is for the "Bananas" products. This is represented graphically by non-parallel lines. However, we must consider that it is difficult to compare values for available lysine content between the product formulations when they are very different. The "Vegetable and Beef" products contain ten times as much available lysine per 100 g food than the "Bananas" products do, and have standard deviations of approximately the available lysine content of the "Bananas"

products (see Table 6). This large difference in available lysine content and in standard deviations causes the significant interaction. When calculated on a percent basis, the extent of the differences between the instant and the jar products for both product formulations equals out (VB 87 = 26%, Ba 87 = 25%; VB 85 = 29%, Ba 85 = 40%). The separated analysis of the main effects presented in Table 6 shows highly significant differences ($p<0.0001$) in available lysine content between the product formulations, "Vegetable and Beef" and "Bananas". The same results have been found for the processing methods with the exception that the "Bananas" products processed in 1987 are significantly different at a larger alpha level ($p<0.0039$). For the processing years, we do not see significant differences except for the jar "Bananas" product ($p<0.0150$).

We can conclude that the "Vegetable and Beef" products have a significantly higher available lysine content per 100 g of food than the "Bananas" products. We can also conclude that the instant products have a significantly higher available lysine content per 100 g of food than the jar products. Finally, we must conclude that there is no significant difference in available lysine content for the different processing years. However, the analysis shows that the "Bananas" products processed in 1985 tend to be lower in available

Table 6

Mean Available Lysine Content[1] of Selected Baby Foods in mg/100 g Food with Standard Deviations and P-Values

Product Analysis	Vegetable and Beef	Bananas	P-Value
Instant 1987	246.4 ± 9.35	24.7 ± 1.43	0.0001
Instant 1985	244.3 ± 16.32	23.0 ± 1.10	0.0001
Jar 1987	181.9 ± 8.58	18.4 ± 2.40	0.0001
Jar 1985	173.1 ± 9.77	13.7 ± 1.38	0.0001

Process Analysis	Instant	Jar	P-Value
VB 1987	246.4 ± 9.35	181.9 ± 8.58	0.0001
VB 1985	244.3 ± 16.32	173.1 ± 9.77	0.0003
Ba 1987	24.7 ± 1.43	18.4 ± 2.40	0.0039
Ba 1985	23.0 ± 1.10	13.7 ± 1.38	0.0001

Year Analysis	1987	1985	P-Value
VB Instant	246.4 ± 9.35	244.3 ± 16.32	0.8236
VB Jar	181.9 ± 8.56	173.1 ± 9.77	0.2260
Ba Instant	24.7 ± 1.43	23.0 ± 1.10	0.1096
Ba Jar	18.4 ± 2.40	13.7 ± 1.38	0.0150

[1] Based on the analysis of four samples in duplicate

lysine content than products processed in 1987. Jar products processed in 1985 also tend to be lower in available lysine content than the jar products processed in 1987.

It can be seen from Table 3 that the protein content of the instant products is considerably higher than that of the jar products, which may account for differences in available lysine content per 100 g of food. Therefore, the values have been adjusted for protein content. These adjusted values are displayed in Table 7. Adjusting the available lysine content of the baby foods for protein content changes the results considerably.

While all three main effects show significant differences, we also have three significant two-way interactions (Table 5) for the mg lysine per g nitrogen content of the baby food. These interactions are shown graphically in Appendix XI. Graphs 1 and 2 (Appendix XI) show the relationship of the available lysine content between the jar and the instant product for the two product formulations. It can be seen that the difference in available lysine content between the jar and the instant product is larger for the "Bananas" product than it is for the "Vegetable and Beef" product, particularly for the 1987 products. This difference is responsible for the significant interaction between the product formulation and the processing method. As shown in Graphs 3 and 4 (Appendix

Table 7

Mean Available Lysine Content[1] of Selected Baby Foods in mg/g N with Standard Deviations and P-Values

Product Analysis	Vegetable and Beef	Bananas	p-value
Instant 1987	384.8 ± 14.77	237.5 ± 13.63	0.0001
Instant 1985	381.5 ± 25.41	221.3 ± 10.63	0.0001
Jar 1987	437.0 ± 20.69	382.8 ± 49.92	0.0914
Jar 1985	416.3 ± 23.75	286.3 ± 28.63	0.0004
Process Analysis	**Instant**	**Jar**	**p-value**
VB 1987	384.8 ± 14.77	437.0 ± 20.69	0.0063
VB 1985	381.5 ± 25.41	416.3 ± 23.75	0.0927
Ba 1987	237.5 ± 13.63	382.8 ± 49.92	0.0014
Ba 1985	221.3 ± 10.63	286.3 ± 28.63	0.0053
Year Analysis	**1987**	**1985**	**p-value**
VB Instant	384.8 ± 14.77	381.5 ± 25.41	0.8323
VB Jar	437.0 ± 20.69	416.3 ± 23.75	0.2358
Ba Instant	237.5 ± 13.63	221.3 ± 10.63	0.1090
Ba Jar	382.8 ± 49.92	286.3 ± 28.63	0.0153

[1] Based on the analysis of four samples in duplicate

XI) there is a smaller difference in the available lysine content between the jar and the instant products for the 1985 products than for the 1987 products. This indicates that there is a significant interaction between storage effect and processing method. Graphs 5 and 6 (Appendix XI) reveal that the significant interaction found between product formulation and storage effect is due to a smaller difference in the available lysine content of 1987 "Vegetable and Beef" and "Bananas" products than for the 1985 products.

How do these observations correspond with the statistical analysis of the separate main effects? A highly significant difference was found in the available lysine content of the two product formulations, except for 1987 jar products ($p<0.0914$). This is also illustrated in Graph 6 (Appendix XI), where the lines are closest for the 1987 jar products. Similar results were found for the two processing methods. The available lysine content of all instant and jar products was significantly different for all processing methods except for "Vegetable and Beef" processed in 1985 ($p<0.0927$). This is illustrated once again in Graph 4 (Appendix XI) where the two lines for the 1985 "Vegetable and Beef" products are closest together. No significant difference was found in the available lysine content of comparable 1985 and 1987 products, except for the jar "Bananas" product ($p<0.0153$). In fact, the

results of storage effect analysis for available lysine per g N are the same as for available lysine per 100 g food. Only 1985 and 1987 jar "Bananas" products show significant differences in available lysine content; there is also a trend for all 1985 "Bananas" products to be lower in available lysine content than 1987 products. There is also a weaker trend for the 1985 jar products to be lower in available lysine content than products processed in 1987.

Since the total lysine content of each baby food was determined on only one sample which was subsampled in duplicate, it is not possible to subject it to statistical analysis. The results of the total lysine analysis expressed as mg lysine per g nitrogen are presented in Table 8. It is important to note that the total amount of amino acids was assumed to be equal to the protein content. No additional protein analysis was done on these samples. This might be an underestimation of the actual protein content for the following reasons: 1) Some amino acids, such as tryptophan are unstable under acid hydrolysis conditions and will decrease the total amino acid content. 2) Protein is usually assessed by determining the nitrogen content, which includes nitrogen from non-protein sources such as amines. Thus the protein content assessed by the Kjehldal method is probably higher than the total amino acid content assessed by amino acid

Table 8

Total Lysine Content[1] (mg/g N) of Selected Baby Foods

Baby Food	Total Lysine
VB I 1987	363.7
VB I 1985	393.2
VB J 1987	441.3
VB J 1985	438.1
Ba I 1987	440.1
Ba I 1985	453.7
Ba J 1987	.....
Ba J 1985	.....

[1] Based on the analysis of one sample in duplicate

..... No data available

analysis. Therefore, no attempt is made to calculate the difference of total and available lysine and discuss it as available lysine losses. Only a relative comparison between the products is made. It can be seen from Table 8 that the jar "Vegetable and Beef" products are higher in total lysine content than the instant products, a finding similar to that for available lysine. While for the instant "Vegetable and Beef" product the total and available lysine is not much different, the instant "Bananas" have apparently a much lower available than total lysine content. This might be due to extensive Maillard browning in the "Bananas" products which did not ocurr or ocurred to a lesser extent in the "Vegetable and Beef" products. Higher losses of available lysine in the "Bananas" formulations are also consistent with the results of Adewusi and Oke (1984) who found that the content of reducing sugars is important with regard to losses of available lysine in foods. The "Bananas" formulations likely have more reducing sugars than the "Vegetable and Beef" products and hence greater losses of available lysine due to Maillard browning.

Instant products were found to be higher in available lysine per 100 g food than jar products. It should be noted that the higher amounts of available lysine per 100 g food in the instant products are likely due to their higher protein

content. This source of variation in the available lysine of the baby foods is controlled for when the results are expressed in terms of mg available lysine per g nitrogen. Under the assumption that protein composition is similar for the jar and the instant products, it can also be concluded that drum-drying, a method that heats the food to a higher temperature than retorting, is more detrimental in regard to the available lysine content than the canning procedure. This assumption can be safely made for the "Bananas" product because bananas are the only ingredients containing protein in this product. In the case of the "Vegetable and Beef" product, we have a mixture of protein sources. Since these different proteins might contain different amounts of lysine, this conclusion cannot safely be made for the "Vegetable and Beef"products, but reflects only one possible reason.

These results are in agreement with Dexter et. al. (1984) who demonstrated that lysine bioavailability in foods decreases as a function of the processing temperature. In regard to storage, our results are not statistically significant, but 1985 products tend to be lower in available lysine content than the 1987 products. Whereas this trend is questionable for the instant "Vegetable and Beef" product (1985 product is 1% lower than 1987 product), it becomes apparent with the jar "Vegetable and Beef" (5%) and the instant "Bananas" (7%)

products, and becomes significant for the jar "Bananas" product (25%). Horvatic and Grüner (1984) also found a small, 6.9%, loss of available lysine in seven different infant formulas over a 12-month storage period.

We also need to consider the serving sizes when evaluating the available lysine content of the baby foods. For the instant product the serving size depends on how the flakes are reconstituted; while according to labels on the jar products a single serving is 128 g of "Vegetable and Beef" and 134 g of "Bananas". Following the reconstitution steps described on the label for the instant products, a single serving is around 100 g for both formulations. Thus, one serving of the instant "Bananas" supplies an infant with about 25 mg of available lysine, and one serving of instant "Vegetable and Beef" supplies 250 mg of available lysine. Since the serving sizes for the jar products are larger than that of the instant products, the amount of lysine fed to the infant is about the same for the jar products, meaning 25 mg for the "Bananas" and 230 mg for the "Vegetable and Beef" formulations. The RDA for 4-6 months old infants is 99 mg lysine per kg body weight per day. Assuming a body weight of 6 kg, one serving of instant "Vegetable and Beef" will supply 42%, the jar "Vegetable and Beef" 39%, and the "Bananas" products 4.2% of the RDA for this age group. Since the

"Bananas" products are considered desserts or snacks, while the "Vegetable and Beef" products are considered full dinners, these results indicate that these baby foods provide an adequate supply of available lysine to infants.

5.3. Vitamin B-6 Analysis

Vitamin B-6 was determined using a microbiological method. The coefficient of variation for this method is 0.14 and was established by using six identical samples taken in triplicate. The vitamin B-6 content of each baby food was determined by analyzing three instant or jar products, which were subsampled in triplicate. Percent recoveries ranged from 93% to 112% for PN, 61% to 69% for PL and 93% to 98% for PM; vitamer recoveries were similar for all baby foods.

The results of the vitamin B-6 analysis of the individual samples are presented in Appendix XII, and a standard curve used in the analysis is shown in Appendix V. The results of the statistical analysis are displayed in Table 9. According to the R-Square value of 0.986, the statistical model accounts for any significant differences that were found. However, it is again necessary to analyse the results in greater detail, since it is not possible to draw conclusions about main

Table 9

P-Values for the Model Describing Differences in Vitamin B-6 Content of Selected Baby Foods

Source of Variation	P-Values
Product Formulation	0.0001
Processing Method	0.0001
Storage Effect	0.0029
Prod.Form.*Proc. Meth.	0.0001
Prod.Form.*Stor.Effect	0.0457
Proc.Meth.*Stor.Effect	0.0813
Prod.F.*Proc.M.*Stor.E.	0.1759

effects when interactions exist. A more detailed analysis was done as for available lysine. The graphs showing the interactions are presented in Appendix XIII. The results of the statistical analysis on the individual main effects are presented in Table 10.

Again, as for the available lysine analysis we have three significant main effects and three significant two-way interactions (Table 9). From Graph 1 and 2 (Appendix XIII) we can see that the highly significant ($p<0.0001$) interaction between product formulation and processing method is due to the much larger difference in vitamin B-6 content of instant and jar "Bananas" than for the "Vegetable and Beef" products. The slightly larger difference seen in Graph 3 (Appendix XIII) in vitamin B-6 content for the 1987 than the 1985 products indicates the reason for the significant ($p<0.0813$) interaction between processing method and storage effect. Graph 5 and 6 (Appendix XIII) show a somewhat larger difference in the vitamin B-6 content of "Vegetable and Beef" and "Bananas" 1987 products than they do for the 1985 products, which is the reason for the significant ($p<0.0457$) interaction between product formulation and storage effect.

The results of the analysis of the separate main effects (Table 10) show that the jar 1985 product contains significantly more vitamin B-6,alpha level ($p<0.0059$), than the other

Table 10

Mean Vitamin B-6 Content[1] (ug/100g) of Selected Baby Foods with Standard Deviations and P-Values

Product Analysis	Vegetable and Beef	Bananas	p-value
Instant 1987	80.7 ± 6.95	212.8 ± 16.74	0.0002
Instant 1985	74.7 ± 4.07	182.7 ± 12.15	0.0001
Jar 1987	56.2 ± 2.19	79.8 ± 2.65	0.0003
Jar 1985	53.1 ± 4.47	71.7 ± 4.01	0.0059

Process Analysis	Instant	Jar	p-value
VB 1987	80.7 ± 6.95	56.2 ± 2.19	0.0043
VB 1985	74.7 ± 4.07	53.1 ± 4.47	0.0035
Ba 1987	212.8 ± 16.74	79.8 ± 2.65	0.0002
Ba 1985	182.7 ± 12.15	71.7 ± 4.01	0.0001

Year Analysis	1987	1985	p-value
VB Jar	56.2 ± 2.19	53.1 ± 4.47	0.3488
VB Instant	80.7 ± 6.95	74.7 ± 4.07	0.2646
Ba Jar	79.8 ± 2.65	71.7 ± 4.01	0.0436
Ba Instant	212.8 ± 16.74	182.7 ± 12.15	0.0655

[1] Based on the analysis of three samples in triplicate

products. This is illustrated in Graph 6 (Appendix XIII), where the difference in the vitamin B-6 content of "Bananas" and "Vegetable and Beef" products is smallest for the 1985 products. Even though this interaction between product formulation and storage effect is significant on the $p<0.05$ level, considering the p-values (0.0001-0.0003) of the separate analysis, it is negligible, since for all treatment combinations, "Vegetable and Beef" has a lower vitamin B-6 content than "Bananas".

Instant products are significantly higher in vitamin B-6 content than the jar products. The p-values also indicate the reason for the highly significant ($p<0.0001$) interaction between the processing method and the product formulation; the "Bananas" products are significantly different on a lower alpha level ($p<0.0001$ and 0.0002) than the "Vegetable and Beef" products ($p<0.0043$ and 0.0035). Finally, there is no significant difference in the vitamin B-6 content of 1985 and 1987 products, except for jar "Bananas" ($p<0.0436$). However, 1985 "Bananas" products in general tend to be lower in vitamin B-6 content. It should be mentioned at this point that storage effect analysis for the available lysine content of the baby foods showed very similar results.

It is difficult in the case of vitamin B-6 to make a statement about the effect of the two processing methods

because it is not possible to relate it to a relative term. In fact, it is not possible to separate a processing effect from a "dilution" effect caused by the addition of various ingredients with little or no vitamin B-6 in both the jar "Bananas" and the jar "Vegetable and Beef" product. Thus we cannot directly determine whether differences in the vitamin B-6 content of instant and the jar products are due to processing or due to differences in formulation of the instant and jar products. In addition, the label of the jar "Bananas" product specifies "fully ripened bananas". Hardin et al. (1989) reported that the vitamin B-6 content of bananas increases during the ripening process. Thus, if fully ripened bananas were used for jar but not for instant products, a difference in vitamin B-6 content will be introduced. Thus, the only statement that can be made is that the instant products are higher in vitamin B-6 content, but the reasons are uncertain.

The vitamin B-6 content of fresh bananas is reported to be between 0.37 and 0.51 mg per 100g (Souci et al. 1986, Hardin et al. 1989; Orr 1969); thus a B-6 content of approximately 0.2 mg/100 g food for instant "Bananas" indicates that there is some vitamin B-6 degradation due to drum-drying. However, it is not possible to make a statement about the percent loss of vitamin B-6 due to processing because the

vitamin B-6 content of the raw material is not known. It is also not possible to make the same statement for the "Vegetable and Beef" product or the jar "Bananas", because of the addition of various food ingredients. However, the vitamin B-6 content of the ingredients for the "Vegetable and Beef" products are reported in the literature, and some inferences can be made using these values. The vitamin B-6 content of canned carrots is reported to be between 0.022 (Souci et al. 1986) and 0.03 mg/100g food (Orr 1969), canned tomatoes contain 0.09 mg/100g, canned peas contain 0.02 mg/100g and beef (strained baby food) contains 0.2 mg/100g of vitamin B-6 (Orr 1969). Thus, our vitamin B-6 results appear to be within reported ranges for similar food products.

The RDA for infants 0-0.5 year of age is 0.3 mg of vitamin B-6 per day (Food and Nutrition Board 1984). Once again, we need to consider the serving sizes of the products, which are 100 g for instant formulations and 128 and 134 g for the jar "Vegetable and Beef" and "Bananas", respectively. Thus, a serving of instant "Bananas" supplies approximately 70%, jar "Bananas" and instant "Vegetable and Beef" approximately 26% and jar "Vegetable and Beef" approximately 18% of the RDA for this age group. While this is satisfactory for the "Bananas" products which can be considered a snack or

dessert, it must be considered somewhat inadequate for the "Vegetable and Beef" dinner products.

5.4. Pyridoxyllysine Analysis

As it has been outlined in Chapter 3.3.4. on pyridoxyllysine methodology, several attempts were made to directly determine pyridoxyllysine content of the baby foods. Whereas pyridoxyllysine was always detectable in spiked samples, it was not possible to detect it in the baby food when 1, 2, 4 and 20 g samples were used (Figure 9 and 10). The pyridoxyllysine peak of the spike was well resolved in HPLC chromatograms for the small sample sizes (Figure 11), whereas in the 20 g sample the spike was visible in the form of a shoulder in the decay curve of interfering peaks (Figure 12). Several theories can be suggested to help explain why pyridoxyllysine was not detectable in the baby food. The most likely reason is that pyridoxyllysine is present in quantities that are below the detection limits of the methods employed in this study. Gregory and Kirk (1978b) reported that 50% of pyridoxalphosphate could be lost in the form of bound pyridoxyllysine after thermal processing and storage. In an earlier study, only 10% of the total PLP was found to be bound in a

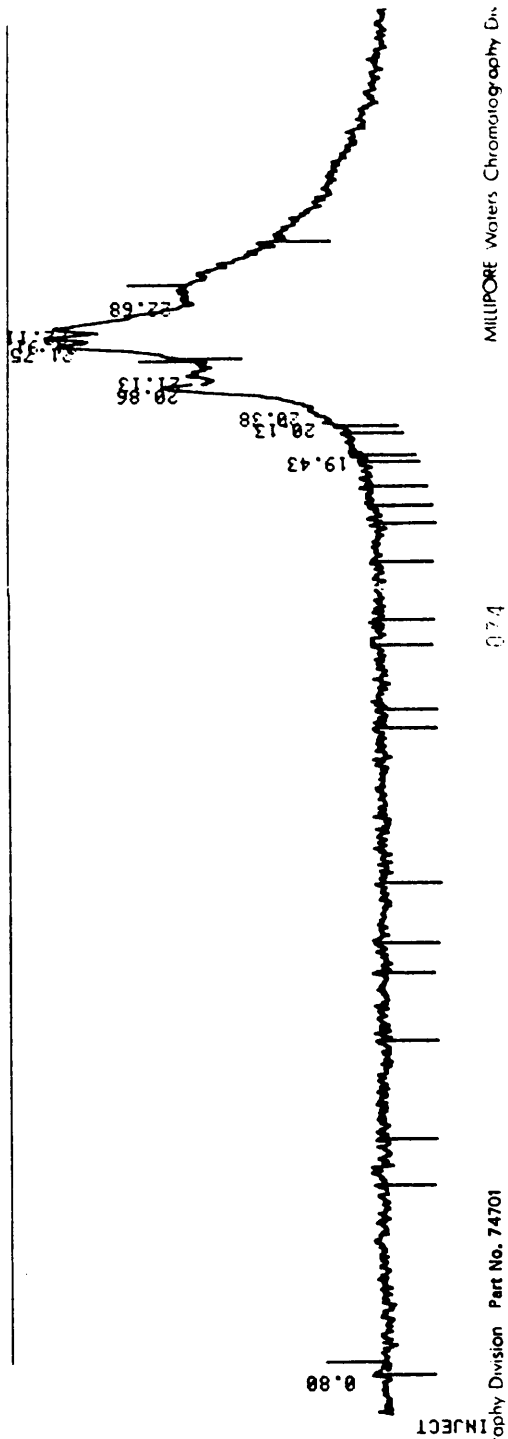

Figure 9: HPLC Chromatogram of Instant "Vegetable and Beef" Product (Sample Size 2g) Without Pyridoxyllysine Spike

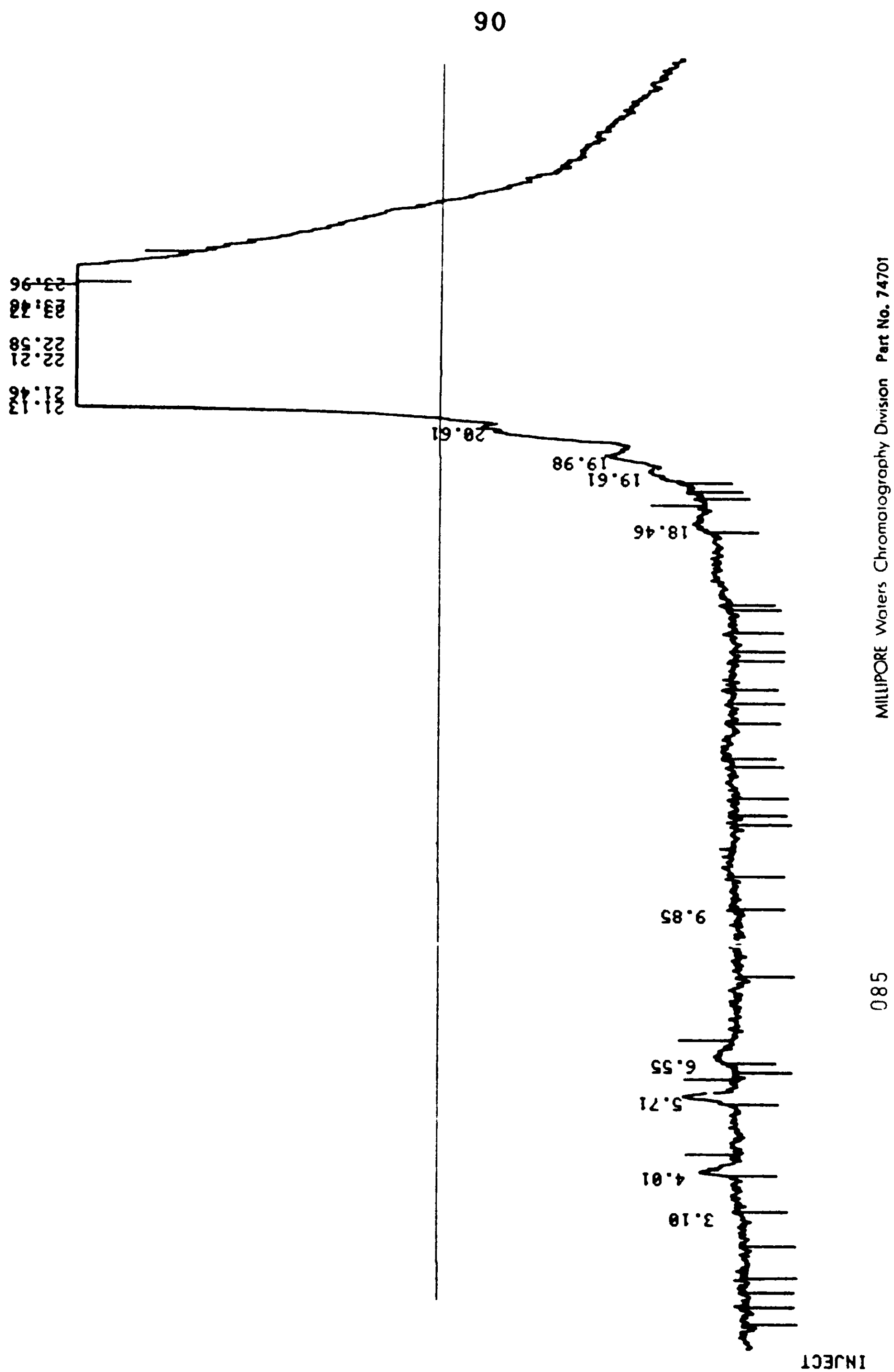

Figure 10: HPLC Chromatogram of Instant "Vegetable and Beef" Product (Sample Size 20 g) Without Pyridoxyllysine Spike

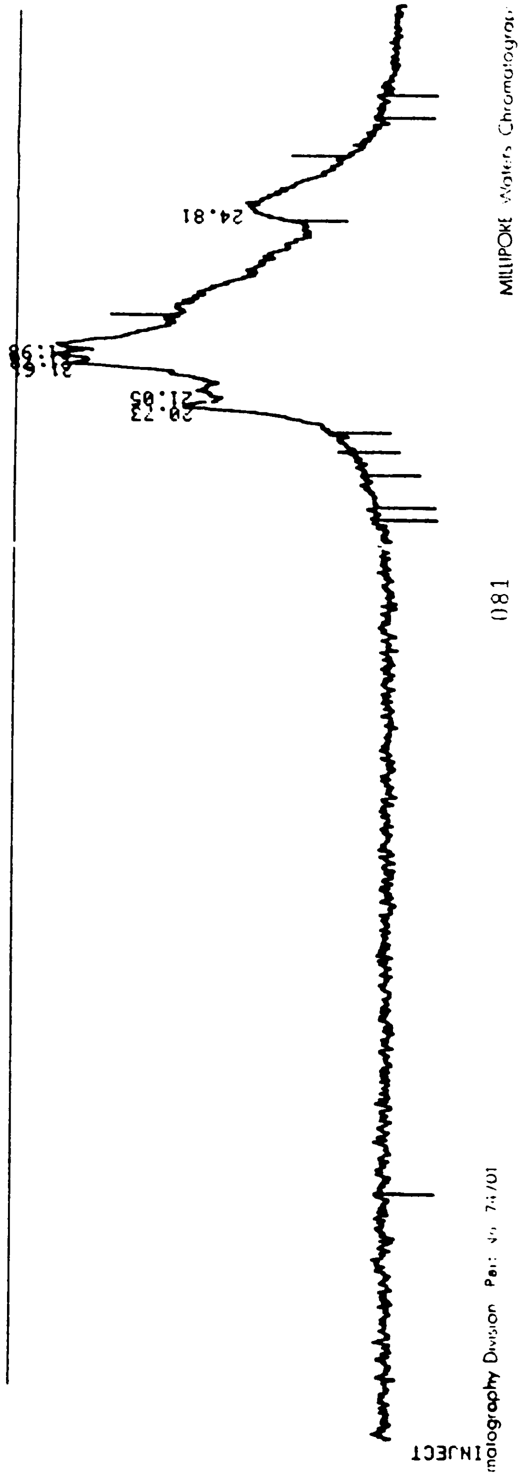

Figure 11: HPLC Chromatogram of Instant "Vegetable and Beef" Product (Sample Size 2g) With 0.5 ug/ml Pyridoxyllysine Spike

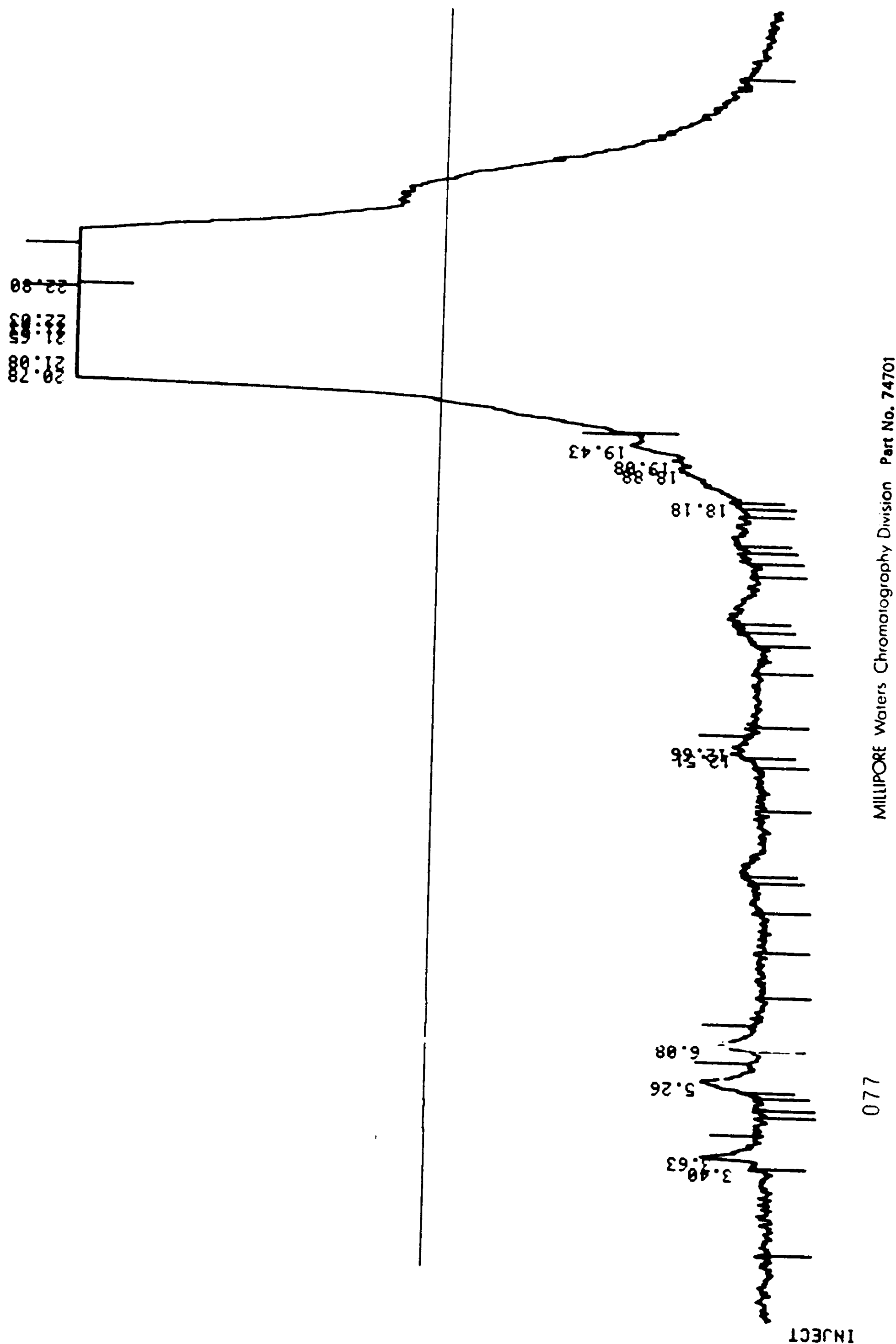

Figure 12: HPLC Chromatogram of Instant "Vegetable and Beef" Product (Sample Size 20g) With 5 ug/ml Pyridoxyllysine Spike

non-reducible form, considered to be pyridoxyllysine (Gregory and Kirk 1977). In a more recent study on pyridoxyllysine in food systems, Gregory et al. (1986) used model food systems with a vitamin B-6 content comparable to endogenous levels found in foods, and detected up to 25% of PLP/PL bound in the form of pyridoxyllysine. In our case, pyridoxalphosphate is the lowest of the three major B-6 vitamers (PN, PMP, PLP) found in foods, and is probably not higher than 10% of the total B-6 in the "Bananas" products and 30% in the "Vegetable and Beef" (Average percentage of total vitamin B-6 contributed by PL: Carrots 20%, Tomatoes 30%, Beef 40%, and Bananas 10% (Orr 1969)). Thus, if the 50% figure reported by Gregory et al. (1978b) were applicable to our baby foods, we could expect about 12 ug pyridoxyllysine per 100g food for instant "Vegetable and Beef", 10 ug/100g for instant "Bananas", 9 ug/100g for jar "Vegetable and Beef" and 4 ug/ 100g for jar "Bananas". In our trials we used 1g, 2g, 4g and 20g samples which were extracted into 2 ml of solvent. A 1g sample of instant "Vegetable and Beef" would result in 60 ng pyridoxyllysine per ml extract. Since the lowest detectable limit is 125 ng/ml and the lowest quantifiable limit is 167 ng/ml (for HPLC chromatogram of pyridoxyllysine standards see Appendix VII), a 2g sample should have contained detectable amounts and a 4g sample quantifiable amounts of pyridoxyllysine if the 50%

figure were applicable. A 20g sample should contain up to 1200 ng/ml of pyridoxyllysine. However, a 1200 ng/ml peak might actually be more difficult to detect in this case because of simultaneous increase in the area of interfering peaks when using a 20 g sample. Although these calculations do not provide conclusive evidence that pyridoxyllysine is below detection limits in the baby food, it does suggest that PLP/PL have not interacted with lysine to form pyridoxyllysine to the extent reported by Gregory and Kirk in 1978 (1978b) (50%), but perhaps rather to the extent reported in 1977 (10%) or in 1986 (10-25%) (Gregory and Kirk 1977, Gregory et al. 1986). This is conceivable because Gregory and Kirk (1977) also noted that their data suggested that glucose-induced browning may retard pyridoxyllysine formation, and the baby food is relatively high in carbohydrates, especially the "Bananas" products. In addition, it is important to consider that the food systems in which pyridoxyllysine was found were model systems that were high in protein and fortified with vitamin B-6, and are not necessarily representative of complex food systems such as the baby food. However, it should also be noted that the thermal processing conditions for these model food systems were similar to the retort conditions used for the jar products.

Pyridoxyllysine peaks observed in the spiked foods were

evaluated for hidden pyridoxyllysine by comparing them to peak heights of pyridoxyllysine standards. The idea was that if every pyridoxyllysine spike in the foods turned out to give a higher peak than expected, one might conclude that this additional peak height is caused by pyridoxyllysine originally present in the food. However, the peak heights of the spiked food samples were the same as the standards.

Pyridoxyllysine might also not be detected if it is not completely released from food proteins, but remains bound to small peptides. However, this possibility is not very likely because 48 hour acid hydrolysis should have cleaved all peptide bonds and released any pyridoxyllysine originally part of proteins.

A third possibility worth considering is that pyridoxyllysine forms a complex with some other food compounds after being released from the protein. If so, then the question arises, why should the food-contained pyridoxyllysine undergo this complex formation and not the spiked pyridoxyllysine. The recovery of the spikes was always between 95 and 100%.

A fourth consideration is the stability of pyridoxyllysine. Although initially, Gregory and Kirk (1978c) reported good stability of pyridoxyllysine under acid hydrolysis conditions, they later reported a 20-25% destruction of pyridoxyl-

lysine (Gregory et al. 1986). Preliminary studies by the author did not detect any considerable pyridoxyllysine destruction under acid hydrolysis conditions which is in agreement with Polyanovskii's (1963) findings who did not detect any significant destruction after 72 hours acid hydrolysis.

Pyridoxyllysine analysis using an amino acid analyzer, resulted in a well resolved peak for the 2.5 umol/ml (797.5 ug/ml) pyridoxyllysine standard (Appendix VI), but no pyridoxyllysine could be detected in the baby food.

Gregory (1980c) stated that small concentrations of pyridoxyllysine could be deleterious to vitamin B-6 when the diet is otherwise deficient in vitamin B-6. Since we were not able to detect pyridoxyllysine in the food by either method, we can conclude that most probably pyridoxyllysine content of the selected baby foods is below 0.25 ug per gram food. At levels of 0.5 ug PN equivalents per g food which is 1 ug/g pyridoxyllysine if 50% B-6 activity of pyridoxyllysine is considered correct, deficiency signs developed more rapidly in rats than when fed a totally B-6-deficient diet (Gregory 1980c). Gregory (1980c) also reported that the deficiency signs were readily overcome by the presence of 0.5 ug PN/g diet. While the foods under study are most probably below the pyridoxyllysine level reported to aggravate deficiency symptoms in rats, they do contain high enough levels of

vitamin B-6 to prevent B-6 deficiency. Thus, there is little or no reason for concern that any pyridoxyllysine possibly present in the instant or jar baby foods might impair vitamin B-6 status if fed to infants.

CHAPTER 6

6. Summary and Conclusions

We would like to consider the results of this study from the viewpoint of Stevenson's (1987) remarks about Karl Popper: "The distinguished philosopher of science Karl Popper, put the emphasis on falsification rather than verification here, holding that the essence of scientific method is that theories are hypotheses, which can never be known for certain but which are deliberately put to the test of observation and experiment, and revised or rejected if their predictions get falsified."

However, it is important that the results of any scientific study are always considered under certain restrictions that pertain to the study. In this case, it was not possible to control for differences in nutrient content brought about by different raw materials. Thus, the differences found between the different products are not necessarily due exclusively to the considered main effects, i.e. product formulation, processing method and storage effect, but include differences due to varying raw materials. Therefore, the differences found between the products are summarized without attempting to draw conclusions that might be incorrect due to variation in nutrient content of raw materials.

The major findings of this study are as follows: "Vegetable and Beef" products are significantly higher in available lysine content but significantly lower in vitamin B-6 content than the "Bananas" products. When expressed on a milligram lysine per 100 gram food basis the available lysine content of instant products is higher than jar products. However, when adjusted for protein content, the jar products are higher in available lysine, which indicates that drum-drying is more detrimental to lysine bioavailability than canning, assuming no difference in the lysine content of the protein sources. In regard to the vitamin B-6 content, the instant products were found to contain more vitamin B-6 than the jar products. However, it is not possible to clearly attribute this finding to differences in the processing methods, because the instant and the jar products even of the same product formulation contain different ingredients. The third major finding is that the products processed in 1985 tend to be lower (although not significantly) in nutrient content than the products processed in 1987. This trend is more apparent for the jar products and for the "Bananas" products than it is for the instant or the "Vegetable and Beef" products, which might be an indication of better nutrient stability for instant and "Vegetable and Beef" products.

It was also estimated that on a per serving basis the instant products supply an infant with about the same amount of lysine, and more vitamin B-6 than jar products. Thus, we must conclude that with regard to available lysine and vitamin B-6 the new instant product is at least equal to the jar product. Here, it ought to be considered, that exact steps followed in the reconstitution of the instant product are the prerogative of the mother. Thus, if she does not follow the instructions on the label, but her own judgement, she might concentrate or dilute the product. After having worked with the product, we feel that a mother will more likely concentrate the instant product rather than dilute it; because when rehydrated according to the label, the product appears thinner than the jar product.

Although the "Vegetable and Beef" products seem to be somewhat low in vitamin B-6 content, the selected baby foods, when part of a complete diet, appear to supply sufficient amounts of the nutrients under study. Since pyridoxyllysine could not be detected in the baby foods at the present time, we have to conclude that it does not impair the quality of the baby foods with regard to vitamin B-6 bioavailability.

CHAPTER 7

7. Suggestions for Further Research

An area of current research interest is the quantification of vitamin B-6 derivatives in foods, which ought to include pyridoxyllysine. This requires a refinement of present methods for determining pyridoxyllysine. It appears that for complex food systems the current pyridoxyllysine extraction procedure needs to be improved. Sample clean up and separation using a variety of chromatographic methods ought to be tried. It is desirable that eventually separation and quantification of the six B-6 vitamers and several vitamin B-6 derivatives in foods can be done using a single procedure.

The bioavailability of vitamin B-6 in pyridoxyllysine has been determined for rats by Gregory (1979 and 1980a), but its bioavailability for humans is still unclear. More research is needed to assess bioavailability of vitamin B-6 derivatives, including pyridoxyllysine for the human.

No studies have been conducted to determine the bioavailability of lysine bound in the form of pyridoxyllysine. Lysine derivatives are classified in three categories, depending on their biological availability (Finot et al. 1980/81). Studies have shown that most of the derivatives belong to the partly available lysine group (Finot et al.

1978, Boggs 1978). It might be possible to assume that the bioavailability of lysine from pyridoxyllysine will be similar to that of pyridoxalphosphate, because the enzyme pyridoxamine-phosphate oxidase that releases pyridoxalphosphate and thus lysine from phosphopyridoxyllysine can be found in various tissues such as liver and brain (Gregory 1980b). Liver is one of the major organs responsible for releasing free lysine from its derivatives (Finot et al. 1980/81). Nevertheless, the bioavailability of lysine from pyridoxyllysine might be considered another area of research, even though the quantities of lysine involved are rather small when compared to more predominant chemical reactions such as Maillard browning.

CHAPTER 8

8. References

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APPENDICES

APPENDIX I

List of Baby Foods Ingredients (as read on the label)

Bananas, Instant:

Dehydrated Bananas, Vitamin C;

Vegetables and Beef, Instant:

Tomatoes, Beef, Carrots, Peas, Rice Flour, Celery, Onions, Dehydrated Potatoes, Oat Flour, Chicken Broth, Soy Protein Isolate, Partially Hydrogenated Vegetable Oil (Cottonseed, Soybean), Thyme, Sage, Celery Seed;

Bananas, Jar:

Water necessary for preparation, Fully-ripened Bananas, Corn Syrup (Nutritive Sweetner), Modified Tapioca Starch, Food Starch-Modified (from Corn), Concentrated Orange Juice, Citric Acid, Vitamin C;

Vegetables and Beef, Jar:

Water, Carrots, Beef, Food Starch-Modified (from Corn), Tomatoes, Oat Flour, Defatted Soy Flour, Dehydrated Potatoes, Celery, Yeast, Onion Powder;

APPENDIX II

List of Vendors

Aldrich Chemicals Company, Milwaukee, WI:
* N-epsilon-Acetyllysine
* Sodiumborohydride

American Type Culture Collection, Rockville, MD
* Saccharomyces Uvarum (ATCC 9080)

Bausch & Lomb, Rochester, NY
* Spectrophotometers Spectronic 2000 and Spectronic 20

BioRad Laboratories, Richmond, CA
* BioRex 70 Cation Exchange Resin, Sodium Form

Difco Laboratories, Detroit, MI
* Bacto YM Agar
* Pyridoxine Y Media

Fisher Scientific Company, Fair Lawn, NJ
* Acetic acid, glacial
* Acetone
* Ammoniumformate
* Ammoniumhydroxide
* Ethanol
* Ethylether
* Formic acid, 88%
* HPLC-grade acetic acid, glacial
* HPLC-grade methanol
* HPLC-grade water
* Hydrochloric acid, conc.
* Hydrogenperoxide, 30%
* Octane-2-ol
* Potassiumhydroxide
* Potassiumpermanganate
* Potassiumphosphate
* Sodiumhydrogencarbonate
* Sodiumhydroxide, 50%
* Sulfuric acid, conc.
* Trichloroacetic acid, 100%

Gelman Sciences, Ann Arbor, MI
* Acrodisc, 2 um

ICN Biochemicals, Cleveland, OH
* Epsilon-Trinitrophenyl-L-lysine monohydrochloride

Meer Corporation, North Bergen, NJ
* Agar agar

Pharmacia Fine Chemicals, Piscataway, NJ
* Sephadex G-10 Resin

Sigma Chemical Company, St. Louis, MO
* Alpha Amino Butyric Acid
* Alpha-Amylase
* Amyloglucosidase
* Methanol
* Pyridoxalhydrochloride
* Pyridoxinehydrochloride
* 2,4,6 Trinitrobenzenesulfonic acid

Spectrum Medical Industries, Inc., Los Angeles, CA
* Dialysis Bags # 132655

Waters Associates, Inc., Milford, MA
* Column Temperature Control System
* Diluent for Amino Acid Analyzer "PICO*TAG"
* Eluent A and B for Amino Acid Analyzer "PICO*TAG"
* Maxima 820 Software
* Micro-Bondapak Octadecylsilane Column (30 cm * 3.9 mm I.D., 10 um porous packing)
* Model 730 Data Module
* Model 720 System Controller
* Model U6K Universal Injector
* Model 45 Solvent Delivery System (2)
* Model 510 Solvent Delivery System (2)
* Model 420 E/AC Fluorescence Detector (Mercury Lamp)
* Model 441 Absorbance Detector
* NEC APC IV Personal Computer
* Pic B7 & B8
* PICO*TAG Column (15 cm * 3.9 mm I.D.)
* Sep-Pak C_{18}
* System Interface Module
* WISP Model 712

Whatman Ltd., England
* Whatman Filter Paper

APPENDIX III

Reagent preparation

Water refers to distilled, deionized water, unless otherwise specified.

Agar Solution (Available Lysine)
Add 1 g of agar to 900 ml of water, heat mixture to boiling, allow to cool, make volume up to 1 l. Preserve the solution by shaking it with 2 ml of octane-2-ol.

Sodiumhydrogencarbonate, 1 M (Available Lysine)
Dissolve 84.01 g in 900 ml water, make volume up to 100 l

TNBS solution (Available Lysine)
Dissolve 1 g of TNBS in 90 ml water, make volume up to 100 ml (make fresh for each determination)

Potassiumpermanganate, 4% (Available Lysine)
Dissolve 4 g potassiumpermanganate in 90 ml water, make up to 100ml

Hydrogenperoxide, 3% (Available Lysine)
To 10 ml hydrogenperoxide, 30% add 90 ml water

YM Agar (Vitamin B-6)
Dissolve 41 g Bacto YM agar in 1 l of water, place 5 ml in each screw top test tube, boil while shaking constantly, autoclave for 15 minutes at 121°C and 15 psi, cool in slanted position at room temperature, store at 5°C.

Pyridoxine Y Media for Inoculum (Vitamin B-6)
Dissolve 0.54 g pyridoxine Y media in 20 ml water (make fresh for each assay)

Saline Solution (Vitamin B-6)
Dissolve 0.9 g NaCl in 100 ml water, pour 10 ml into a test tube, cap, and autoclave 15 minutes at 121°C and 15 psi.

HCl, 0.44 M (Vitamin B-6)
37.8 ml concentrated HCl (appr. 12.1 M) brought to 1 l volume with water

KOH, 6 M (Vitamin B-6)
Dissolve 138 g KOH in 400 ml water, cool down, make up volume to 500 ml.

Pyridoxine Y Media for Sample Assay (Vitamin B-6)
To prepare 100 test tubes, dissolve 20 g pyridoxine y media in 500 ml water, stir to dissolve (make fresh for each assay)

Stock standard (Vitamin B-6)
12.06 mg PN HCl in 100 ml 25% ethanol, store in dark bottle at 5°C.

Working standard (Vitamin B-6)
1 ml of the working standard was diluted to 100 ml, 1 ml of this solution was again diluted to 100 ml, of which 5 ml were diluted to 25 ml. 0.25, 0.5, 1, and 2 ml produced 0.5, 1, 2, and 4 ng pyridoxine per tube (10 ml)

Spiking standards (Vitamin B-6)
12.18 mg PL HCl in 100 ml 25% ethanol and 14.34 ml PM diHCl in 100 ml ethanol. Store in dark bottles at 5°C.

Trichloroacetic acid, 50% (Pyridoxyllysine)
Mix 50 ml water with 50 ml 100% TCA

Potassium Phosphate Buffer, 0.1 M, pH 7 (Pyridoxyllysine)
Combine 390 ml 0.1 M monobasic potassiumphosphate (13.61 g in 1 l HPLC-grade water) and 610 ml dibasic potassiumphosphate (17.42 g in 1 l HPLC-grade water)

HCl, 6 M (Pyridoxyllysine, Total Lysine)
Combine 50 ml concentrated HCl (appr. 12.1 M) with 50 ml HPLC-grade water

Ammonium Formate, pH 4.0 (Pyridoxyllysine)
Dissolve 63.06 g ammoniumformate in 900 ml HPLC-grade water, adjust pH to 4.0 with formic acid, 88%, make up to 1 l volume with HPLC-grade water

Acetic acid, 1 M (Pyridoxyllysine)
Combine 57.5 ml HPLC grade acetic acid, glacial with 942.5 ml HPLC grade water

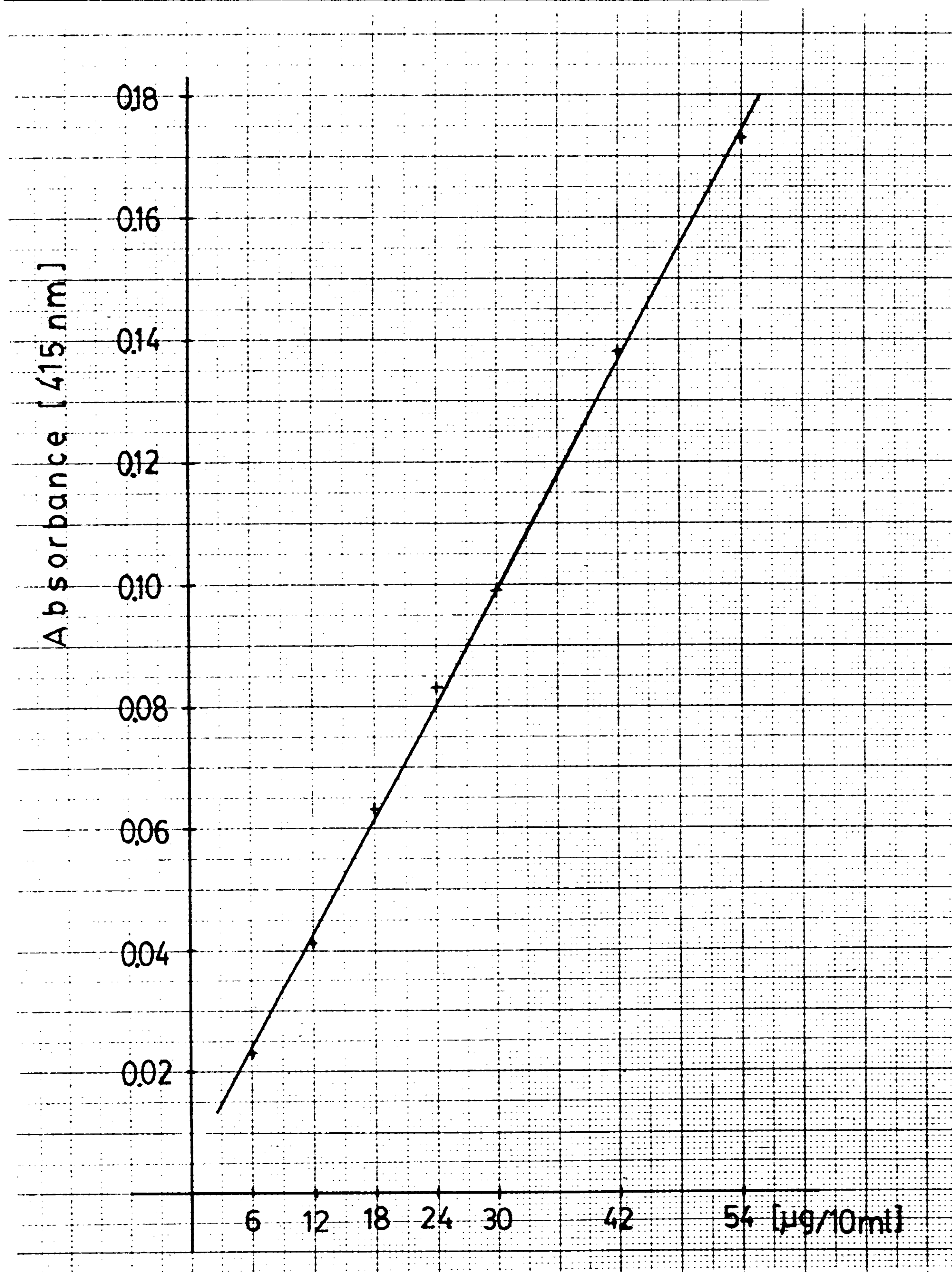
Derivatisation Reagent (Total Lysine)
Combine 490 ul methanol (HPLC grade),70 ul triethylamine, 70 ul water and 70 ul phenylisothiocyanate; make up fresh each time.

Alpha amino-n-butyric acid (AABA) 5 um/ml (Total Lysine)
Weigh 0.2578 g AABA into a 500 ul flask and dilute to volume with 0.1 M HCL

Redry (Total Lysine)
Combine 800 ul methanol (HPLC grade), 800 ul water and 400 ul triethylamine

APPENDIX IV

Standard Curve for Available Lysine Determination



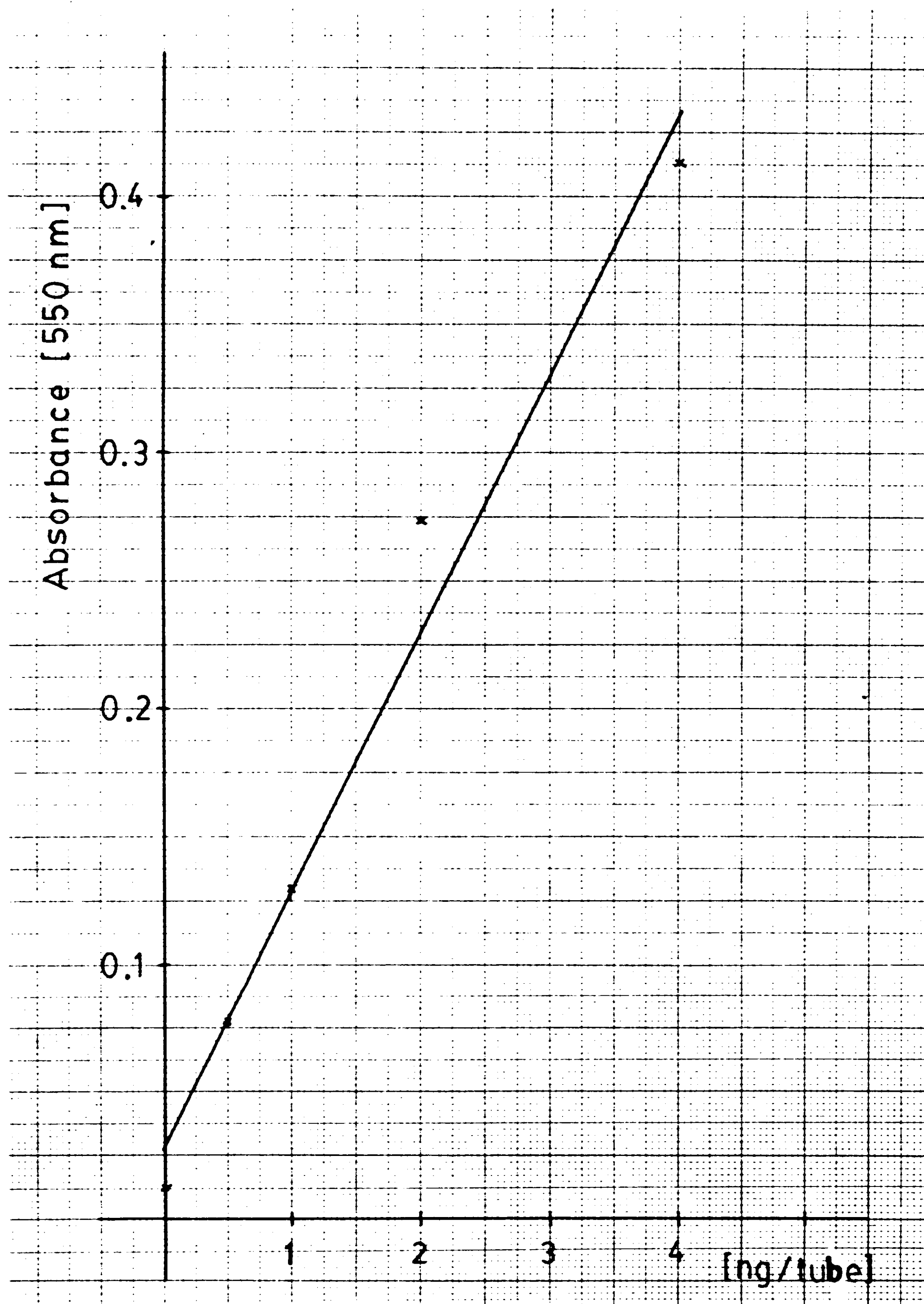
The values of the standard curve are displayed in the following table, each value is the average of two readings.

Concentration (ug/10 ml)	Absorbance (415 nm)
6	0.023
12	0.041
18	0.063
24	0.083
30	0.099
42	0.138
54	0.173

The correlation coefficient of this standard curve is 0.9995.

APPENDIX V

Standard Curve for Vitamin B-6 Determination



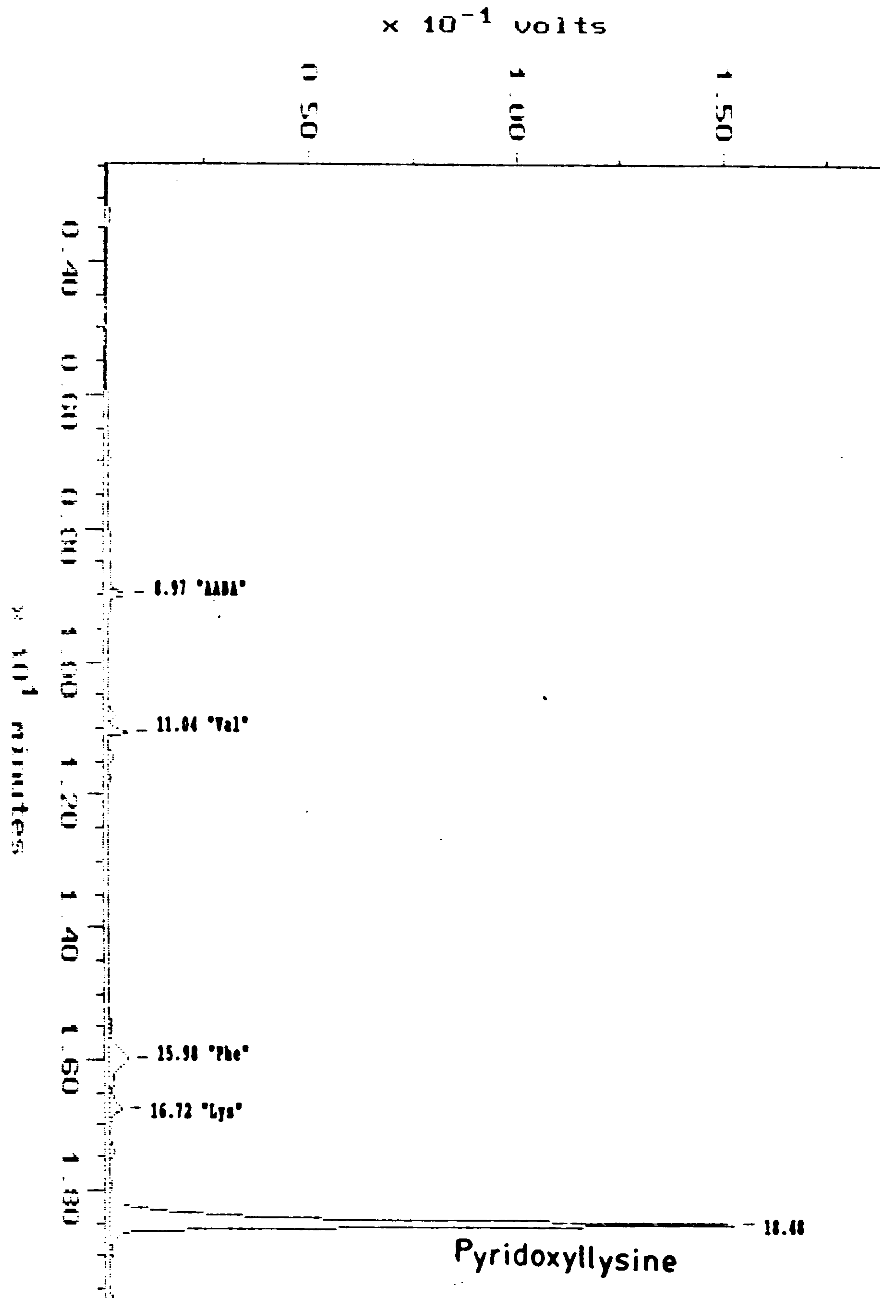
The values of the standard curve are displayed in the following table, each value is the average of three readings.

Concentration (ng/tube)	Absorbance (550 nm)
0	0.013
0.5	0.077
1.0	0.130
2.0	0.273
4.0	0.413

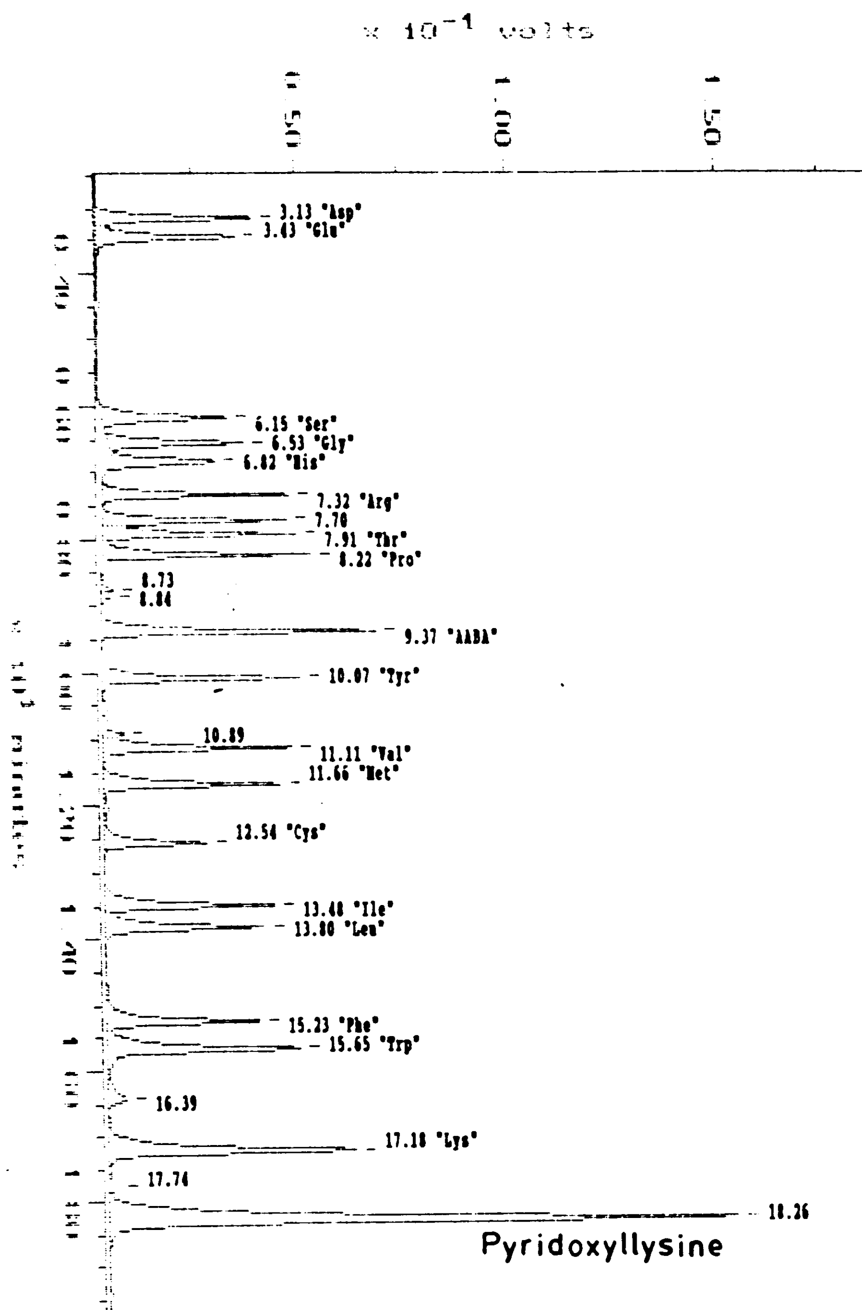
The correlation coefficient of this standard curve is 0.987.

APPENDIX VI

Pyridoxyllysine standard detected by amino acid analysis

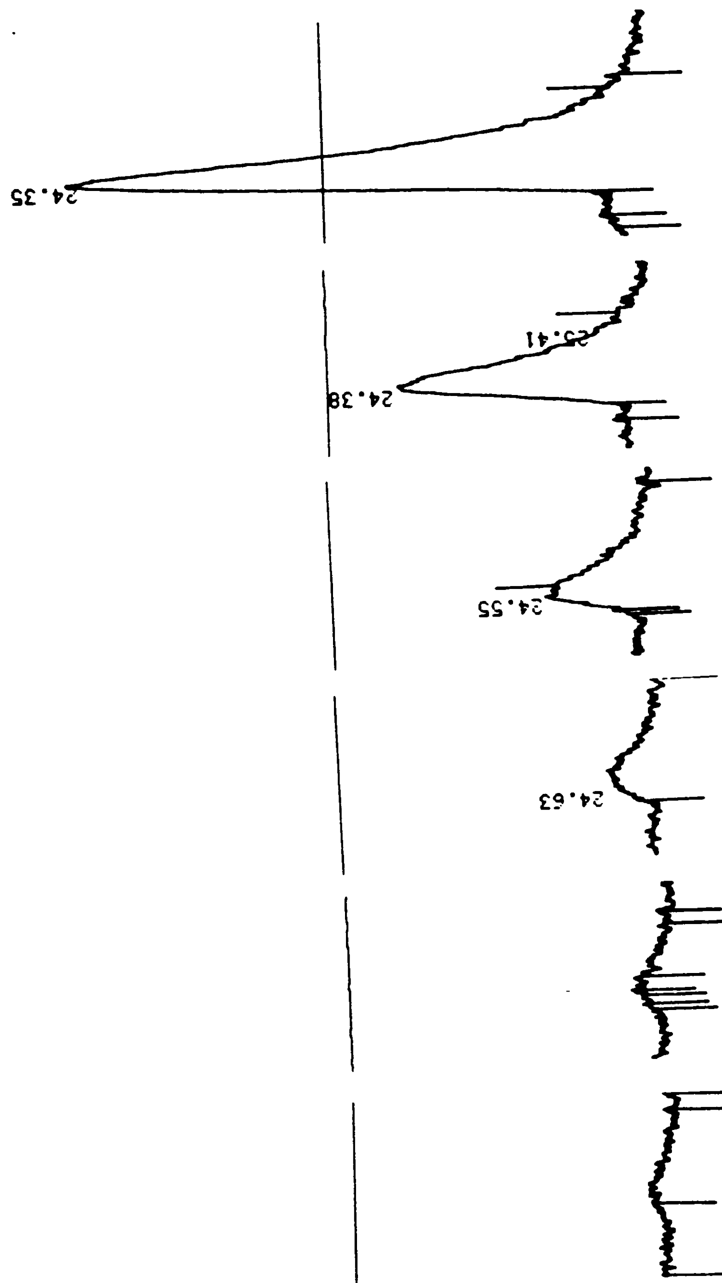


Pyridoxyllysine standard separated from other amino acids by amino acid analyzer



APPENDIX VII

Pyridoxyllysine Standard Curve for HPLC (25 ul of 125 ng/ml, 167 ng/ml, 250 ng/ml, 500 ng/ml, 1000 ng/ml, 2000 ng/ml)



APPENDIX VIII

Proximate Analysis Values

The table shows the individually determined values expressed on a percentage basis for the proximate analysis. Each product was sampled twice with the samples coming from two different jars.

Product	Moisture	Ash	Fat[1]	NDF[2]	Protein	CHO[3]
BaJ87	82.6	0.19	0.47	3.9	0.33	16.41
BaJ87	82.9	0.19	0.37	3.9	0.31	16.29
BaJ85	83.0	0.17	0.49	3.7	0.32	16.02
BaJ85	82.8	0.20	0.39	3.7	0.29	16.32
BaI87	84.9	0.69	0.22	3.1	0.72	13.47
BaI87	84.9	0.71	0.21	3.1	0.66	13.52
BaI85	84.9	0.63	0.22	...	0.62	13.63
BaI85	85.0	0.76	0.16	...	0.59	13.49
VBJ87	87.4	0.30	0.85	2.6	2.62	8.83
VBJ87	87.4	0.32	0.76	2.6	2.50	9.02
VBJ85	88.6	0.28	0.67	2.3	2.68	7.77
VBJ85	88.5	0.26	0.41	2.3	2.63	8.20
VBI87	88.8	0.31	1.58	4.3	3.74	5.57
VBI87	88.8	0.29	1.97	4.3	3.95	4.99
VBI85	88.8	0.24	2.08	...	4.12	4.76
VBI85	88.8	0.32	2.18	...	4.07	4.63

[1] Each value represents the average of two determinations

[2] Neutral Detergent Fiber of each product was determined only once

[3] Nitrogen-free extract $\cong$ carbohydrate determined by difference

... No data available

APPENDIX IX

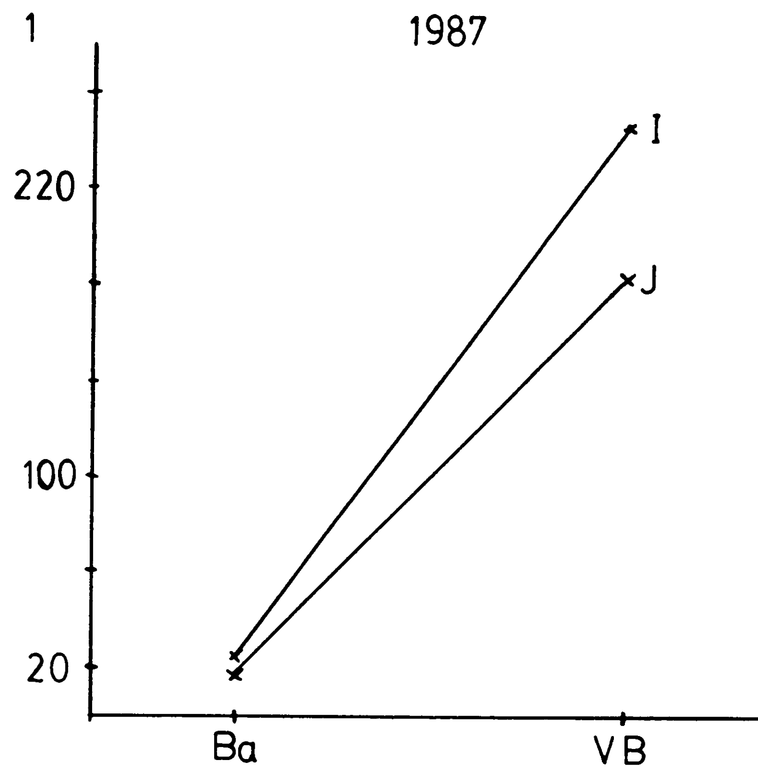
Available Lysine Values

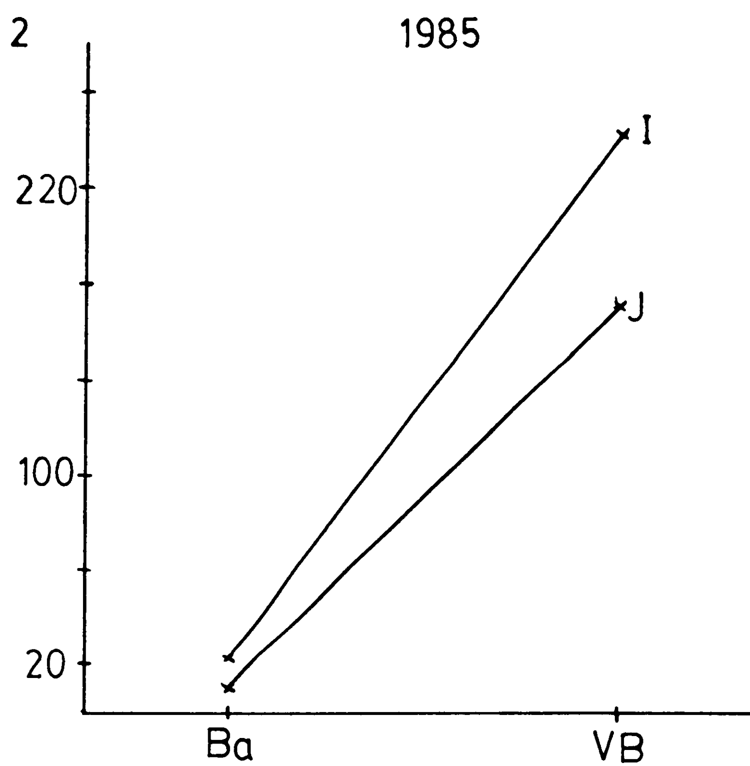
The table shows the individually determined values for available lysine. Each product was sampled four times.

Product	mg Lysine/100 g food	mg Lys/g Nitrogen
VBJ85	165.7	398
VBJ85	164.3	395
VBJ85	184.5	444
VBJ85	177.9	428
VBJ87	184.1	442
VBJ87	173.9	418
VBJ87	176.5	424
VBJ87	193.0	464
VBI85	227.5	355
VBI85	253.0	395
VBI85	234.0	366
VBI85	262.6	410
VBI87	237.7	371
VBI87	239.5	374
VBI87	251.4	392
VBI87	257.1	402
BaJ85	15.3	319
BaJ85	12.4	258
BaJ85	12.8	267
BaJ85	14.4	301
BaJ87	20.4	426
BaJ87	18.5	386
BaJ87	15.0	312
BaJ87	19.6	407
BaI85	23.8	229
BaI85	23.8	228
BaI85	21.5	206
BaI85	23.1	222
BaI87	23.3	224
BaI87	25.3	243
BaI87	23.8	229
BaI87	26.5	254

APPENDIX X

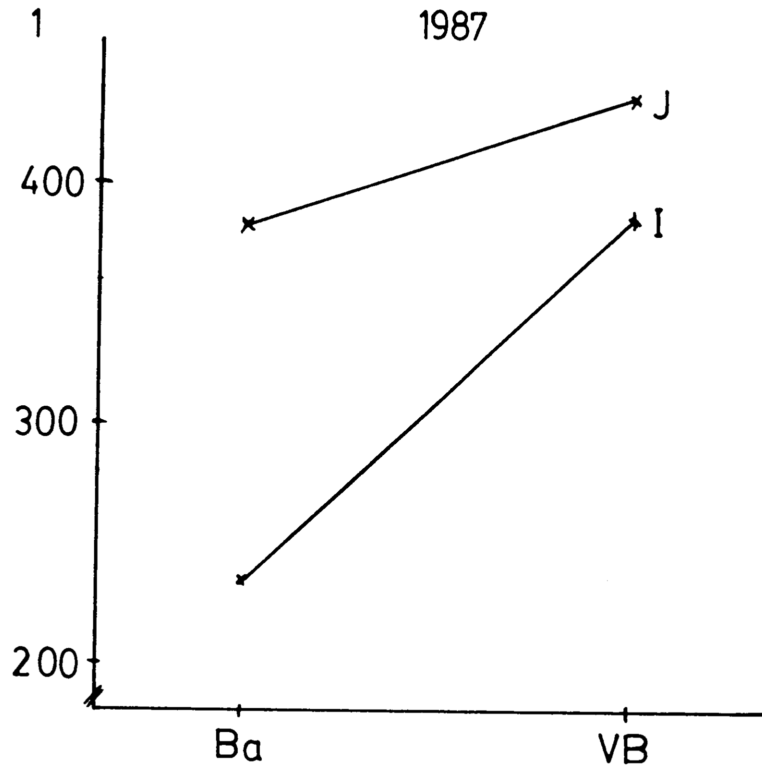
Graphs illustrating the interactions found for the available lysine analysis (mg lysine / 100 g food)

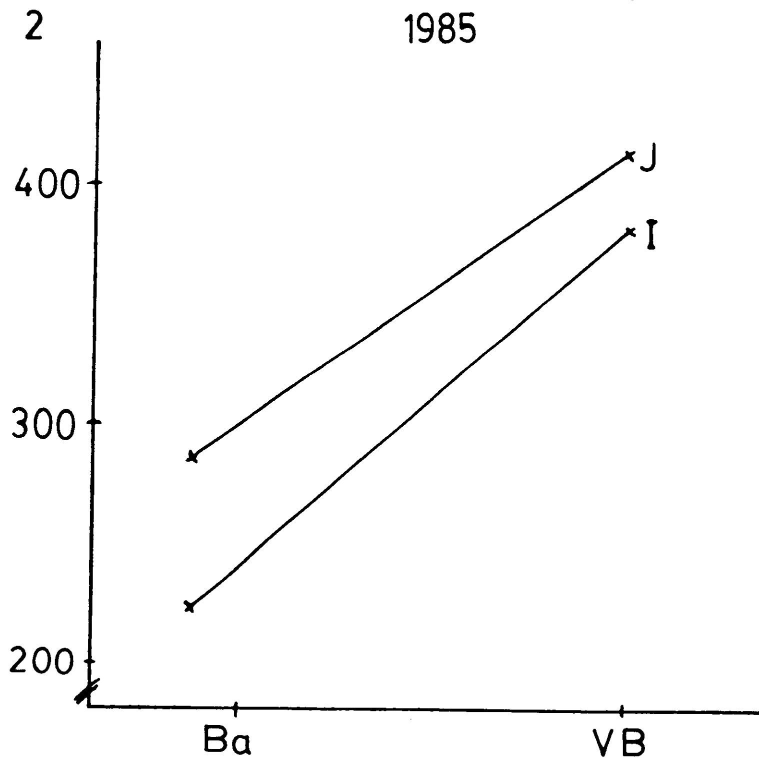


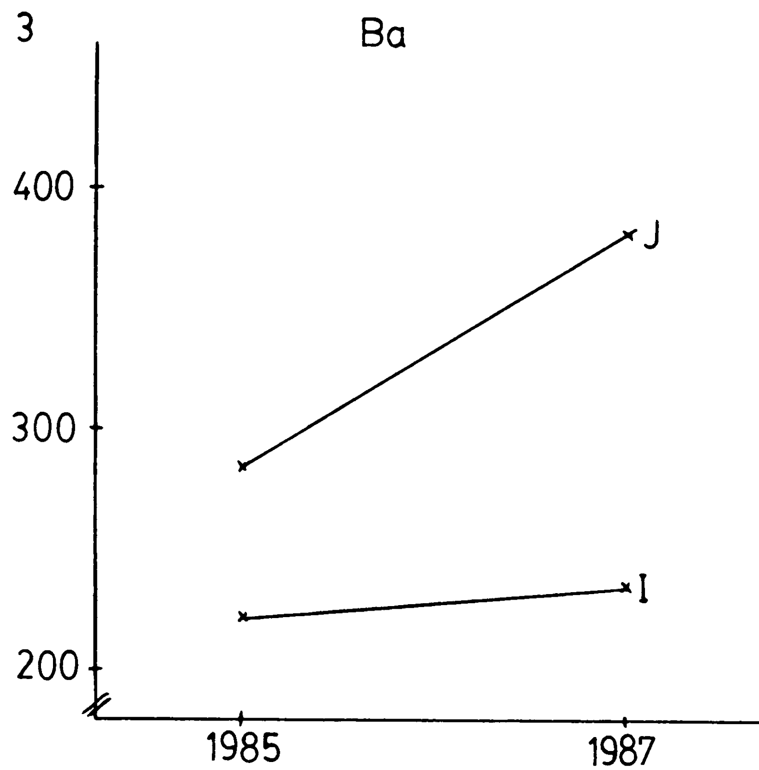


APPENDIX XI

Graphs illustrating the interactions found for the available lysine analysis (mg lysine / g Nitrogen)



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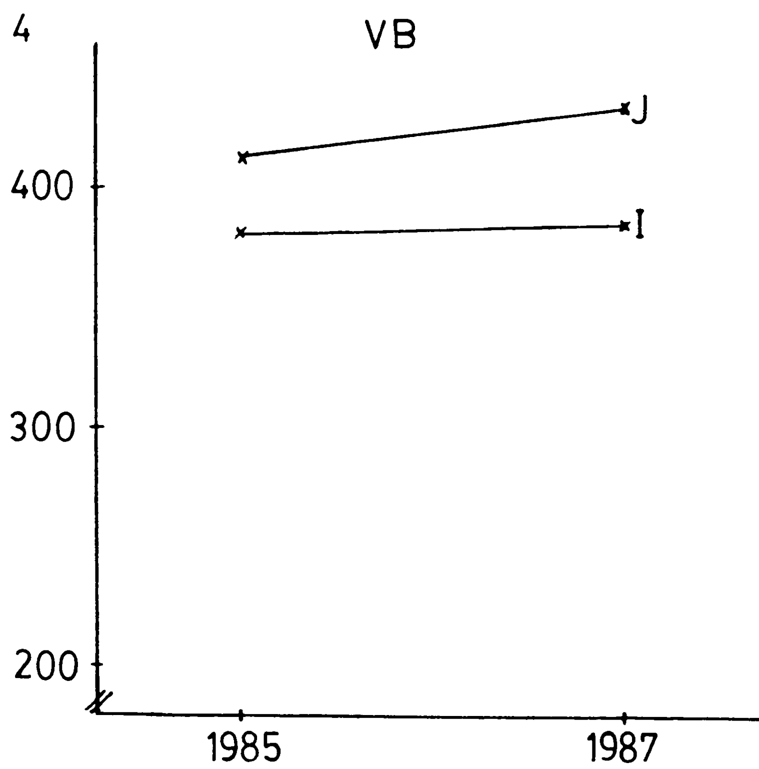
Ba
400
300
200
J
I
1985
1987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VB
400
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1985
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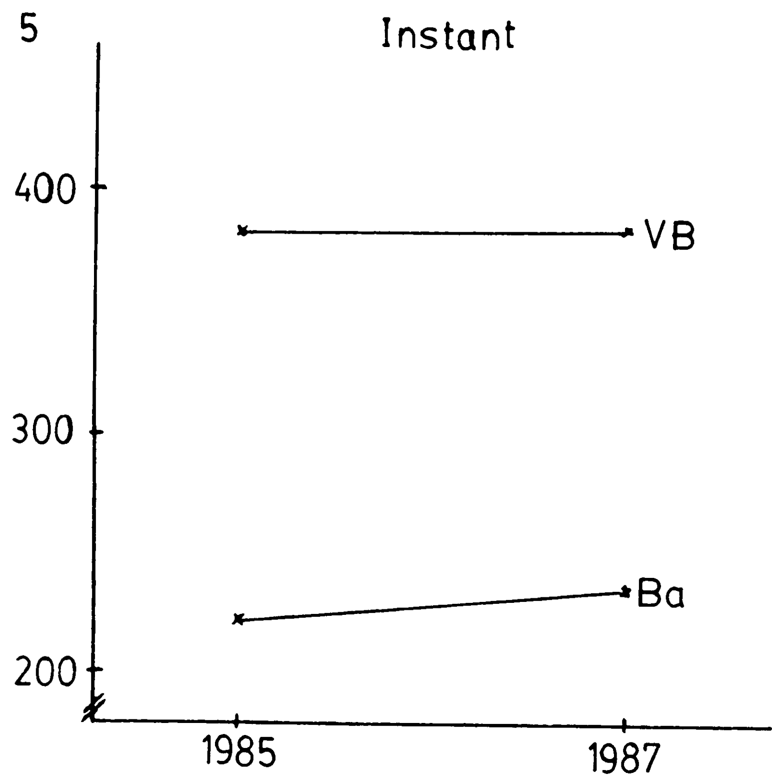
5
Instant
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Ba
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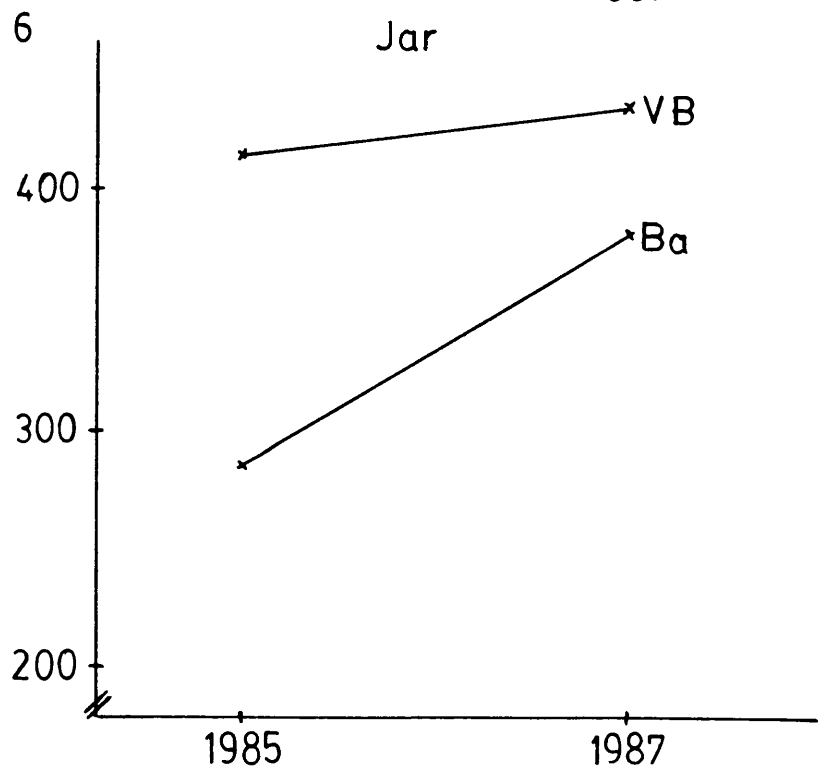
1987


6
Jar
400
300
200
VB
Ba
1985
1987

APPENDIX XII

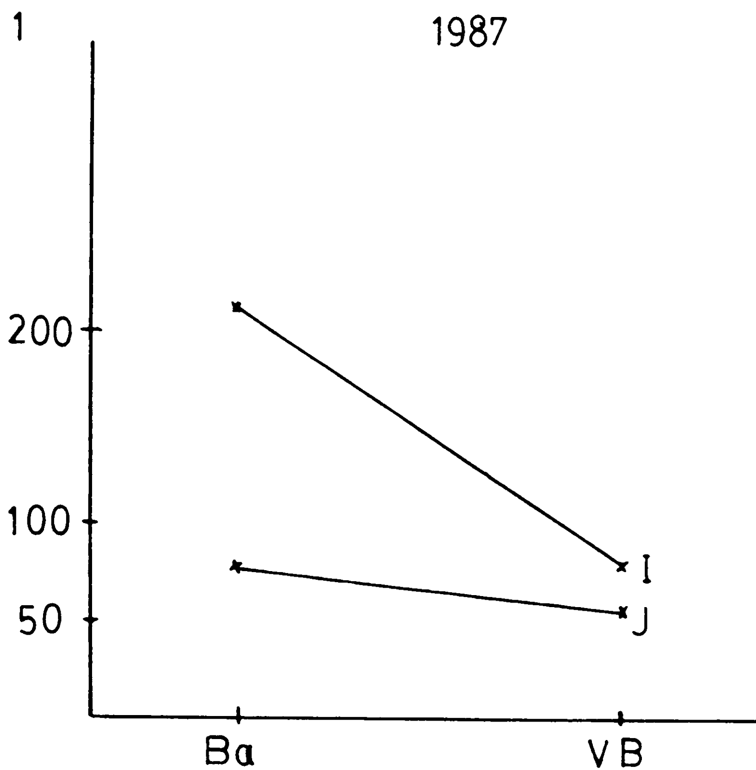
Vitamin B-6 Values

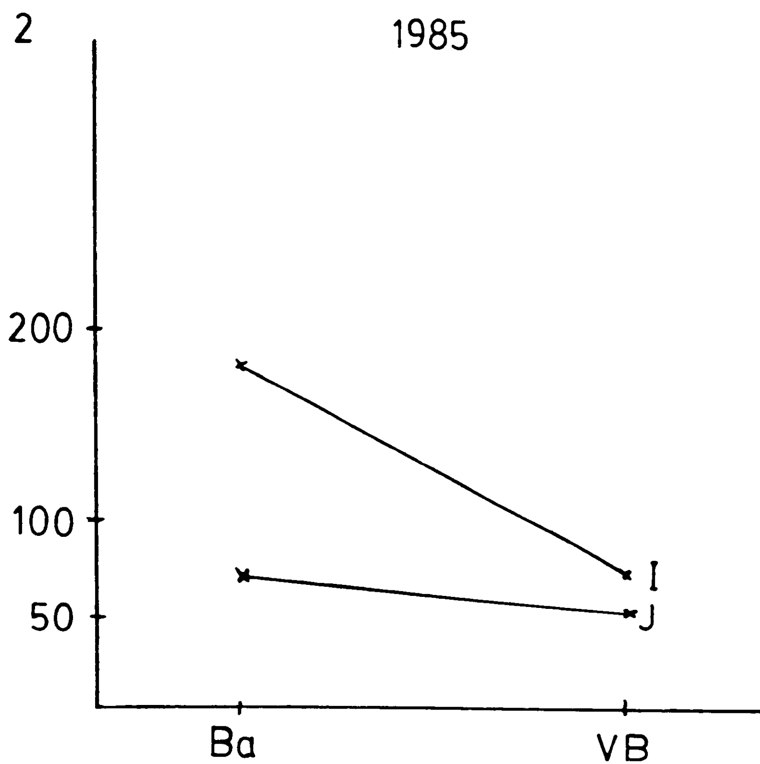
The table shows the individually determined values for vitamin B-6. Each baby product was sampled three times.

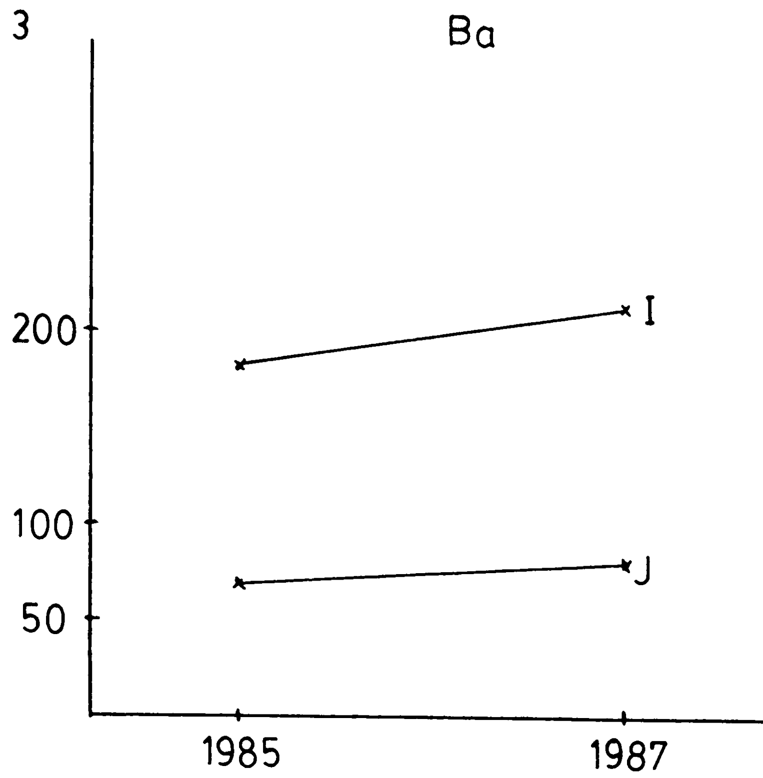
Product	ug vitamin B-6/100g food
VBJ85	49.58
VBJ85	51.66
VBJ85	58.15
VBJ87	54.46
VBJ87	58.64
VBJ87	55.43
VBI85	75.42
VBI85	70.25
VBI85	78.29
VBI87	76.68
VBI87	88.70
VBI87	76.66
BaJ85	68.83
BaJ85	76.26
BaJ85	69.94
BaJ87	77.24
BaJ87	79.49
BaJ87	82.53
BaI85	196.76
BaI85	176.00
BaI85	175.44
BaI87	200.81
BaI87	231.92
BaI87	205.67

APPENDIX XIII

Graphs illustrating the interactions found for the vitamin B-6 analysis (ug vitamin B-6 / 100 g food)



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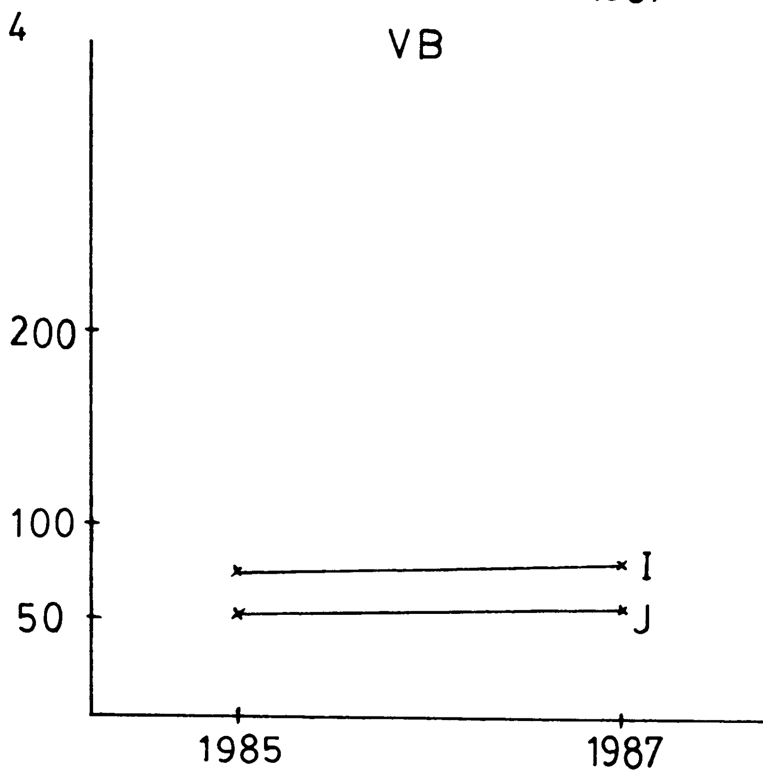
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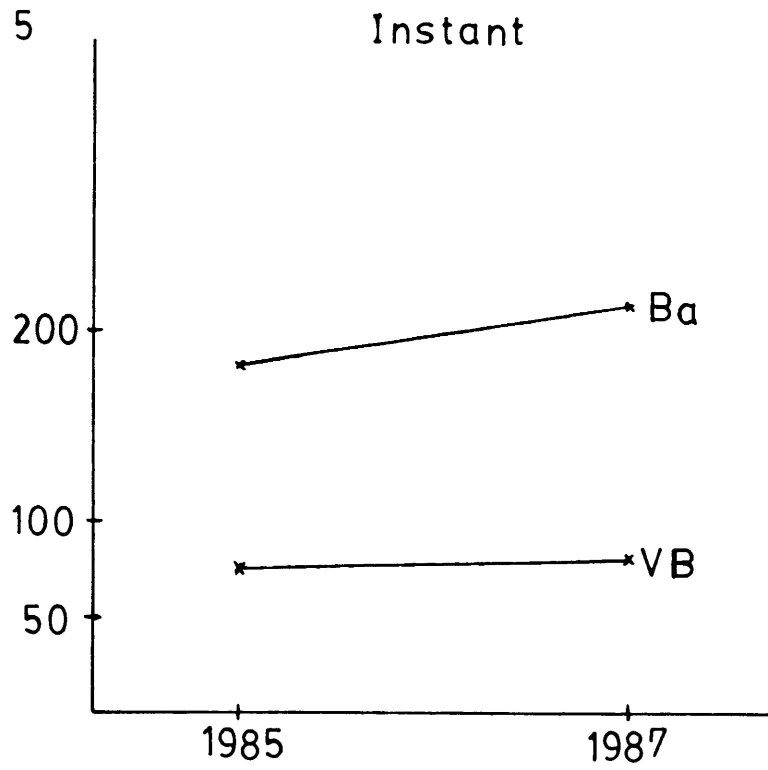
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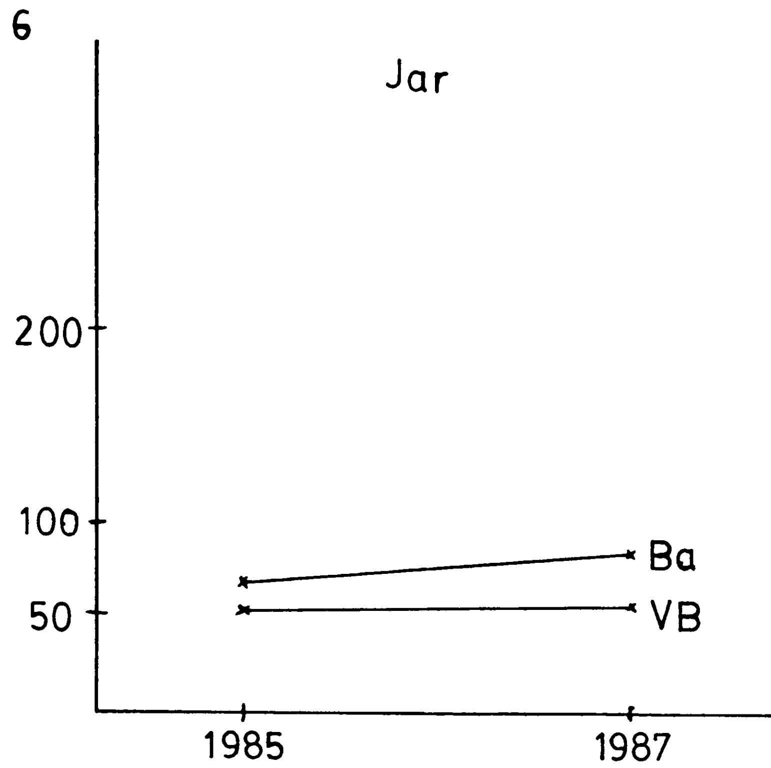
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