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DETERMINING THE TOTAL FOLATE CONTENT IN SOME OF THE COMMONLY CONSUMED FIJIAN FOODS

By

Viola Talemailakeba Lesi

A Thesis Submitted
In Partial Fulfillment of the Requirements for the
Masters of Science Degree

School of Biological and Chemical Sciences,
Faculty of Science, Technology and Environment,
The University of the South Pacific

October, 2009

DECLARATION OF ORIGINALITY

I hereby declare that the work contained in this thesis is my very own and where I have used the thoughts and works of others I have clearly indicated.

Ms Viola Talemailakeba Lesi (s95007473)

Date: 23/10/09

The research in this thesis performed under my supervision and to my knowledge is the sole work of Viola Talemailakeba Lesi unless otherwise stated.

Professor William Aalbersberg,

Principal Supervisor

Date: 23/10/09

ABSTRACT

The total folate content of 24 commonly consumed Fijian foods (8 staple foods, 6 green leafy vegetables, 6 fruits, and 4 other vegetables) was investigated using the microbiological assay method with *L. casei* coupled with the tri-enzyme extraction procedure. Prior to this, the method of analysis was validated; successfully with the standard reference material (SRM) 1846, with the precision determined to be 9%, the method detection limit (MDL) determined as 8×10^{-3} ng/mL and the limit of reporting as 1.6×10^{-2} ng/mL. The range of the total folate content determined for the foods analysed was 11 – 267 µg/100 g. Rourou (*Colocasia esculenta*) contained the highest folate content 267 µg/100 g, total folate values for four of the foods were within the 200 – 267 µg/100 g range, six in the 101 – 199 µg/100 g range , four in the 50 – 100 µg/100 g range and the remaining ten food samples contained less than 50 µg/100 g food. As expected the green leafy vegetables are rich source of folate, with the exception of nama, a poor source of folate. The staple crops were found to be moderate to poor sources of folate. The fruits were found to be poor sources of folate with the exception of tarawau (rich source) and ivi (moderate source). The remaining vegetables were found to be poor source of folate. Though the majority of the foods analysed were moderate to poor sources of folate, it can be concluded that the Fijian population should be able to meet the recommended daily intake (RDI) of folate as their daily diet is mainly modeled in the traditional Fijian diet of leafy green vegetables coupled with a staple crop.

Acknowledgment

The completion of this thesis has been at the most part through the support and prayers of many people. The first person I would like to thank is my supervisor Professor William Aalbersberg for believing in me and giving me this tremendous opportunity. Thank you sir for you guidance and words of encouragement and instilling in me the importance of churning out data that is of the highest quality and nothing less and also importantly, that there is always a way. For this I will forever be indebted.

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To my precious boys; Isimeli, Setoki and Senitiki, thank you for understanding that I had to be away for certain periods of times. This is dedicated to you.

And to my Heavenly Father, thank you for your blessings.

LIST OF ABBREVIATIONS

AOAC	Association of Official Analytical Chemists
ATCC	American Tissue Culture Collection
CV	Coefficient of Variance
DNA	Deoxyribonucleic acid
DHF	Dihydrofolate
dUMP	Uridylate
dTMP	Thymidylate
e.g.	Example
EPBA	Enzyme Protein Binding Assay
FA	Folic Acid
g	Grams
HPLC	High Performance Liquid Chromatography
h	Hour(s)
HCl	Hydrochloric Acid
L. casei	*Lactobacillus casei*
LOD	Limit of detection
LC-MS	Liquid Chromatography Mass Spectrometry
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
L	Liter(s)
MDL	Method Detection Limit
MA	Microbiological Assay
mL	Milliliter(s)
min	Minute(s)
M	Molar
ng	Nanogram(s)
nm	Nanometers(s)
NTD	Neural tube defects
PABA	para – Aminobenzoic acid
PBA	Protein Binding Assay
RDA	Recommended Daily Allowances

RDI	Recommended Daily intakes
R^2	Regression Coefficient
rpm	Revolutions Per Minute
s	Seconds
SD	Standard Deviation
NaOH	Sodium Hydroxide
SRM	Standard Reference Material
THF	Tetrahydrofolate
UIL	Upper Intake Limit
UV	Ultra Violet
^oC	Degrees Celsius
5-CHO THF	5-formyltetrahydrofolate
10-CHO THF	10-formyltetrahydrofolate
5-CH_4 THF	5-methyltetrahydrofolate
µg	Microgram(s)
µL	Microlitre(s)
%	Percentage

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Chapter One

Introduction

1.1 General Background

Folate has attracted recent attention due to its link with a number of illnesses such as anemia, neural tube defects (NTDs), cancer, Alzheimer's disease and cardiovascular diseases (Raven and Johnson, 1996; Raul *et al*., 2005; Ho *et al*., 2003; Steegers-Theunissen, 1995; Kim, 1991; Giovannucci *et al*., 1995). The daily ingestion of sufficient folate is therefore essential.

The initial step taken to decrease the occurrence of NTD was the recommendation by worldwide official bodies, for women to take 400 µg of folate/day but this was not an effective one due to poor compliance (Hannon-Fletcher *et al*., 2004). Folate intake is of great importance 4 weeks before and 12 weeks after conception. It is reported that more than half of pregnancies are unplanned (45 – 50% in Australia) which makes pre-conception supplementation difficult. Studies have shown that peri-conception intake of folic acid (FA) occurs in only 30% of women between the age of 18 – 45, and that majority of women only start doing so once they know they are pregnant (Buttris, 2004). This resulted in the introduction of mandatory fortification of selected food products in Canada, United States of America, in some of the South American countries, and in Saudi Arabia. Voluntary fortification has also been introduced in Australia, New Zealand, United Kingdom, Ireland, Hungary, and Asian countries (Kelly *et al*., 1997)

which is also the case in Fiji. Fortification may also help reduce the occurrence of other folate deficiency related illnesses (French *et al*., 2003; Molloy, 2005).

However, vitamin fortification is disadvantageous as it is untargeted therefore resulting in high levels of folate delivered to at risk groups such as the elderly and individuals being treated with anti-cancer drugs. One of the concerns is that high levels of folate masks the symptoms of vitamin B_{12} deficiency of at risk groups which are typically the elderly, resulting in the permanent damage of the nervous system (Gregory *et al*., 2005). The set safe upper level of folate intake is 1000 µg/day (Buttriss, 2004). A study by Rader *et al*. (2000) indicates that the fortification level in grains is at least 20% higher than the mandated fortification level of 140 µg/100g. Some studies have also indicated that the nutrient level in some fortified foods may actually vary as much as 320% from the value on the label (Whittaker, 2001). This suggests that that the level of 1000 µg/day may be exceeded. Another at risk group are those being treated with anti-cancer drugs which interferes with folate metabolism, as high levels of folate in the diet may interfere with the effectiveness of the drug (Expert Groups on Vitamins and Minerals, 2002). Extensive studies on the adverse effects of increased folate intake need to be done (Shane, 2003). Considering that folic acid is not a natural co-enzyme, the biological effects of its long term use may need to be established (Lucock, 2004).

The other approach to satisfying the Recommended Daily Intake (RDI) is to increase the consumption of natural foods particularly rich in folates. This would make it more important to determine the folate content of our local foods, so that we are able to meet the daily RDI of folate through natural folates. It should also be considered that not

everyone in Fiji and the Pacific has access to fortified foods, particularly people in rural areas and below the poverty line. Determining the folate content would be the first step in meeting the RDI for each age group.

The food composition tables for the Pacific, which is continually being updated by the University of the South Pacific, currently have no values on the folate content of Pacific foods. Knowledge of folate content of foods is required to help control folate deficiency related illnesses. This then should be followed by bioavailability studies which look at the extent of intestinal absorption of the folates in the foods and thus the folate status of the population, but for now this project will only focus on determining the folate content in Fiji foods. This knowledge would enable various countries in the region to determine the folate status of its population and act accordingly. Therefore it is imperative that the folate content in foods in Fiji is determined to start this whole process.

In Fiji, a number of studies have focused on other vitamins, such as vitamin A, B_1, B_2, B_3 and C (Aalbersberg *et al*., 1996, 2001). These studies have resulted in the adoption of these analyses in the Institute of Applied Sciences (IAS) which in turn has led to the analysis of various Pacific foods and the eventual compilation of the food composition tables. The composition tables are used by Health and Agricultural Ministries and the National Nutrition Authority to assess the status of nutrient intake in Fiji.

The first step is the setting up and the validation of the appropriate method for the analysis of folates in foods. Thus this study's primary aim was to set up and validate the microbiological assay of total folates in foods in the IAS.

1.2 Literature Review

The main role of folate is as a co-enzyme in which it acts as an acceptor and donator of 1-carbon units in important cell reactions such as; the synthesis of the purines and pyrimidines which are the base constituents of DNA, the de novo biosynthesis of methionine and the interconversion between glycine and serine (Ball, 1998). A lack of folate in diets and consequently the cells, results in inadequate DNA replication and therefore impaired cell division which may be the reason why folate has been linked to a number of illnesses.

Initial interest in folate was through its role in preventing the occurrence of neural tube defects (NTDs) (Molloy, 2005). This has intensified in recent years as other studies have also linked the deficiency of this vitamin to other illnesses such as cancer, Alzheimer's disease and cardiovascular diseases (Raven and Johnson, 1996; Raul *et al*., 2005; Ho *et al*., 2003; Steegers-Theunissen, 1995; Kim, 1991; Giovannucci *et al*., 1995). The daily ingestion of folate is therefore essential.

This review will look at current literature in regards to folate history, chemistry and occurrence, role, bioavailability, deficiency related illnesses, sources, fortification and methods of analysis.

1.2.1 The Discovery of Folate

The curative effect of folate was first observed by a research group led by Willis in 1931 from prepared yeast extract (Ball, 1998). From then on, other groups also isolated substances from liver and yeast extracts which had similar effect on anemia and was

identified to be a growth factor for *Lactobacillus casei*. At that time, folate was still unknown, so was known by different names: Vitamin M, Vitamin B_c and the *L. casei* factor (Ball, 1998). In 1941 a research group led by Mitchell purified it from spinach and named it folic acid which comes from the Latin word folium meaning leaf. The structure of folic acid (FA) was eventually elucidated in 1946 by a research group led by Angier (Ball, 1998).

1.2.2 Folate Chemistry and Structure

Folate is the general name given to this group of water-soluble B_9 vitamins that perform similar tasks but differ from each other due to slight variation in their structures as will be discussed later (Forssen, 2000). It is not stored in the body and needs to be continually replenished through the diet. The parent structure (Fig. 1), chemically known as pteroylglutamic acid, is also referred to as folic acid (FA), and is composed of three parts, a bicyclic pterin linked by a methylene bridge to para-aminobenzoic acid (PABA) which is linked to a molecule of L-glutamic acid (Forssen, 2000).

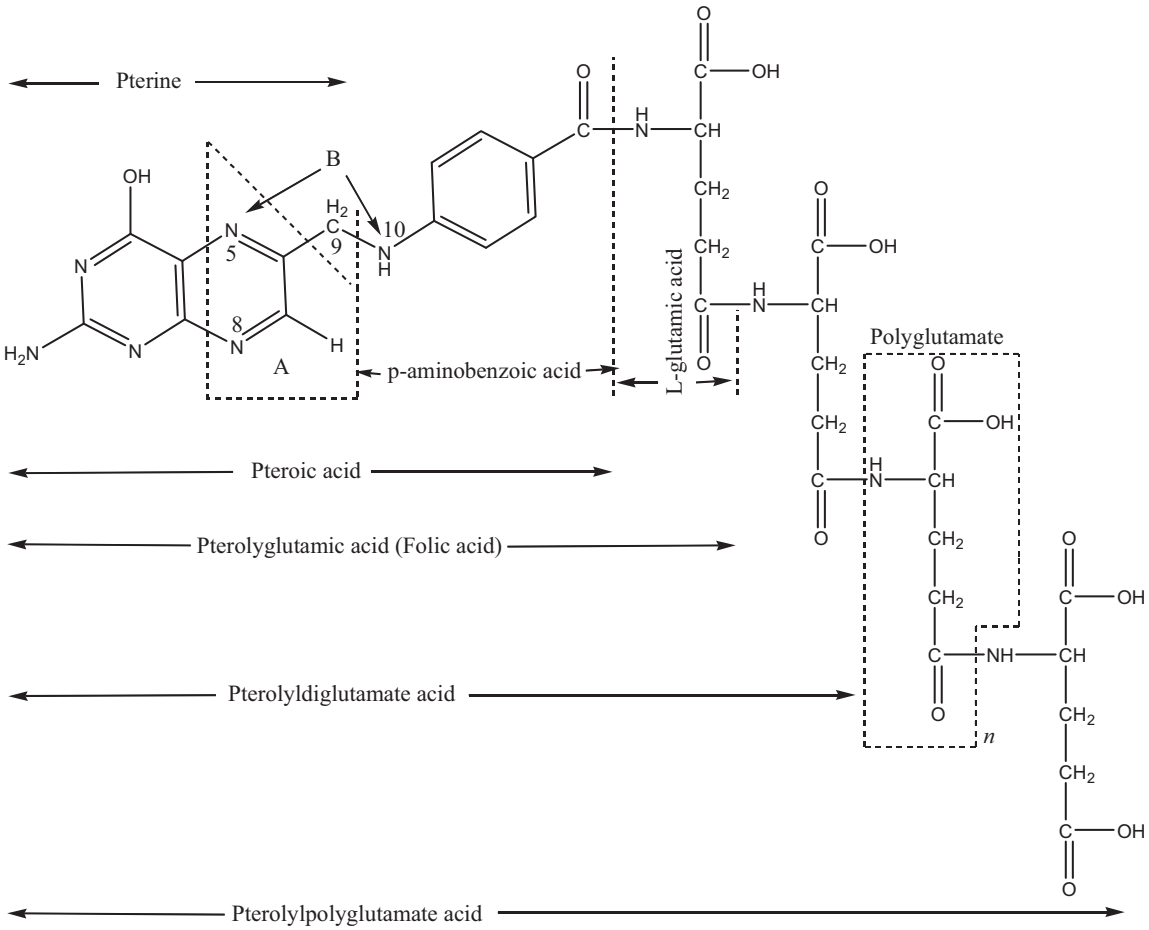


Figure 1.1 Structural relationships of folates (Eittenmiller and Landen, 1999)

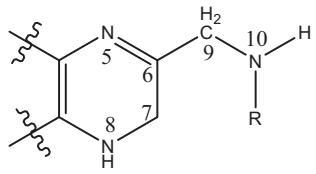
There are an estimated 150 derivatives of folate (Baugh and Krumdieck, 1971 cited in Basu and Dickerson, 1996). This arises from the three possible structural alterations to the parent structure:

I. The pteridine ring is reduced to give 7, 8-dihydrofolate (DHF) or 5, 6, 7, 8-tetrahydrofolate (THF). The pteridine ring or nucleus is made up of two rings; the pyrazine ring is the part of the pteridine nucleus that undergoes reduction to form dihydro- and tetrahydro glutamic acid.

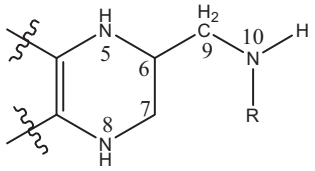
II. The differences in the covalently bonded one carbon units attached to either N5 or N10 or both N5 and N10 positions of the pyrazine ring (Table 1.1). The known one-carbon units are methyl, formyl, formimino, methylene and methenyl.

Table 1.1 Common one-carbon units that can be attached at N5 and N10, or both N5 and N10 of the pyrazine ring (Eittenmiller and Landen, 1999).

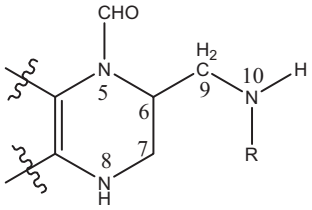
Name	***Abbreviation***	***Position***	
		N-5	***N-10***
Pteroylglutamic acid	folic acid	-	-H
7,8-dihydrofolate	7,8-H_2folate	-H	-H
5-methyl-5,6-dihydrofolate	5-CH_3-5,6-H_2folate	-CH_3	-H
5,6,7,8 tetrahydrofolate	H_4	-H	-H
5-methyltetrahydrofolate	5-CH_3-H_4folate	-CH_3	-H
5-formyltetrahydrofolic acid	5-CHO-H_4folate	-CHO	-H
10-formyltetrahydrofolate	10-CHO-H_4folate	-H	-CHO
5,10-methenyltetrahydrofolate	5,10-CH=H_4folate	=CH-bridge	-
5,10-methylenetetrahydrofolate	5,10-CH_2H_4folate	-CH_2-bridge	-
5-formiminotetrahydrofolate	5-CHNH-H_4folate	-CHNH	-H



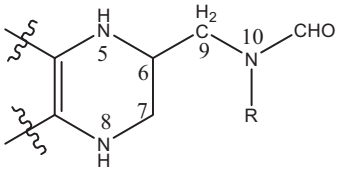
(A) 7,8-dihydropteroyl monoglutamic acid (DHF)



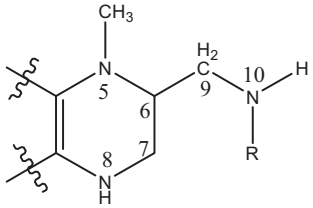
(B) 5,6,7,8-tetrahydropteroylmonoglutamic acid (THF)



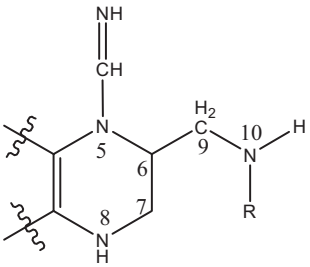
(C) 5-formyl-THF (5-CHO-THF)



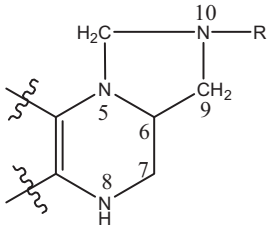
(D) 10-formyl-THF (10-CHO-THF)



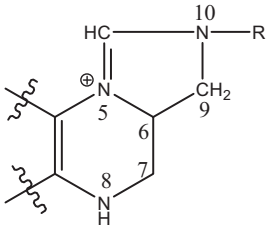
(E) 5-methyl-THF (5-CH_3-THF)



(F) 5-formimino-THF (5 CH=NH-THF)



(G) 5,10-methylene-THF (5,10-CH_2-THF)



(H) 5,10-methenyl-THF (5,10 =CH_2-THF)

Figure 1.2 Structures of tetrahydrofolates, unsubstituted or substituted with single carbon units attached at either N5 or N10 or both (Ball, 1998)

III. The number of glutamate residues added to the end of the molecule via a gamma–peptide linkage to form polyglutamyl folates. All folates are found in the polyglutamate form in nature, containing five to seven glutamate residues linked together by gamma-peptide bonds.

The most common forms of folate in foods are: tetrahydrofolate (H_4 folate), 5-methyltetrahydrofolate (5-CH_3-H_4 folate), 5-formyltetrahydrofolate (5-CHO-H_4 folate) and 10-formyltetrahydrofolate (10-CHO-H_4 folate) (Koontz *et al*., 2005). Approximately 90% of the polyglutamate form that occurs in natural foods is 5-methyl THF and the majority of the remainder in the 10-formyl form (Expert Group on Vitamins and Minerals Secretariat, 2002). Due to the presence of plasma conjugase (the enzyme required to hydrolyze folate polyglutamates to folate monoglutamates), only monoglutamates are present in the blood (Ball, 1998).

Folate is pale yellow to yellow-orange in colour, is stable in alkaline solution but lesser in acidic solution, and is virtually insoluble in water, alcohol, acetone, chloroform and ether, but is soluble in hydrochloric acid and sulphuric acid (Davis, 1986; Herbert, 1999; Expert Group on Vitamins and Minerals Secretariat, 2002).

The term folic acid (FA) has become synonymous with the vitamin to the general public, due to health promotional campaigns when it is in fact the synthetic form of this vitamin. Unlike the reduced natural forms of folate, folic acid is fully oxidized hence it is the most stable form of the vitamin and has been reported to be able to remain intact up to a temperature of 100ºC when protected from light and at pH 5.0 to 12.0 (Eittenmiller and

Landon, 1999). This is the form used for food fortification in the food industry and in vitamin supplements.

1.2.3 Sources of Folate and the Recommended Daily Intake

The major sources of folate are green leafy vegetables, liver, beans, egg yolk, and wheat germ, yeast and cereals (Arcot and Shrestha, 2005). Foods are grouped as being rich, moderate or poor sources of folate, with folate concentrations of >100 μg, 50-100 μg and 15-50 μg per serving respectively (Witthoft and Jagerstad, 2004). Folate is also present in yeast, yeast extracts and beer (Expert Group on Vitamins and Minerals Secretariat, 2002).

The recommended daily intake (RDI) is 400 μg for adults, 600 μg in pregnant women and 600 μg for those that are breastfeeding (Patring *et al*., 2005; Yates *et al*., 1998). In many countries, cereals and other supplements are fortified with folic acid (the most stable and synthetic form of folate) as a precaution against folate deficiency in the diet. However the actual levels in fortified foods may vary as much as 320% from the value stated on the label (Whittaker *et al*., 2001). This has resulted in New Zealand and Australia also estimating the Upper Intake Limit (UIL) of folate as shown in the table 1.2.

Table 1.2 New Zealand and Australian RDI and UIL for folate

Gender group	*Age*	*Folate (folate equivalent µg/day)*	
		RDI	*UIL*
Infants	*0 - 6 months*	*-*	*BM*
	7 - 12 months	*-*	*BF*
Children	*1 - 3 yrs*	*150*	*300*
	4 - 8	*200*	*400*
	9 - 13	*300*	*600*
Adolescents	*14 - 18*	*400*	*800*
Adults	*> 19*	*400*	*1000*
Pregnant women	*14 - 18*	*600*	*800*
	19 - 50	*600*	*1000*

** BM – amount normally received from the breast milk of a healthy woman,*
** BF – amount in breast milk and food.*
(Draft NHMRC; 2004a, cited in Thompson; 2005)

1.2.4 Folate Stability

Vitamins can be highly labile, it is therefore important to have a good knowledge of the stability of folate as losses can occur during sample preparation, extraction, storage and analysis. Folate loses its biological activity through the oxidative cleavage of the C-9-N-10 bond induced by heat, UV light and oxygen (Eittenmiller and Landen, 1999). Therefore it is essential that analysts ensure that exposure to light and atmosphere is minimized during analysis. This cleavage results in folate being split into two biological inactive compounds, pteridine and p-aminobenzoylglutamate which are not able to be converted into biological active forms (Murphy *et al*., 1976 and 1978; Scott, 2001, cited in McKillop *et al*., 2002). This is of particular importance when dealing with reduced naturally occurring folates. This may not be a problem for FA which is fully oxidized. This cleavage is minimized by the presence of one–carbon substituents at the N-5 and

N-10 of the naturally occurring folates and the presence of reducing agents such as ascorbic acid, 2-mercaptoethanol and dithiothrietol during analysis (Eittenmiller and Landen, 1999). The former two are by far the most commonly used reducing agents used for stabilizing folate (Arcot and Shrestha, 2005). Further precautionary measures are the flushing of tubes with nitrogen gas to remove oxygen and the use of yellow light to minimize photodegradation (Quinlivan *et al*. 2005).

THF being unsubstituted is extremely unstable. The presence of adducts at either or both N-5 and N-10 increases the stability of some of the folate derivatives comparable to that of FA as indicated in some of the studies mentioned in Eittenmiller and Landen (1999):

I. 5-MethylTHF – has a half-life of 21 min at 100ºC as long as the pH is within 5-12 (Chen and Cooper, 1979). At 25ºC, in the absence of antioxidant it is stable at pH 7.3 and 3.5, however in the presence of an antioxidant it is stable at pH 9 and 7.3 with the antioxidant having no protective effect at pH 3.5. (Lucock *et al*., 1993).

II. 5-FormylTHF- has similar stability to folic acid as long as the neutral pH is maintained (Pain-Wilson and Chen, 1979).

III. 5, 10-methenylTHF is not susceptible to atmospheric oxidation in acidic condition (Stokstad and Foch, 1967).

IV. 10-FormylTHF- its stability is comparable to folic acid at neutral pH (Gregory, 1985; cited in Eittenmiller and Landen, 1999).

Folate losses also occur during food processing and storage. This depends on a number of factors such as differences in food matrices, oxygen availability, chemical environment and the form of folate present in the food (Eittenmiller and Landen, 1999). Folate is water-soluble and losses may occur through leaching during cooking and canning (Dang *et al*., 2000; Eittenmiller and Landen, 1999). Presence of Fe^{3+}, high level of dissolved oxygen and food additives also reduce the stability of folates (Eittenmiller and Landen, 1999).

1.2.5 Biological Role of Folate

The principal function of folate coenzyme is to accept or donate one-carbon units in key metabolic pathways (Bailey and Gregory, 1999). These one carbon units are methyl-, formyl-, formimino-, methylene- and methenyl-, which originate from the catabolism of the amino acids glycine, serine and histidine (Witthoft and Jagerstad, 2004).

Folates provide the carbon atoms for the synthesis of purines (adenine and guanine). There are seven reactions involved in the synthesis of purine of which reaction one and six require the involvement of two folate coenzymes (Basu and Dickerson, 1989). The two active coenzymes are 10-formyl-THF and 5,10-methenyl-THF where the two molecules act as formyl donors, incorporating the one carbon units which becomes the carbon 2 and 8 of the developing purine rings respectively (Lucock, 2000) as shown in Fig 1.3. This is considered to be the most important role of folate and its derivatives.

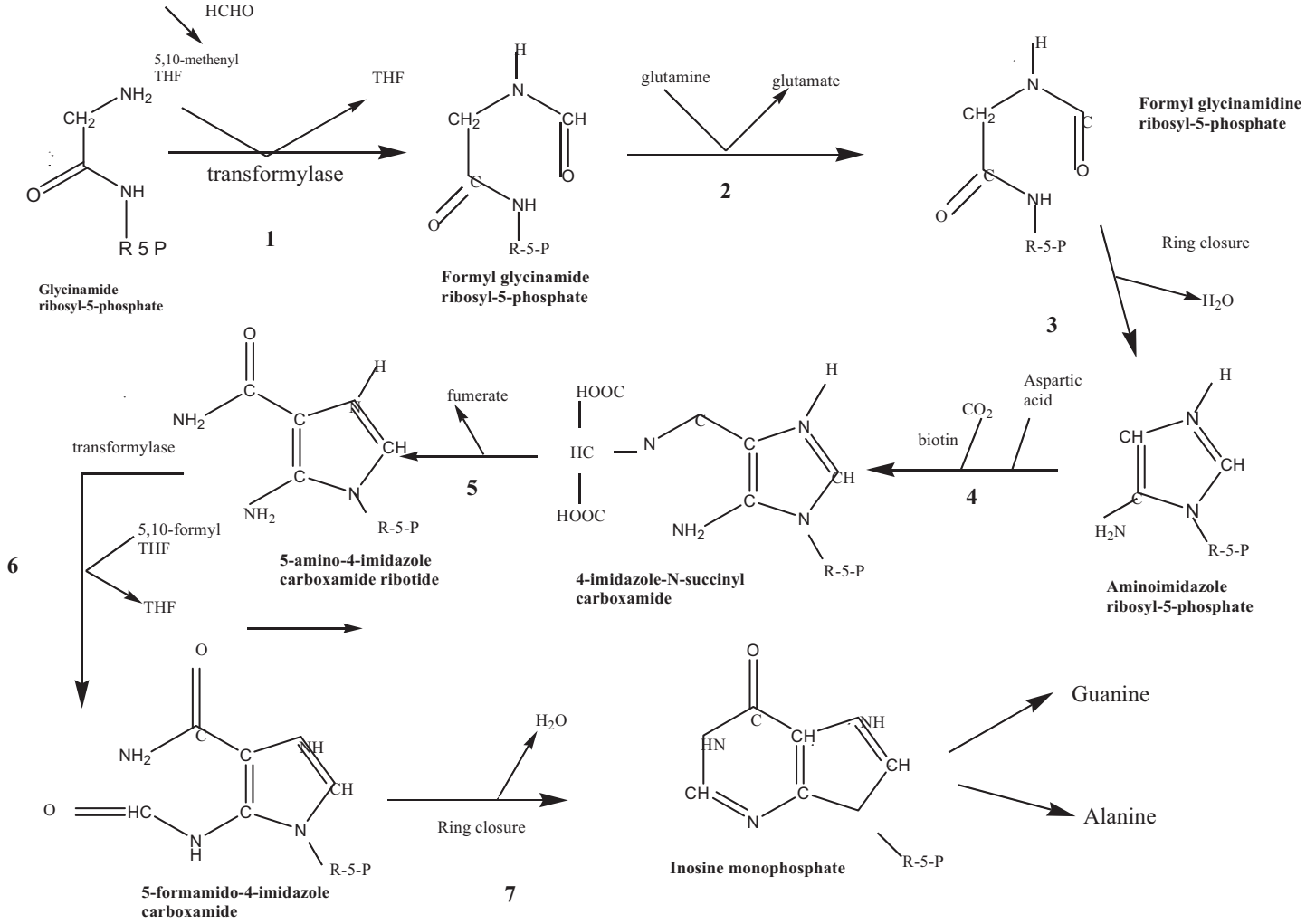


Fig 1.3 The role of folate in the biosynthetic pathway for purines (Basu and Dickerson, 1996)

Folate is also involved in the synthesis of thymidylate, which is a pyramidine. The folate co-enzyme 5, 10-methylen-THF donates the methyl group which results in the conversion of uridylate (dUMP) to thymidylate (dTMP) (Rampersaud *et al*., 2002).

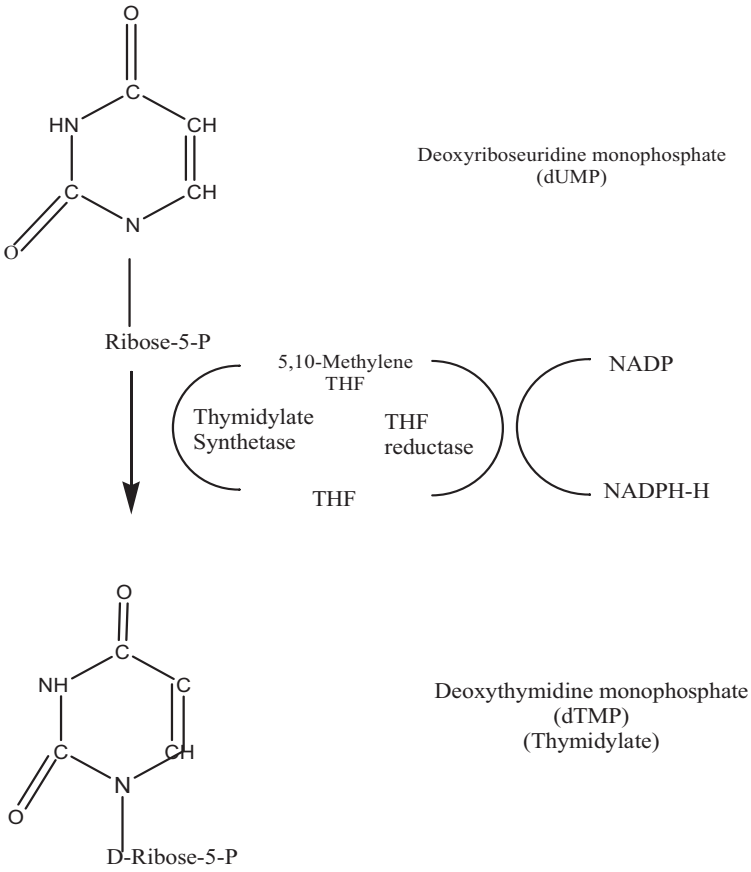


Fig 1.4 The role of folate in the biosynthesis of thymidylate (Basu and Dickerson, 1996)

Folate also plays an important role in the synthesis of certain amino acids. It provides the methyl group that is required for the synthesis of methionine from homocysteine. Methionine is a very important amino acid, for not only is it essential for the synthesis of proteins, it is also the precursor of S-adenosylmethionine, which acts as a source of methyl group for more than 100 enzymatic reactions that have critical roles in metabolism (Combs, 1998). 5-methylTHF is converted to THF by the vitamin B_{12} dependant enzyme methionine synthetase, releasing the methyl group to homocysteine which in turn generates methionine. Deficiency in the vitamin B_{12} results in the folate being trapped as methyl folate which is why vitamin B_{12} deficiency results also in folate

deficiency. This also leads to high homocysteine level in the blood which studies have indicated to be a risk factor for cardiovascular diseases (Raul *et al*., 2005).

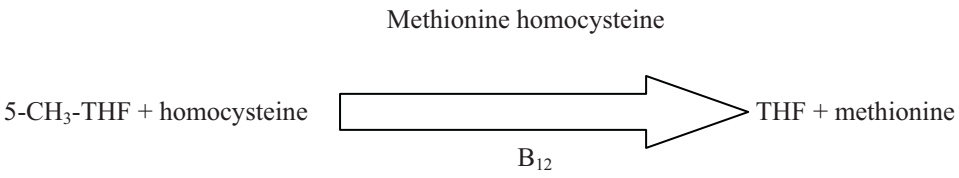


Fig 1.5 The involvement of vitamin B_{12} as a coenzyme in the demethylation of 5-CH_3-THF (Ball, 1998).

Therefore folate is very important for growth, reproduction and maintenance of cells (Opladen, 2005).

1.2.6 Folate Absorption, Transport and Excretion

After consumption of food, the folate must undergo hydrolysis where it is converted from the polyglutamate form to monoglutamate form. The enzyme responsible for this is γ-glutamylcarboxypeptidase but is normally known by its trivial name conjugase (Basu and Dickerson, 1989). This enzyme is located at the lumen and brush border of the intestinal mucosa. The monoglutamates are then absorbed at the jejunum which is pH dependent with the optimum pH being 6.3 (Basu and Dickerson, 1989). During absorption the monoglutamates are also converted to 5-Methyl-THF. The folates are then transported via the venae portae to the liver for storage, where most of it is excreted to the bile. The monoglutamates which are predominantly 5-methyl-THF are metabolized to the polyglutamate form by the enzyme polyglutamate synthetase upon entering the cells as this is the preferred form for storage. The polyglutamate forms will

have to be hydrolyzed to the monoglutamate form by conjugase before they can be transported out of the cells (Expert Group on Vitamins and Minerals Secretariat, 2002).

The total amount of folate lost through urine daily is 5-40 μg (Herbert, 1968 cited in Basu and Dickerson, 1998), ≤ 10 μg/daily for a normal person (Chanarin 1990 cited in Expert Groups on Vitamins and Minerals Secretariat, 2000) and can either be in the biologically active form or as breakdown compounds (Herbert, 1968 cited in Basu and Dickerson, 1998).

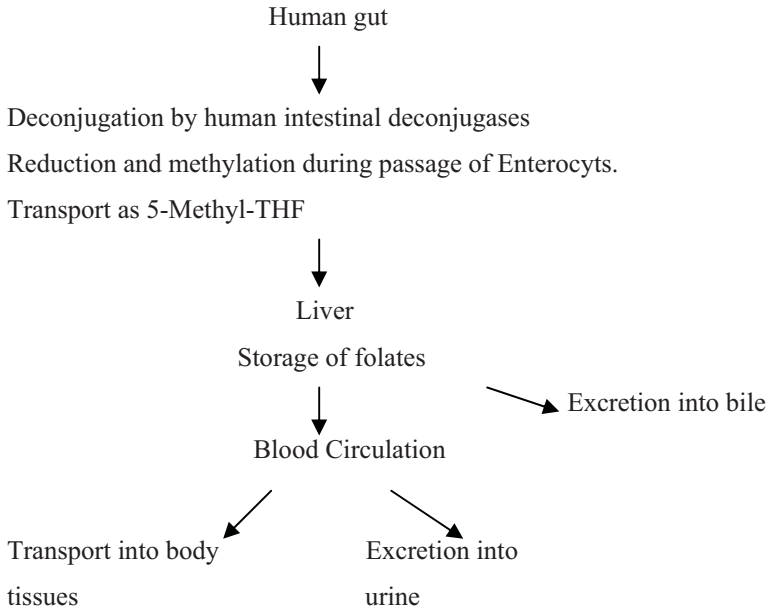


Fig 1.6 Human folate metabolism (Witthoft and Jagerstad, 2004).

1.2.7 Folate Bioavailability

Bioavailability looks at the extent of the actual intestinal absorption of folate. Initial studies on bioavailability were conducted on animals such as rats, chickens and monkeys, however may not have been appropriate and now are not commonly used due to the differences in the mechanisms involved in the intestinal conjugase of the animals

mentioned above to that of humans (Gregory, 1995; Halsted, 1999 cited in Gregory, 2001).

There are three approaches that can be taken to determine the bioavailability of foods (Witthoff and Jagerstad, 2004; Expert Groups of Vitamin and Mineral Secretariat, 2002; Gregory, 2000). The first is the use of short term protocol. In this approach the volunteers are subjected to only one dose of the vitamin supplement, fortified food or food sample, with changes in plasma or serum folate measured 1-2 h after ingestion. The values obtained in this protocol is based on one to two plasma values; its disadvantage lies with the fact that it would be impossible to ascertain from this if the changes in the plasma or serum folate are due to the rate or extent of absorption (Gregory, 1998). The second approach is the use of the long term protocol which runs a course of 3-6 weeks duration. This method allows for comparisons to be made on different diets with the same folate content but different sources of folate. Here the volunteers are often divided into groups, with each group receiving different treatments, e.g. a group could receive folate supplement dosages, fortified folate, and another from meals prepared from natural sources of folates. This method measures the serum folate, the plasma homocysteine levels and folate levels in urine, with serum folate considered to be very sensitive. The third approach is the stable isotope protocol which is also the more recent of the protocols. It involves the isotopic labeling of folates which are then incorporated into the diet which are subjected to the volunteers. The advantage this protocol has over the other two is that a differentiation can be made on the isotopically labeled folates and the endogenous folates in the body.

Bioavailability studies are used to determine the effectiveness of the diet of a population, or a particular diet given to test subjects, the bioavailability of different foods and the influence various cooking methods have on the absorbance of folate in foods by the intestine, information that can be eventually used to determine the folate status of a population. Studies have shown varied bioavailability in foods and diets. Herbert (1987) estimated the bioavailability of folate in a typical North American diet to be 50-75%. Sauberlich *et al.* (1987) have estimated the bioavailability of a mixed diet to be no more than 50%. It was this study by Sauberlich *et al.* (1987) that led the mandatory fortification of all enriched cereal – grain products in the US (Winkels *et al*., 2007). However a study by Winkels *et al*., (2007) which was in agreement with an earlier study by Brower *et al*., (1999) suggests that a diet in food rich in folate can efficiently meet the RDI, and are bioavailble by 73 – 80% that of FA. FA in fortified foods is 85% bioavailable, 1.7 times more than natural folate (US institute of Medicine cited in Expert Group of Vitamin and Mineral Secretariat, 2002).

There are various factors that may affect the bioavailability of food folates. These include consumption of alcohol and foods high in fibre, presence of conjugase inhibitors in the food and oxidative cleavage during digestion (Eittenmiller and Landen, 1998; Witthoft and Jagerstad, 2004).

Studies on the bioavailability of natural sources of folates should be of absolute importance now considering that the other two alternatives for addressing the folate status of the public are problematic, as seen in the limited compliance in folic acid supplementation and safety concerns associated with folic acid fortification (Hannon-

Fletcher *et al*., 2004) and also taking into consideration the studies by Brower *et al*. (1999) and Winkels *et al.* (2007).

1.2.8 Folate Deficiency and Related Illnesses

As discussed earlier in section 1.2.5, folate plays a significant role in the synthesis of DNA, therefore any deficiency in the vitamin may result in the interference in DNA replication and thus all cell division. Therefore folate deficiency would be most noticeable in cells where the most cell multiplication occurs hence its link to megaloblastic anemia, the occurrence of NTDs and some forms of cancers.

1.2.8.1 Major Illnesses

1.2.8.1.1 Megaloblastic Anemia

The main symptom for folate deficiency is the occurrence of megaloblastic anemia (Raven and Johnson, 1996). The red blood cells are not dividing well, so there are large, immature, fragile red bloods cells (RBC) with a short life span present in the blood. These red blood cells have inadequate hemoglobin, hence reduced oxygen carrying capacity, resulting in anemia (Lucock, 2000). This form of anemia can also be caused by vitamin B_{12} deficiency but this differs from folate deficiency megaloblastic anemia in that it has an additional effect of causing nerve damage (Sherwood, 2004). The two vitamins are essential in DNA and red blood cells (RBC) synthesis, their deficiency therefore leads to the formation of defective red blood cells (Sherwood, 2004). Although it is iron deficiency anemia that is more commonly associated with pregnancy, it is

megaloblastic anemia which is frequently associated with twin pregnancy (Basu and Dickerson, 1996).

1.2.8.1.2 Neural Tube Defects

Folate deficiency in pregnant women just before and after conception may result in neural tube defects (NTDs), where the neural tube does not close up after 28 days of gestation (Steegers-Theunissen, 1995). It is one of the major causes of infant mortality, occurring in one quarter of a million babies per year (Molloy, 2005). A study by Lancaster and Jansen in 1969 showed the occurrence of fusion defects in babies born at the CWM Hospital's maternity ward (Jansen, 1991), which occurred 3-4 times more in Indians than in Fijians. The two most common forms of this lesion are spina fida and anencephaly. In spina fida (open spine) the spinal cord is affected and occurs in 50% of the NTDs cases. Anencephaly is the more lethal of the two, where the brain or skull is underdeveloped resulting in death either before or after birth. It occurs in 30% of NTDs (Green, 2002). It is now accepted that 50-70% of affected births can be prevented through maternal ingestion of folic acid during pregnancy based on an extensive 20 years study (Molloy, 2005).

1.2.8.2 Minor Illnesses

1.2.8.2.1 Vascular Diseases, Alzheimer's Disease, Depression

Methionine, another essential amino acid, is synthesized in the body through the methylation of homocysteine (Basu and Dickerson, 1996). Folate participates in the synthesis of methionine from homocysteine. Methyltetrahydrofolate is the required one

carbon-carrier for this pathway to convert homocysteine to methionine. When there is a deficiency in folate there is a decreased synthesis of methionine and homocysteine builds up in the blood.

A high level of homocysteine in the blood has long been linked to the occurrence of vascular diseases as it results in clotting and the thickening of the arterial walls (Raul *et al*., 2005). It has been reported that cardiovascular diseases are a major cause of death in Fiji (Ministry of Information, 2004), with the incidence of stroke rising in the last four decades (Maharaj and Panapasa, 2002). It is therefore important that the folate content in Fiji foods be determined to help assist in the minimizing this problem.

The amino acid homocysteine has also been reported to be a risk factor for Alzheimer's disease (Lao *et al*., 2004). Studies by Ho *et al*. (2003) showed that neuronal cultures which were deprived of folate underwent neurodegenerative changes characteristic of Alzheimer's disease. It has also been reported that a high level of homocysteine may lead to a development of Alzheimer's disease within eight years.

The high level of homocysteine in the blood as a result of folate deficiency has also been linked to depression (Bottiglieri, 2005, Reynolds, 2002). A study by Learner *et al*. (2006), where all patients admitted to a psychiatric hospital were examined for serum cobalamin and folate levels, showed a positive correlation between low serum folate and depression. It suggested that this may be due to the correlation between S-adenosylmethionine (also an antidepressant in humans) and CSF 5-HIAA (a metabolite of serotonin), used as an indicator of serotonin levels in the brain (Liu, 1998; Young,

2007). Folate deficiency reduces the S-adenosylmethionine level in the brain resulting in the decreased levels of CSF 5-HIAA, indicating the low levels of serotonin (Botez *et al.,* 1982 cited in Liu, 1998), hence occurrence of depression..

1.2.8.2.2 Cancer

Folate plays an important role in DNA replication and cell division as it is involved in the de novo biosynthesis of purine and thymidylate (Kim, 1991), the latter being of particular interest here, as it is involved in the synthesis of thymine, one of the four bases of DNA. The occurrence of low folate levels in the body results in high uracil content, which leads to the misincorporation of uracil in DNA in place of thymine (Rampersaud *et al*., 2002). Cells have a repair mechanism to deal with this, as uracil is not a usual component of DNA. However, overloading of this repair mechanism leads to the occurrence of nicks in DNA, leading to strand breakage, genetic instability and DNA damage (Rampersaud *et al*., 2002).

Recent epidemiological and clinical evidence suggest that folate deficiency may be linked to the occurrence of cancer. Disturbances in folate metabolism have shown to promote tumor growth. An assessment over a period of 20 years in a Western Australian town has associated folate deficiency with the occurrence of breast and prostrate cancer (Rossi *et al,* 2006). Giovannucci *et al*. (1995) have also linked folate deficiency with prostate cancer.

1.2.9 Folate Deficiency in Fiji

In Fiji there is a common occurrence of anemia. This could possibly be caused by folate deficiency as the data obtained do not specify the type of anemia it is. The National Nutrition Survey in both 1980 and 1993 showed the high prevalence of anemia in the general population. The 1980 survey showed that anemia occurred in 83% of the children between the ages of 7 – 15 years, 43.9% in Indian women compared to 42.6% of Fijian women, 36.1% in Indian males and 37.9% in Fijian males. The 1993 survey showed that anemia occurred in 40% of children under the age of five. It affected more Indian women at a rate of 38% compared to the 26% in Fijian women. In the case of pregnant women, the occurrence of anemia was also high for Indian women occurring at a rate of 62% compared to 52% in Fijian women. The annual reports for the years 2003 – 2004 of the CWM Hospitals Anderson ante-natal clinic shows an alarming increase in the number of anemic cases in women that visited the clinic. The occurrence of anemia was 50% in 2003, 56% in 2004 and 66% in 2005. Having a high anemic population is not good for any government as this will decrease the productivity of the workforce. A study on female workers in a garment factory in Nasinu showed that the productivity of anemic women was decreased by 11% (National Food and Nutrition Centre, 1999).

Folate deficiency as previously mentioned has been linked to the high level of homocysteine in the body which is a risk factor for cardiovascular diseases. It has also been reported that cardiovascular diseases are a major cause of death in Fiji (Ministry of Information, 2004), with the incidence of stroke rising in the last four decades (Maharaj and Panapasa, 2002). This may be an indication of the poor folate status of the general population, however this will need to be verified through bioavailability studies.

Further work is needed by the Ministry of Health with the assistance of relevant authorities from the University of the South Pacific to ensure future reports will specify the type of anemia it is (folate, cobalamin or iron deficient anemia) and to conduct research that look at the homocysteine level in the body as another means of determining the folate status of the population. Determining the folate content in foods in Fiji is imperative; to help combat the occurrence of folate-deficiency related illnesses in Fiji.

1.2.10 Fortification

Fortification may be an effective way of ensuring the intake of the RDA of folate. This is in light of the fact that there are limited data on the folate content of Fiji foods, with the only other study conducted on the food folate content in Fiji conducted by Devi (2008). In 2004, through the collective funding of UNICEF, AusAid and the Flour Mills of Fiji, the Flour Fortification Project was launched in Fiji. The amount of FA added to FMF flour is 160 µg/100g. The US government as a preventative measure for neural tube defect has fortified their flour with FA but at an optimal level so as to ensure that there is no overexposure of FA of non-risk groups. The other countries where mandatory FA fortifications have been introduced are Canada, in some of the South American countries, and in Saudi Arabia. Voluntary fortification has also been introduced in Australia, New Zealand, United Kingdom, Ireland, Hungary, and Asian countries (Kelly *et al.,* 1997).

There have been a reported decrease of 30-50% in the occurrence of NTDs in the US and Canada since the compulsory fortification of cereal grain products has been put in place (Dietrich *et al*. 2005; Molloy, 2005). Fortification of flour has not only decreased

the occurrence of neural tube defects, as it was intended to, there has also been reports on the decreased childhood cancer since fortification (French *et al*. 2003), and a drop in plasma homocysteine has also been observed (Molloy, 2005).

However folate fortification may be problematic as it is untargeted and result in at risk groups receiving very high levels of folate. Of particular concern are those suffering from vitamin B_{12} deficiency which is a common occurrence in the elderly, as the intake of folate fortified foods may mask the symptoms of B_{12} deficiency, resulting in the permanent damage of the nervous system. Folate is only able to carry out its role with the interaction of the vitamin B_{12}, and they share identical deficiency symptoms. Folate may correct the anemia but not the changes in the nervous system caused by vitamin B_{12} deficiency (Gregory *et al*., 2005). The changes in the nervous system are only detected in the later stages when it is incurable. There is also the possibility that high doses of FA supplements can often reverse the effect of anticonvulsants in epileptic patients. Chien *et al*. (1975) conducted a study to determine the effects of intravenous FA on epileptics treated with anticonvulsant drugs. The study observed that in some individuals treated with anticonvulsant drugs, seizure activity was induced. Therefore epileptic patients may need to be cautious with folic acid supplements.

Some researchers have suggested that there should be a long term monitoring of fortification programmes to ascertain that fortification is indeed beneficial and that minimum to no risks are involved (Molloy, 2005). Thus it may be safe to assume that the ingestion of natural folates is the way to go, again emphasizing the need for determining the folate content of foods in Fiji, so as to ensure that the general public are

able to make informed decisions on the type and amount of food to be eaten to satisfy the RDI of folate.

1.2.11 Folate Analysis

Determining the folate content in foods can be difficult due to its multiple forms and low availability in foods (Arcot *et al.,* 2005). There are three main steps involved: sample preparation and purification, deconjugation and detection.

1.2.11.1 Extraction

In natural foods, folate is bound to protein and polysaccharide matrices. Folate is extracted by first heating the food extracts followed by the enzyme treatment.

1.2.11.1.1 Heat Treatment

The food sample is homogenized in a buffer solution before being heated. Various temperatures have been used, however the most commonly used extraction temperatures are 100^{o}C and 121^{o}C (Arcot and Shrestha, 2005). This step ensures that any folate that are physically bound to the food matrix are released.

Heat treatment and digestion with conjugase can be enough when determining the folate content of fortified foods as the folic acid is only physically bound (Tamura, 1998; Rader *et al*., 1998; Shresta *et al*., 2000). However this is not the case when analyzing natural foods as the folates are normally chemically bound to the food matrix. Further steps are needed to ensure the complete release of folates, ensuring there is no underestimation of folate content in foods.

1.2.11.1.2 Enzyme Treatment

Initially, the folate extraction method only comprised of two steps which included heat treatment to release the folate from binding proteins followed by conjugase treatment for the hydrolysis of polyglutamyl folate to monoglutamyl folate. This was the traditional folate extraction method which was also referred to as the single enzyme extraction procedure as it only required the use of only one enzyme.

However in 1990, separate studies by Martin *et al.* and De Souza and Eitenmiller both showed a significant increase in folate content when using the tri-enzyme extraction procedure which involved the use of two additional enzymes α-amylase and protease indicating that this procedure ensured a complete extraction of folate in the food. Treatment with α-amylase and protease ensures the complete release of all folate chemically bound to carbohydrate matrix and protein matrix respectively. It is now the most commonly used method for extraction of folates particularly in foods with high protein, starch and carbohydrate content (Chang and Gage, 2003).

Conjugase is added to hydrolyze pteroylpolyglutamate to pteroylmonoglutamate. This step is referred to as deconjugation. Folates in food are in the polyglutamate form. The techniques used to detect the folate content in food do not respond to this form of folate, only to folate in monoglutamates to tri-glutamate forms. In the deconjugation step, the food samples are treated with conjugase, the enzyme that hydrolyses polyglutamate to mono- or di-glutamates. There are four main sources of conjugase (Gregory 1989). Chicken pancreas has neutral optimum pH and yields folate di-glutamate. Human plasma and rat plasma both have an optimum pH of 6.2-7.5 and yield monoglutamates

as the end product. Hog kidney conjugase has an optimum pH of 4.5-4.9 with mono-glutamates as the end product. Chicken pancreas conjugase is the most commonly used conjugase though it is reported to have very high endogenous folate content (Pederson, 1998; Arcot and Shrestha, 2005). Research by Shrestha in 2003 has shown that there was a difference in folate content when spinach was deconjugated for 3 h and 16 h (Arcot and Shrestha, 2005). Human plasma is cheaper and more easily available than the other conjugase source, it is required in small quantities, has low endogenous content and no additional purification is required, but surprisingly not many of the studies have used human plasma as the conjugase source (Dang *et al*. 2000). This could be due to the unreliable and sometimes very low conjugase activity in both commercial (Sigma) and fresh source (Arcot and Shrestha, 2005). It is also reported to have a short shelf life (Shrestha *et al*., 2000). Rat serum can be easily prepared commercially however the purification steps required to remove the endogenous folate can be tedious and it is susceptible to inhibitors that may be present in the food samples (Arcot and Shrestha, 2005).

Many researchers have stated that the tri-enzyme treatment is essential for the treatment of food prior to folate analyses (Aiso and Tamura, 1998; Rader *et al*., 1998; Martin *et al*., 1990). Tamura *et al*. (1997) reported that there was a significant increase in folate values when extracts were treated with conjugase, α-amylase and protease (tri-enzyme treatment) compared to extracts that were treated with conjugase only (single enzyme treatment). This has also been reported by Rader *et al*. (1998) and Shrestha *et al*. (2000).

A comparative study on the folate content of spinach using the single and tri-enzyme treatment showed the folate yields increase by between 22% and 51%, when using the tri-enzyme treatment (Aiso *et al*., 1998 and Martin *et al*., 1990, Hyun and Tamura 2005). Another study showed the analysed folate content of samples increased by as much as 271% when using the tri-enzyme method (Tamura, 1997). Shrestha *et al*. (2000) showed a similar trend where the analysed folate content of bread increased as high as 130% compared to single enzyme extraction. Extracts also tend to be clearer and more homogenous than the original digestion mixture and require less filtration, when the tri-enzyme treatment is used (Rader *et al.,* 1998). This is especially important when using the microbiological assay as the quantification method, as the method is turbimetric.

However, other independent researches have contradicted this. A group of researchers working separately on spinach have reported that tri-enzyme treatment is not always necessary (Pandarangi, 2004; Iwatani *et al*. 2002; Tamura, 1998). Studies by Pandarangi and Laborde (2004) found that treatment with protease only, followed by conjugase (dual enzyme treatment) is effective enough for determining the folate content in spinach. Konings *et al*. (2001) also came up with the same conclusions. In a similar study by Iwatani *et al*. (2003), the tri-enzyme treatment had no significant impact on the folate content of spinach when comparing with the single enzyme treatment. It should be considered that lower values obtained when treating food with tri-enzyme could be due to prolonged exposure to heat when treating with α-amylase incubation, as folate undergoes oxidative degradation during analyses (Tamura, 1998). Therefore an optimization of the extraction and detection of the food sample needs to be carried out before the actual analysis.

1.2.12 Quantification of Folates

The three principal methods for folate quantification are: High Performance Liquid Chromatography technique, Bio-specific procedures which includes enzyme protein binding assay (EPBA), radio-binding assay and immunoassays, and Microbiological assay.

1.2.12.1 High Performance Liquid Chromatography

The High Performance Liquid Chromatography (HPLC) method is used for either qualitative or quantitative analysis of folate. This method is able to identify and determine the amount of the different folate derivatives in the food sample, an advantage which HPLC has over the other two methods, which are only able to detect the total folate contents in food (Arcot and Shrestha, 2005). This technique involves two steps: separation and purification of the deconjugated food samples and quantification. The most commonly used methods for the separation and purification of folates are ion exchange liquid chromatography and reversed phase chromatography. According to Eittenmiller and Landon (1999) reversed phase liquid chromatography is the preferred and widely publicized method of use with a C-18 column. However Arcot and Shrestha (2005) mentions the preference of affinity chromatography columns by many researchers for the sample purification. In affinity chromatography the biological specifity of folate binding proteins (FBP) is applied (Arcot and Shrestha, 2005). The columns are prepared with immobilized FBP. However the immobilized FBP does not bind to folate in the form of 5-formyltetrahydrofolate, therefore samples with this derivatives will require prior conversion to 10-formyltetrahydrofolate. UV absorbance or fluorescence is used for detection and quantification, although folate values detected

through UV absorbance correlate more with those detected in the microbiological assay (Arcot and Shrestha, 2005). Although HPLC is a better analytical method compared to the other two methods, the purification step is necessary which can be tedious and complicated. Finglass *et al*. (1999) suggest that more work should be done on the optimization of this method as results are often contradictory when using this method. This method also needs to be optimized for other forms of folate other than $5CH_3$-THF.

1.2.12.2 Bio-specific Procedures

There are two types of bio-specific procedures, the enzyme protein binding assay (EPBA) and immunoassays. The protein binding assay uses an enzyme labeled folate binding protein (Finglass *et al*., 1998; Arcot and Shrestha, 2005). Free folic acids are immobilized on the walls of the wells in microtitre plates, which is followed by the addition of the sample combined with the enzyme labeled folate binding protein. The enzyme labeled folate binding protein will bind onto the folate in the samples, if not much folate is present in the sample, more enzyme will bind onto the immobilized folic acid on the surface of the wells and vice versa. The unbound materials are removed by a simple washing procedure. A substrate that changes colour when it comes into contact with the enzyme is added into the wells, with the colour changes measured spectrophotometrically (Prescott *et al.*, 1999). The greater the extent of colour change the lesser the folate content in the food sample analyzed. The immunoassay method uses antibodies instead of enzymes, but has the same principles as the EPBA procedure (Arcot and Shrestha, 2005). Although this method is very sensitive, rapid and inexpensive, the responses between the kits differ and they have a short shelf-life, are only suitable for monoglutamates and are generally not suitable for determining folate

content in food samples (Arcot and Shrestha, 2005). Shane *et al*. (1980) had suggested that this technique had varied response to the different forms of folate. Stralsjo (2002) states that this method can be used after separation by the HPLC method to identify foods that mainly contain $5CH_3$-H_4folate, but it needs to be considered that it is difficult to identify the foods that contain predominantly $5CH_3$-H_4folate before analyses. He states that this method would work as an alternative for the two methods HPLC and Microbiological assays, for the folate quantification of foods that mainly contain $5CH_3$-H_4folate.

1.2.12.3 Microbiological Assay

This was the method used in this project as it is the official method recommended by the Association of the Official Analytical Chemists (AOAC), for compliance purposes (Rader *et al*., 1998). The microbiological assay (MA) method is based on the fact that some bacteria require certain vitamins for growth. In this case the folate content in foods is determined by using folate dependant bacteria *Lactobacillus casei,* which is a lactic acid bacterium. Lactic acid bacterium is used widely in microbiological assays as it grows readily in synthetic and semi-synthetic media and in limited amount of air (as in test tubes) and is non-pathogenic (The Association of Vitamin Chemists, 1951). It is the most commonly used microorganism for this as it responds to most forms of folate; mono-, di-, tri- and to some extent polyglutamates, as opposed to the other two methods of detection (Arcot and Shrestha, 2005). The samples are inoculated with *L. casei* and incubated for the recommended time. The bacterium feeds on the folate in the sample tubes and grow, which will be evident in the turbidity produced. The turbidity produced is proportional to the amount of folate in the food sample. The turbidity produced is

measured using an UV spectrophotometer. A calibration curve is drawn from the optical density of the standard tubes. To determine the folate content in the food samples, its optical density values are interpolated onto to the calibration curve using the equation that comes with the curve.

This procedure also has low set up costs. The disadvantages associated with this method are it can be time consuming and tedious, requires microbial expertise and only measures total folate. This method is based on the assumption that the bacterium is totally dependant on folate for growth but if a factor other than folate influences its growth, it invalidates the assay (Arcot and Shrestha, 2005). In this project the microbiological assay (MA) method will be validated and used to determine the folate content in the chosen food samples. In doing so, the data obtained will add to the nutrient data base that has so far been compiled at the Institute of Applied Sciences and will also serve to put into place an analytical procedure for the analysis of folate at USP.

1.3 Objectives

The objectives of this study were:

I. To validate the microbiological assay method for folates at Institute of Applied Science, USP.

II. To determine the folate content of the chosen food sample.

III. Add to the food composition table of the South Pacific foods.

Chapter 2

Materials and Method

The procedure used for the preparation of *Lactobacillus casei* culture and the microbiological assay are based on the method by Arcot and Shrestha (2005) which was adapted with some modification from Wilson and Horne (1982).

2.1 Chemicals

All the chemicals used were of analytical grade. The sample preparation and assay were carried out in subdued light and all the glassware was wrapped in aluminum foil to minimise exposure to light. Distilled water was used. Refer to Appendix I for the detailed description of media and reagent preparation.

2.2 Preparation of Standards, Bacterial Cultures and Samples

2.2.1 Preparation of Standard

FA was used as the standard for this procedure mainly due to its stability over the 16 ± 2 h of incubation time which is used in this procedure (Ambrosis, 2006). The stock folic acid solution (0.2 mg/mL) was prepared by dissolving 100 mg of folic acid in 500 mL 0.01M NaOH in 20% ethanol. The solution was transferred to several 1 mL eppendorf tubes and stored at -80^{o}C. This can be stored for a period of 6 months. The working standard (200 μg/mL) is prepared on the day of assay, one of the eppendorf tubes is thawed and diluted by transferring 0.50 mL of the stock solution to 500 mL 0.01 M

NaOH in 20% ethanol. This is followed by the preparation of the assay standard (1ng/mL) by transferring 0.50 mL of the working standard to 100 mL of the Dilution Buffer (section 1.6.2 in Appendix 1).

2.2.2 Inoculum Preparation: Glycerol- Cryoprotected Method

The lyophilized *Lactobacillus casei* subspecies Rhamnosus (ATCC 7469) stored in sterile glass vials was obtained from the School of Microbiology and Immunology of the University of New South Wales in Sydney, Australia. The lyophilized culture was aseptically transferred from the glass vial to sterilized Lactobacillus broth (10.0 mL), vortexed and incubated at 37°C for 22-24 h.

On the next day prior, to the end of the incubation of the bacteria in the broth, the folic acid casei medium was prepared. Folic acid Casei medium(9.40 g) and 50 µg of ascorbic acid were accurately weighed and dissolved in 200 mL of distilled water, and mixed with 0.30 mL of working standard solution of folic acid in a media bottle. This medium was then sterilized at 121°C for 10 min and cooled immediately in a running water bath.

At the end of the incubation period of the bacteria in the broth, the folic acid casei medium was inoculated with 0.50 mL of *L. casei* and incubated in a water bath set at 37°C for 20 – 22 h. The appearance of a white mucilaginous cottony mass was indicative of the end of incubation period. The medium was cooled in an ice bath. An equal volume of sterilized and cool 80% glycerol solution was poured into the culture solution, and mixed well by swirling for 2 min. The chilled *L. casei*-glycerol mixture was transferred to eppendorf tubes and stored at -80^{o}C. Prior to an assay, one of the

eppendorf tubes is thawed. All bacterial transfers and inoculations were carried in the laminar flow hood, with sterilized pipette tips. This was to ensure that there was no contamination.

2.2.3 Optimization of the *L. casei* Culture

After the preparation of the bacterial batch, it was optimized to ascertain the culture volume and inoculum load that was ideal for the culture prepared. An eppendorf tube containing the cultures was thawed. The culture volumes of 0.15 mL, 0.20 mL, 0.25 mL and 0.50 mL were transferred into 50.0 mL volumetric flasks, with the final volume made using sterilized 0.85% sterile saline.

Various inoculum volumes ranging from 20 µL, 25 µL, and 50 µL of the different bacterial dilutions mentioned above were tried out. Test tubes in triplicates with the following volumes of the assay standard (1.8.3 of Appendix 1): 0.20 mL, 0.40 mL, 0.60 mL, 0.80 mL, and 1.0 mL were subjected to the different dilution ratios of the bacterial culture and incubated at 37ºC for 16 h. The dilution ratio that gave an ideal curve, had absorbance values within the range of 0.1 to 1.0, and with a low un-inoculated blank, was used for the assay.

2.2.4 Preparation of Food Samples

A total of 24 foods were analyzed for folate content. Foods that were likely to make a significant contribution to the diet of Fijian people were chosen. This was in line with the 2004 National Nutrition Survey by the National Food and Nutrition Center which indicated that the Fijian people's food consumption has not differed from the traditional

pattern of food consumption i.e. staple foods supplemented by a dish of greens either cooked on its own or cooked with animal protein (Thaman, 1990). Though most of the green dishes are often cooked with meat (fish, kai or lamb etc.) this study is only focused on determining the folate content in plant foods as folate occurs in significant amount in plants as opposed to meat based foods. Some of the foods were chosen based on their availability throughout the year and frequency of consumption. The other lot was chosen due to its abundance at different seasons, making them possible alternative sources of folate at those seasons. The foods were also chosen after consultation with the student in the School of Chemistry who was also working on folates at the time this project was carried out, so as not to duplicate the foods but some were done in common to compare results.

Each of the materials were sampled from the Suva market with care taken in ensuring that they were from three different suppliers/geographical location (e.g. Beqa, Tailevu and Naitasiri) and a composite of the samples analyzed. The foods were chopped in subdued light and with care also taken to minimise contact with air. This was done on the same day they were sampled. The chopped foods were put into 125 mL beakers and freeze-dried. The wet weight and dry weight were recorded. The foods were freeze-dried as this ensured that the foods could be stored over a long period of time without losing any of its properties, enabling repeat analysis.

2.3 Extraction

2.3.1 Heat Treatment

The extraction procedure was modified slightly from Arcot and Shrestha (2006). Exactly 1.0 g of the freeze-dried food was measured and transferred to a 125 mL conical flask covered with aluminium foil. To this, 25.0 mL of the extraction buffer was added and mixed well by swirling for 2 min. The homogenate was heated in a 100ºC water bath for 10 min and cooled. In situations where the free folate / folic acid is required to be determined, 10.0 mL of the homogenate is to be transferred into a centrifuge tube and centrifuged for 30 min at 4,000 rpm, with the supernatant collected amber bottles and stored at -20ºC until the assay. However in this project, the total folate in selected foods is determined using the tri-enzyme procedure.

2.3.2 Tri-enzyme Treatment

The procedure by Arcot and Shrestha (2006) was essentially followed with a slight modification. The homogenate (10.0 mL) from the heat treatment was further subjected to the tri-enzyme treatment. The pH of the food extracts were adjusted to 4.5 using 0.1 M HCl. To each conical flask, 1.60 mL of the protease was added, vortexed and incubated at 37ºC for 16 h. At the end of the 16 h, the protease was deactivated by heating at 100ºC for 5 min, before cooling immediately in running tap water. The food extracts were further treated with 1.60 mL of the α-amylase and incubated at 37^{o}C for 4 h. At the end of the incubation period, the enzyme was deactivated by heating at 100ºC for 5 min, before cooling immediately in running tap water. The pH of the food extract was adjusted to 7.2 using 0.1 M NaOH. The rat serum (50 µL) was added to the food

extracts, vortexed and incubated at 37ºC for 3 h. At the end of the incubation period the enzyme was deactivated by heating at 100ºC for 5 min. The food extracts were then transferred into centrifuge tubes and centrifuged at 4,000 rpm for 15 min. The supernatant was transferred into amber bottles and stored at -20ºC until the assay. This food extract was used to determine the total folates in the food and can be kept for up to one month.

2.4 Microbiological Assay

2.4.1 Preparation of Tubes for Assay

The test tubes (12 x 110 mm) were cleaned thoroughly with detergent and anti-bacterial liquid, and air dried overnight at 70°C.

2.4.2 Preparation of Standard Tubes for Assay

The assay standard solution (1 ng/mL) was pipetted into 5 sets of triplicate test tubes in the following volumes: 0.20 mL, 0.40 mL, 0.60 ml, 0.80 mL, and 1.0 mL. Appropriate volumes of the dilution buffer were added to each test tube to bring the volumes up to 1.50 mL. The basal media (1.50 mL) was than added to the test tubes to make final volume in the tubes 3.0 mL. Refer to Table 2.1.

2.4.3 Preparation of Samples for Assay

The sample extracts (which included the un-deconjugated food extracts and SRM 1846) were diluted with the dilution buffer, in such a way that the final concentration of the solutions was between 0.20 to 1.0 ng/mL. The dilution with absorbance value within the

folic acid standard range was determined by preparing three different dilutions for each sample extract at the initial assay. The enzyme blanks were also diluted the same way. The diluted sample extract (0.50 mL) was transferred into triplicate test tubes, followed by 1.0 mL of the dilution buffer. The volumes in the test tubes were then made up to 3.0 mL by adding the basal media. Refer to Table 2.1.

2.4.4 Assay

All the test tubes were then covered with plastic caps and autoclaved for 5 min at 121°C, and cooled quickly in a cold water bath to minimize colour formation and reduce long exposure to high temperature.

Once cool, each test tube (standards, samples, blanks and recovery tubes) was inoculated with 15 µL of the diluted cryoprotected culture except the tubes labeled un-inoculated blank as they were required for zeroing on the spectrophotometer. The test tubes were vortexed for a few seconds and incubated in a water bath set at 37°C for 16 ± 2 h. At the end of the incubation period the assay tubes were removed from the water bath. The spectrophotometer was switched on at least 30 min before the start of the turbidity measurement. The wavelength was set at 540 nm. The spectrophotometer reading was zeroed by inserting the un-inoculated blank.

The assay tubes were vortexed before their contents were transferred into a cuvette and its optical density reading taken. Only the stable readings after 30-60 s were recorded. The absorbance of the inoculated blank and enzyme blank was read, followed by the standards in increasing order of concentration, followed by the sample tubes.

Table 2.1 Preparation of folic acid standards, sample, blank and recovery tubes.

Description	*Volume of Assay Standard (ng/mL)*	*Volume of Diluted Food extract (mL)*	*Volume of Enzyme Blank (mL)*	*Volume of Dilution Buffer (mL)*	*Volume of Basal Media (mL)*	*Total Volume (mL)*
Standard 1	*0.20*	-	-	*1.30*	*1.50*	*3.0*
Standard 2	*0.40*	-	-	*1.10*	*1.50*	*3.0*
Standard 3	*0.60*	-	-	*0.90*	*1.50*	*3.0*
Standard 4	*0.80*	-	-	*0.70*	*1.50*	*3.0*
Standard 5	*1.0*	-	-	*0.50*	*1.50*	*3.0*
Inoculate Blank	-	-	-	*1.50*	*1.50*	*3.0*
Un-inoculate Blank	-	-	-	*1.50*	*1.50*	*3.0*
Enzyme Blank	-	-	*0.50*	*1.0*	*1.50*	*3.0*
Recovery	*0.20*	*0.50*	-	*0.80*	*1.50*	*3.0*
Food samples	-	*0.50*	-	*1.0*	*1.50*	*3.0*

2.5 Quantification

Using the average absorbance values as the ordinate and the concentration of the folic acid solution as the abscissa in logarithmic scale in MS EXCEL, a calibration curve was drawn. The regression curve [y = ln(x) + c] and the R-square value of the calibration curve were also determined using this programme. This equation was used to interpolate the absorbance of the sample tubes to the standard curve. The R-square values were calculated on the standard. Assays with R-square values lower than 0.98 were rejected.

The sample tubes with absorbance values outside the range of standard folic acid solution were discarded. In cases where all three of the different dilutions of the food extracts were outside the folic acid standard absorbance range, the assay was repeated by

either increasing or decreasing the dilutions of food samples depending on the absorbance values obtained. The folate content of the sample in ng/mL was determined by interpolating the optical densities into the regression equation. This value was multiplied by the dilution factor to express the results as µg per 100 g of food.

2.6 Validation of Microbiological Assay Method

The MA method was validated prior to sample analysis.

2.6.1 Accuracy

The Standard Reference Material (SRM) 1846 was included in all the assays. SRM 1846 is a milk-based infant formula powder that was developed by Analytical Systems Research Corporation, Indianapolis, USA. This product was developed primarily for the validation of methods that are used to determine calories, vitamins, minerals, and trace elements in infant formulas and similar matrices. The values obtained were compared with the reference value of 129 ± 28 µg/100g of food in the SRM. If the values fall within the acceptable range of the SRM, the assay was accepted.

2.6.2 Precision

Precision is defined as the measure of random errors or the closeness of agreement between independent tests and may be expressed as repeatability and reproducibility (ISO 5725 -1986E). Repeatability is the closeness of agreement between independent test results obtained using the same method on identical test material in the same laboratory by the same analyst using the same equipment within short intervals of time.

Reproducibility is the closeness of agreement between test results. The former was conducted in this project.

Precision was tested by analyzing an in-house reference material twenty times within a time period, with the data collated used to determine the mean, standard deviation and the coefficient of variation. This was to check for the repeatability of the value. The coefficient of variation determined was then compared with that produced by the Horwitz equation at equivalent mass fraction to indicate the validity of the data collated. Horwitz *et al*. (1980), after examining results of 50 AOAC collaboration studies found that there was a strong empirical relationship between the concentration of the analyte and the precision, which is independent of the analytical method used i.e. the Horwitz equation is an empirical formula that give a good measure of expected repeatability by an experienced analyst. The Horwitz function is widely used as benchmark for the performance analytical methods (Thompson, 2004). The coefficient of variation (CV) of the data collated under repeatability or reproducibility conditions should not exceed the level calculated by the Horwitz equation:

$$CV = 2^{(1 - 0.5 \log C)}$$

Where C is the mass fraction expressed as a power (exponent) of 10.

Table 2.2 Examples of Values calculated using the Horwitz equation

Mass Fraction C	***Equivalents***	***log C***	***1 – 0.5 log C***	***Reproducibility CV (%) = $2^{(1 - 0.5 \log C)}$***
1		*0*	*1.00*	*2*
0.01	*1g/100g*	*-2*	*2.00*	*4*
0.001	*1 g/kg, 100mg/100g*	*-3*	*2.50*	*6*
0.0001	*1,000, 0000 μg/kg, 10 mg/100g*	*-4*	*3.00*	*8*
0.00001	*10, 000 μg/kg*	*-5*	*3.50*	*11*
1 x 10^{-6}	*1 000 μg/kg (1ppm)*	*-6*	*4.00*	*16*
1 x 10^{-7}	*100 μg/kg*	*-7*	*4.50*	*23*
1 x 10^{-8}	*10 μg/kg*	*-8*	*5.00*	*32 (*)*

(*) For mass fractions lower then 100 μg/kg, the application of the Horwitz equation gives unacceptably high values.

Therefore, CVs for the concentrations lower than 100 μg/kg, shall be as low as possible. The reporting units for total folate is μg/100 g, which is equivalent to 10 μg/kg (refer to Table 2.2), therefore the % CV determined must not exceed 32%, to render the data collated valid.

2.6.3 Recovery Calculation

A triplicate set of recovery tubes was also included in all the assays. This was included to test the bias of the analysis. The diluted food extract (0.5 mL), 0.8 mL of the dilution buffer were pipetted into the tubes and spiked with the 0.2 mL of the assay standard solution (0.2 ng).

The % recovery was calculated using the formula given below:

$$\% \text{ Spike Recovery} = \frac{(C_{(\text{sample + spike})} - C_{\text{sample}}) \times 100}{C_{\text{spike}}}$$

Where $C_{(\text{sample + spike})}$ = the measured concentration of spiked sample.

C_{sample} = concentration of the test sample.

C_{spike} = theoretical concentration of spiked sample.

The assays with 95-105% recovery were accepted.

2.6.4 Enzyme, Inoculated and Un-inoculated Blanks

Three sets of blanks were included in all batches of assay. The un-inoculated blank tubes comprising of 1.50 mL of the basal medium and 1.50 mL of the dilution buffer and not inoculated with the diluted bacterial culture was included so that it could be used to zero the spectrophotometer. The inoculated blank comprising of 1.50 mL of the basal medium and 1.50 mL of the dilution buffer and inoculated with the diluted bacterial culture was included in order to account for any folate that may be present in any of the reagents. The enzyme blank comprising of 0.50 mL of the enzyme blank, 1.0 mL of the dilution buffer and 1.50 mL of the basal medium and inoculated with the diluted bacterial culture was included to account for any folate that may be present in the enzymes used.

Chapter 3

Results and Discussion

3.1 Method Validation

Traditionally, peer review had been relied upon for the quality control of analysis procedures. However, this all changed in the 1990s, when a number of researchers and sponsors began to question its validity, resulting in the implementation of quality control systems hence the validation of test methods prior to analysis (Robins *et al*., 2006). Having a quality system in place has a lot of benefits in that it gives credibility of staff and results, satisfaction of client needs, comparability of results.

Whether a test procedure is established to be the standard method, whenever a laboratory is taking up that procedure for the first time, it needs to be validated, as different laboratories will have sets of conditions that differ from each other. This procedure was validated by testing for its accuracy (by analyzing the SRM 1846), precision, and recovery.

3.1.1 Accuracy

The first step in validating a test procedure is to test for how accurate the procedure through the analyses of the Standard Reference Material (SRM) 1846, a spray-dried infant formula. The reference folate content of this SRM is 129 ± 28 μg/100 g of food with the mean value determined 143 ± 7.9 μg/100 g as indicated in Table 3.1.

Table 3.1 Mean total folate values obtained using microbiological assay analysis of SRM 1846 – Infant milk

Standard Reference Material	*Average Determined Value (µg/100 g)*	*Expected Value (µg/100 g)*
SRM 1846 – Infant formula	*143*	*129 ± 28*

* SD = 7.9
* %CV determined = 5.7
* Value is mean of four analyses.

3.1.2 Precision

This was done by analyzing the infant food, Cerelac by Nestle, twenty times so that a better picture of its precision is seen, as it is a microbiological method which is known for its variation. The results obtained are shown on Table 3.2.

Table 3.2 Precision data

In-house reference	*Reference Value (on Packet) µg/100 g*	*Mean Value Determined µg/100 g*	*Standard deviation*	*Coefficient of variance (%)*
Cerelac by Nestle	*22*	*35*	*9*	*27*

* Value is mean of values obtained from 20 replicate assay

The coefficient of variation of the data collated was within the range of 32% as calculated by the Horwitz equation at equivalent mass fraction (1 x 10^{-8}), deeming the data collated to be acceptable. The label on the package stated that the folate content in the infant food was 22 µg/100 mL. However as indicated in Table 3.2, the average total folate content of the Cerelac was determined to be 35 ± 9 µg/100 g from the precision analysis carried out. There are two possible reasons as to why the determined value of folate in the Cerelac is much higher than the value indicated on the label of the product.

Firstly, the data on nutrient table contents of such products may have been estimated by the compilers of the table, from known data of similar food components or may have been calculated by summing up the nutrients of all the ingredients of which the food is composed of (Schakel *et al*., 1997) rather than from actual analyses. International studies have also indicated that the actual levels of nutrients in fortified foods were considerably higher than the label value (Whittaker *et al*., 2001). In this study by Whittaker *et al*. (2001) analysed iron and total folate content of 28 breakfast cereals were found to be considerably higher than the label values with the analysed iron values higher by 80 – 190% and the total folate values higher by 98 – 320%.

Secondly the use of the microbiological assay procedure coupled with the use of tri-enzyme extraction has been reported to increase the detected folate content in foods from 12% to as much as 320% (Martin *et al*., 1990; Rader *et al*., 1998; Tamura *et al*., 1997; Aiso and Tamura, 1998; Shrestha *et al*., 2000; Whittaker *et al.,* 2001). It must be considered though that other studies have indicated that the use of tri-enzyme extraction procedure when analyzing fortified foods such as cereals or the Cerelac product analysed in this project, may not be necessary, as it may actually decrease the folate content determined (Shrestha *et al*., 2000; Hyun and Tamura, 2005). In fortified foods the FA is only physically bound to the food matrix and not chemically bound, therefore the tri-enzyme extraction may not be necessary.

3.1.3 Method Detection Limit

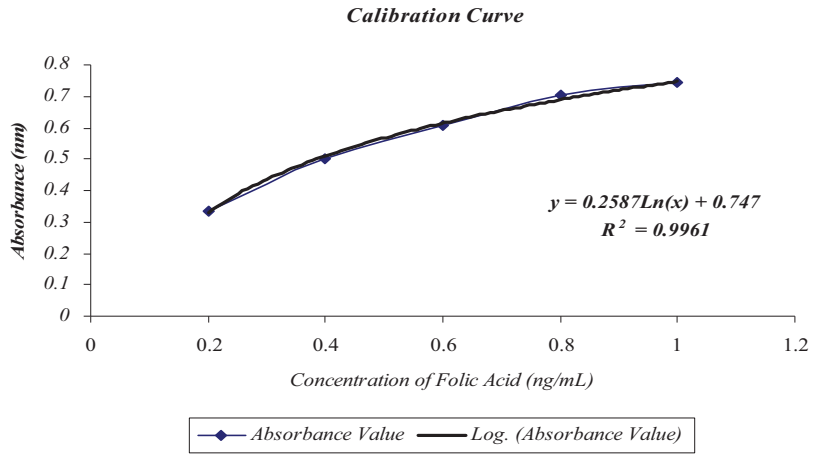
The method detection limit (MDL) was determined by decreasing the volume hence the concentration of the assay standard FA solution (1ng/mL) added to the tubes. The

concentration of the assay standard was systematically decreased from 0.1 ng/mL to 5 x 10^{-4} ng/mL, to ascertain if the bacteria were still able to pick out the folate even at such low concentrations and grow. As indicated in Table 3b (Appendix 3), no turbidity was produced when the concentration of folic acid was decreased to 6 x 10^{-3} ng/mL, therefore the MDL determined in this project is 8 x 10^{-3} ng/mL with the limit of reporting determined to be 1.6 x 10^{-2} ng/mL. These low levels reinforce the fact that MA procedure is highly sensitive.

3.1.4 Calibration Curve

The absorbance values of the five sets of triplicate standard tubes were used to draw the calibration curve from which the folate content of the food samples were interpolated. The R-squared values obtained from the calibration curves were ≥ 0.98. Assays whose R-squared value less than 0.98 were rejected. Shown on the following page is the calibration curve of one of the assays with the R-squared value ≥ 0.98.

Fig 3.1 Typical calibration curve of standard folic acid



3.1.5 Recovery

As part of method validation, recovery studies accompanied all assays, with values within the acceptable range of 95 – 105% as specified by Shrestha *et al*. (2000) indicating the reliability of the method.

3.2 Folate Analysis of Foods

3.2.1 Total Folate Content in Foods

3.2.1.1 Staple Foods

The staple food in Fiji (Jansen, 1991) has remained the same over the years, however, cassava, a non traditional crop (a native plant of the tropical Americas), has at present replaced yam and dalo as the most commonly consumed staple food. The dominant staple foods (kakana dina) are shown in Table 3.3. Before the arrival of the European, dalo and yams were the most important traditional root crops; dalo mainly on the windward or wetter areas of Fiji (such as Rewa) and yams on the leeward or drier side of Fiji (such as the Lau group, Lomaiviti and the Yasawas). Breadfruit, a common tree species in village backyards, is a seasonal staple food. The two main seasons of breadfruit are from November to February and from April to June. Sweet potatoes or kumala is a fast growing root crop and can be ready for consumption in as little as three months. Cassava or tavioka is consumed particularly in the urban areas (where a large portion of Fiji's population is concentrated) due to its ability to grow in soils with poor conditions and less laborious in cultivation hence their easy availability and at low costs

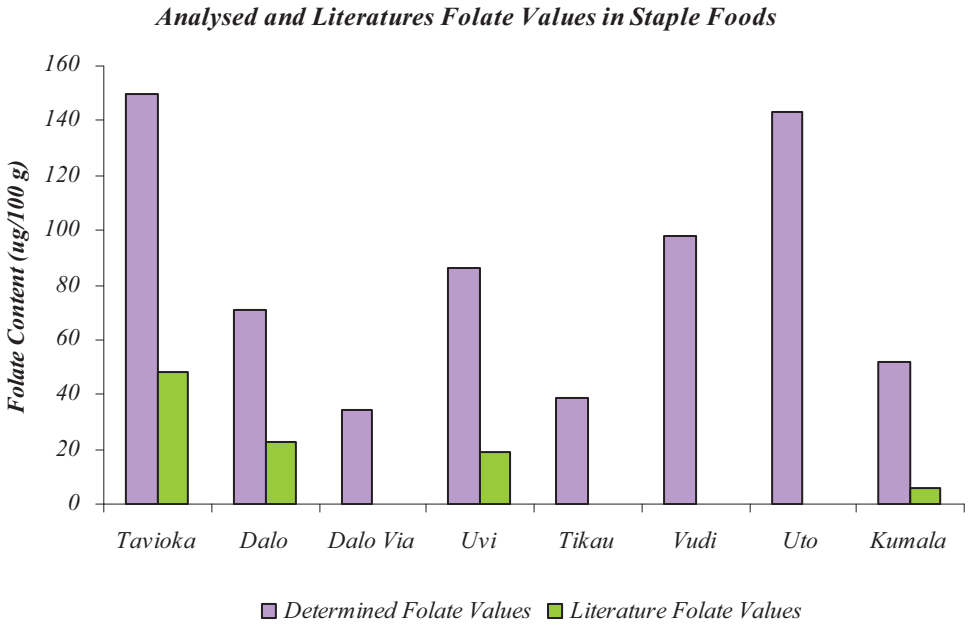
Table 3.3 Total folate content of analysed staple foods (µg/100 g ± SD)

Scientific Name	Common Name	% Moisture	Determined Folate Content (µg/100 g wet wt)	Reference Folate Content (µg/100 g wet wt)	References
Manihot esculenta	Cassava / Tavioka	60	150 ± 13	18 48	USDA Devi 2007
Colocasia esculenta	Taro / Dalo	66	71 ± 19	23	USDA
Cyrtosperma chamissonis	Swamp Taro/ Dalo Via	64	34 ± 8	-	-
Dioscorea alata	Yam / Uvi	74	86 ± 11	12, 19	USDA
Dioscorea nummularia	Wild Yam / Tivoli or Tikau	73	39 ± 2	-	-
Musa spp	Plantain / Vudi	78	98 ± 11	-	-
Artocarpus altilis	Breadfruit / Uto	71	143 ± 36	-	-
Ipomoea batatas	Sweet potato / Kumala	71	52 ± 12	6	USDA

* Values are expressed as mean of triplicate determination.

The total folate content of the staple foods ranged from 34 – 150 µg/100 g of sample on a wet weight basis. The staple crop with the highest total folate content was cassava (tavioka) at 150 ± 13 µg/100 g wet wt, with swamp taro (dalo via) the least at 34 ± 8 µg/100 g wet wt. The literature data for four of the staples analysed were not available but were available for the remaining four samples as indicated on Table 3.3. A comparison of the analysed folate values between the present study and the reported data in the literature is presented in Fig. 3.2. As clearly indicated in Fig 3.2, the literature folate content, were significantly lower than the analysed total folate content.

Fig. 3.2 Analysed and literature folate values in staple foods analysed



3.2.1.2 Green Leafy Vegetables

A traditional Fijian meal is generally made up of the staple crops mentioned in 3.2.1.1 and supplemented by a dish of greens either cooked on its own or with a meat product. The greens that have been chosen in this study are the commonly consumed ones. Taro leaves or rourou are obtained from taro plants that have been planted for the sole purpose of the use of their leaves. Bele is easy to grow, and is often grown as a border plant in village gardens or plantations and commonly grown in backyards of urban households. Amaranth was introduced into Fiji and is either planted in gardens or grows wild in backyards. Watercress is either grown or grows wildly, and is now sold more often in the markets. These plants have been chosen because of their high occurrence and easy availability.

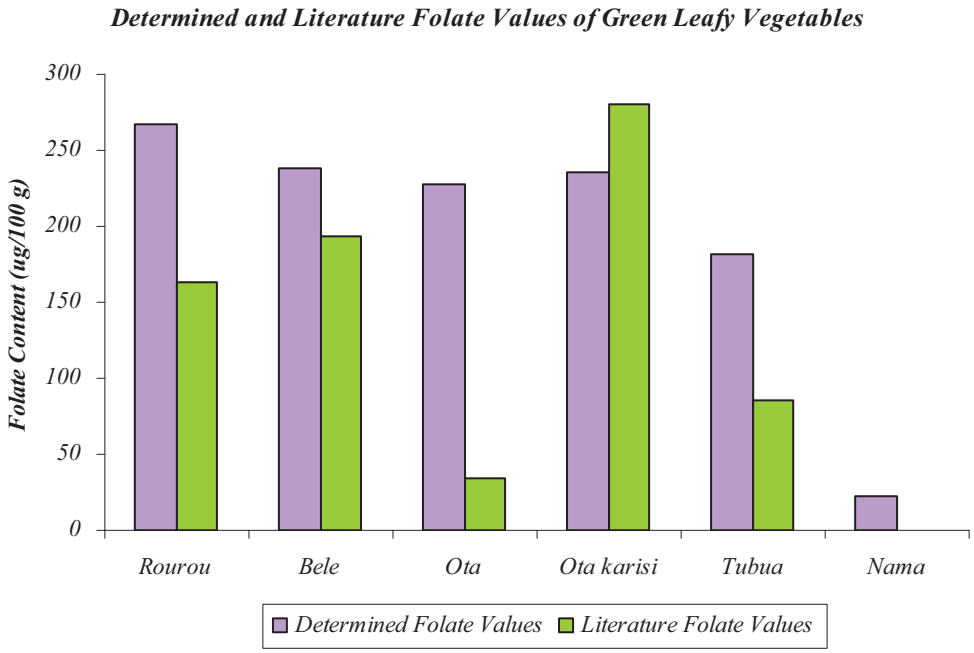
Table 3.4 Total folate content of analysed green leafy vegetables (μg/100 g ± SD)

Scientific Name	Common Name (English / Fijian)	% Moisture	Analysed Folate Content (μg/100 g wet wt)	Reference Folate Content (μg/100 g wet wt)	References
Colocasia esculenta	Rourou	83	267 ± 6	101, 163 6	USDA, Devi 2007
Hibiscus manihot	Bele	86	238 ± 25	194 177	USDA, Devi 2007
Anthyrium esculentum	Ota	91	228 ± 24	34.4 3	USDA, Devi, 2007
Roripa nasturitium-aquaticum	Watercress / Ota karisi	90	235 ± 15	280 200	Iwitani *et al*., 2002
Amaranthus sp	Amaranth /Tubua / Moca	89	182 ± 4	85 57	Iwitani *et al*., 2002, Devi, 2007
Caulerpa racemosa	Sea grapes / Nama	95	22 ± 2	-	-

* Values are expressed as mean of triplicate determination.
* These are the green leafy vegetables commonly consumed by the indigenous population.

The analysed total folate content of the green leafy vegetables ranged from 22 – 267 μg/100 g of sample on a wet weight basis, except for sea grapes (nama) which was relatively low compared to the other vegetables as indicated in Table 3.4. Rourou (*Colocasia esculenta*) had the highest analysed total folate content at 267 ± 6 μg/100 g wet wt, and the remaining four vegetables with folate content within the 22 - 238 μg/100 g wet. Literature values for five out six of the green leafy vegetables analysed were available. As clearly indicated in Fig 3.3, the analysed total folate content of four (rourou, bele, ota and tubua) were significantly higher, except ota karisi, whose analysed total folate content was slightly lower than the literature value.

Fig.3.3 Analysed and literature folate values on green leafy vegetables analysed



* Values are expressed as mean of triplicate analysis ± SD.
* These are the green leafy vegetables commonly consumed by the indigenous population.

3.2.1.3 Fruits

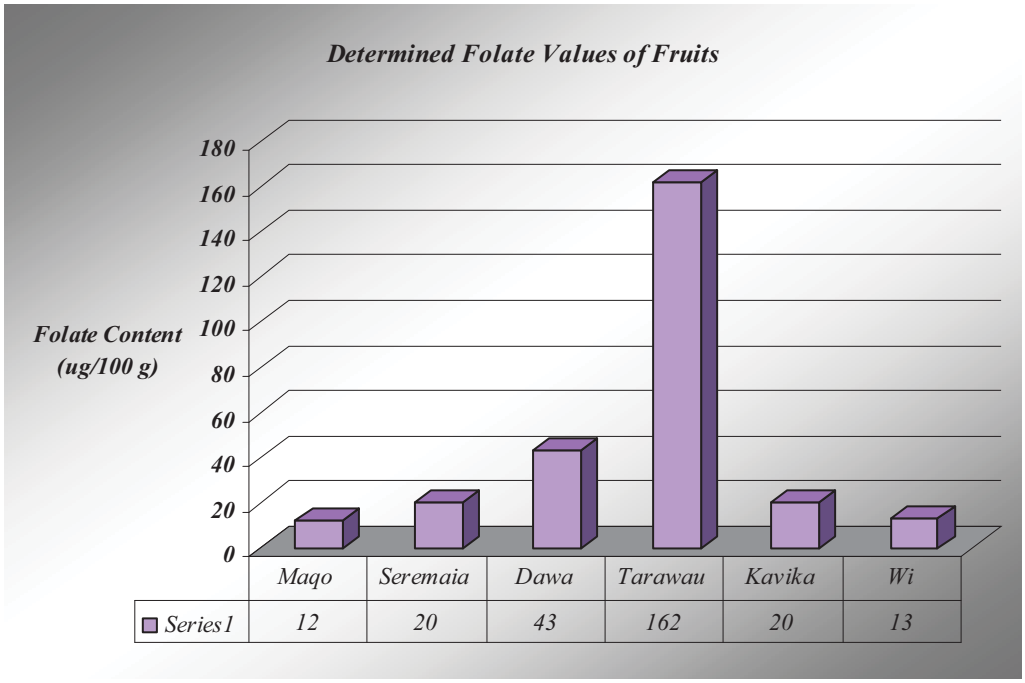
The analysed total folate contents of the fruits analysed were relatively low, with all total folate values < 50 µg/100 g of sample wet wt. However the total folate content of tarawau (162 ± 7 µg/100 g) was significantly higher compared to the other fruits analysed. Only the literature value for mango was available which was significantly lower than the analysed total folate content.

Table 3.5 Total folate content of analysed fruits (µg/100 g ± SD)

Scientific Name	Common Name	% Moisture	Determined Folate Content (µg/100 g wet wt)	Reference Folate Content (µg/100 g wet wt)	References
Mangifera indica	Mango / Maqo	93	12 ± 4	6.5	USDA
Annona muricata	Soursop / Seremaia	92	20 ± 0.55	-	-
Pometia pinnata	Oceanic lychee / Dawa	87	43 ± 3.7	-	-
Dracontomelum vitiense	Tarawau	72	162 ± 7	-	-
Syzygium malaccense	Malay apple / Kavika	94	20 ± 3	-	-
Averrhoa carambola	Wi	89	13 ± 2	-	-

* Values are expressed as mean of triplicate determination.
* These fruits are available in large quantities and cheaply when in season, and are consumed in large quantities by the indigenous population.

Fig.3.4 Analysed total folate values of fruits analysed



3.2.1.4 Other Vegetables

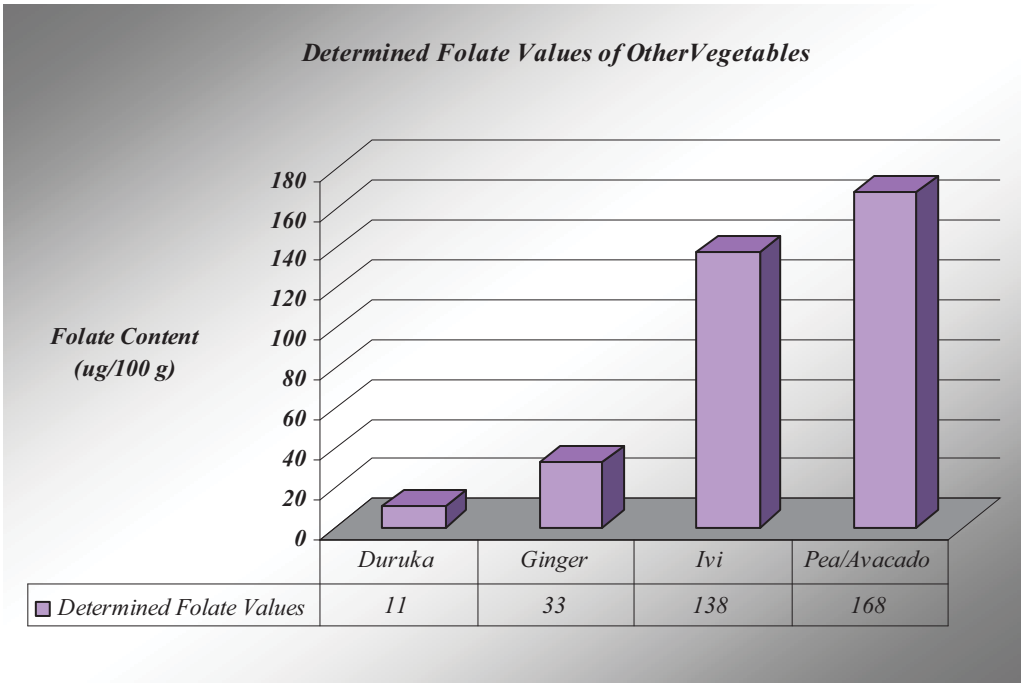
The analysed total folate content of the food samples in this category ranged from 11 – 168 µg/100 g wet wt. Avocado was the only food in this category that had a literature value, this was significantly lower from the analysed value as indicated in Table 3.6. Duruka, a Fijian delicacy, which is in season from April to June, is a relatively poor source of folate (11 ± 0.5 µg/100 g wet wt) along with ginger (33 ± 1 µg/100 g wet wt). However ivi and avocado are relatively rich sources of folate, 138 ± 9 and 168 ± 27 5 µg/100 g wet wt respectively.

Table 3.6 Total folate content of other vegetables analysed (µg/100 g ± SD)

Scientific Name	Common Name	% Moisture	Determined Folate Content (µg/100 g wet wt)	Reference Folate Content (µg/100 g of food)	References
Saccharum edule	Duruka	89	11 ± 0.5	-	-
Zingiber officinale	Ginger	92	33 ± 1	-	-
Inocarpus fagifer	Tahitian chestnut / Ivi	76	138 ± 14	-	-
Persea americana	Avocado / Pea	90	168 ± 27	113.4, 40	USDA

* Values are expressed as mean of triplicate determination.
* These foods are seasonal and consumed in large quantities when they are in season.

Fig.3.5 Analysed folate values of other vegetables analysed



3.2.2 Discussion

The range of the analysed total folate contents of the: staple foods was 34 to 150 µg/100 g on a wet weight basis; green leafy vegetables was 22 to 268 µg/100 g on a wet weight basis; fruits were all < 50 µg/100 g on a wet wt basis except tarawau (162 ± 7 µg/100 g of wet wt) and for other vegetable two had values < 50 µg/100 g on a wet weight basis (duruka: 11 ± 0.5 µg/100 g of wet wt, ginger: 33 ± 1 µg/100 g of wet wt) and two were over > 100 µg/100 g on a wet weight basis (ivi:138 ± 14 µg/100 g of wet wt, avocado: 168 ± 27 µg/100 g of wet wt).

The literature value of 11 out of 24 foods analysed was available. Of these, the analysed total folate content of ten of the foods (cassava, taro, uvi, kumala, rourou, bele, ota,

tubua and mango) was significantly higher than the literature value, with the exception of watercress (ota karisi) whose analysed total folate content was slightly lower than the literature value.

The differences may have been due to a number of reasons. Firstly, the differences may have been due to the differences in the analytical and extraction procedure used particularly for the values obtained from the USDA which did not indicate the analytical procedure used in terms of the analytical method and deconjugation procedure used. This is one of the pressing issues with many food composition tables as they do not indicate the procedure used. It has been reported that the nutrient value of another food, but of the same family, may be used while computing food composition tables as is the case of the USDA nutrient database for standard reference: e.g. (1) folate in Asian pears (*Pyrus pyrifolia*) were taken directly from the pear (*Pyrus communis*) value; e.g. (2) folate values for beef heart was used for the folate value of veal heart, same species but different maturity (Gebhardat, 1992, cited in Schakel 1997).

Four of the foods analysed in this project was also analysed by Devi *et al.* (2008), whose procedure was identical to the one used in this project. The significant difference in the folate content between Devi's analysis (Cassava – 48 µg/100g; rourou - 6 µg/100g; bele – 177 µg/100g and ota - 3 µg/100g) and values obtained in this project (Cassava – 150 ± 8 µg/100g; rourou - 267 ± 6 µg/100g; bele – 238 ± 25 µg/100g and ota – 228 ± 24 µg/100g) may have been due to factors such as season, climate, geography, geology and the variety/species (Mullen *et al*., 1982 cited in Iwitani *et al*., 2002) or the stage of maturation (Gebhardat, 1992, cited in Schakel 1997) of the food sample being analysed.

Another factor may have been the differences in sampling procedure used. In this project food samples were sampled from the Suva market with care taken in ensuring that they were from three different suppliers/geographical location (e.g. Beqa, Tailevu and Naitasiri) and a composite of the samples analyzed, compared to Devi *et al.* (2008) where samples were obtained from one locality with species variation also a factor. Other factors that may have contributed to the differences in analysed total folate content are part of the plant that is being analysed and growing conditions. Seasonal variation has also been identified as a contributing factor to variation in the analysed nutrient content of the same variety as was identified by two separate studies on the variation in the phylloquinone content in green leafy vegetables (Ferland and Sadowski, 1999; Koivu *et al*., 1997; cited in Damon *et al*., 2005).

Two samples (tubua and watercress) were also analysed for total folate by Iwatani *et al*., 2003 where the values obtained were as a result of single enzyme extraction. Watercress was the exception in this project in that the analysed total folate content (205 ± 6.6 µg/100 g) was lower than the literature value (Iwatani *et al*., 2002) of 280 µg/100 g) though not significantly so. Therefore based on the data obtained from this study, the variation in the analysed total folate content when using either single or tri-enzyme extraction is not significant. However in the case of tubua the analysed folate content was significantly higher than the analysed total folate content obtained from the single enzyme treatment by Iwatani *et al.* (2003). This difference may have been due to differences in geographical location and growth condition, maturation stage and variety as mentioned earlier. Therefore it must always be considered that the actual effect of a particular technique will vary from food to food and it is suggested that an initial

optimization of the extraction and detection method be carried out for different food groups (Arcot and Shrestha, 2005).

The results from this study clearly indicated that the green leafy vegetables (with the exception of nama), the three staples: cassava; breadfruit and plantain, tarawau (fruit) and ivi as rich sources of folate. The remaining staples are moderate sources of folate, and to some extent meet the RDI for folate because of their high daily consumption by the Fijian population. The remaining fruits (mango, soursop, dawa, Kavika and wi) and vegetables (duruka and ginger) are relatively poor sources of folate. However due to their high abundance and consumption when they are in season, these fruits may contribute to some extent to the folate intake of the Fijian population. These fruits would be better snacking options as opposed to the highly processed, salty snacks available in supermarkets, and still contribute to folate intake, in addition to the consumption of other natural foods in the Fijian diet. Consumption of four mangoes, kavika and wi, and one quarter of a soursop fruit, would individually contribute to half the RDI for folates in children and should compliment the folate intake from normal meals.

In conclusion, results from this study demonstrates that the Fijian population are easily able to meet their folate RDI by ensuring that their daily menu are modeled to the traditional Fijian menu of staple foods with green leafy vegetable side dish, and snacking on seasonal fruits.

Chapter 4

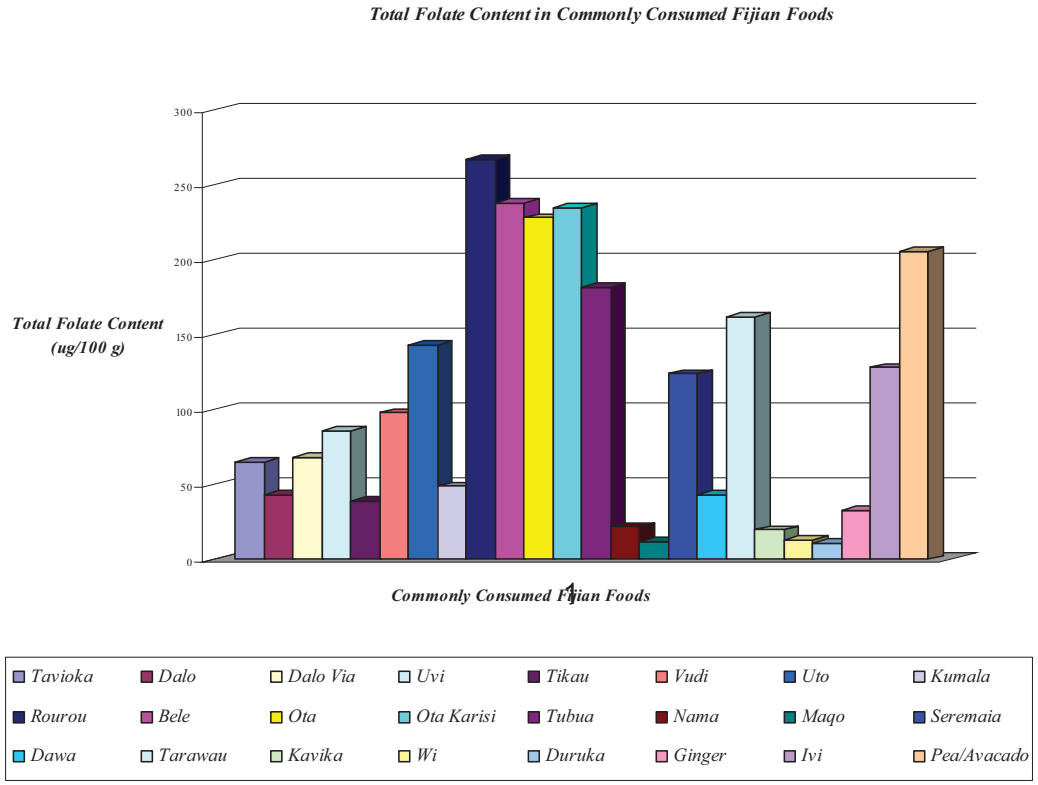
Conclusions and Recommendations

The objectives of this study were met. The analytical method, tri-enzyme extraction coupled with microbiological assay method, was successfully validated. It must be noted that great care must be taken when conducting the analyses from sample preparation (weights, pH alteration, enzyme treatment and the incubation time) as any slight deviation from the required parameters will be greatly magnified due to the low units being used (initial form ng/mL to μg/100g) and the use of microorganisms as an analytical tool.

The total folate content of the 24 food samples analysed ranged from 11 – 267 μg/100 g of sample wet wt. Of these four of the samples had total folate content within the 200 – 267 μg/100 g of sample wet wt range (rourou, bele, ota, ota karisi), six in the 100 – 199 μg/100 g of sample wet wt range (cassava, avocado, tarawau, uto, ivi, and tubua), four in the 50 – 99 μg/100 g of sample wet wt range (vudi, yam, dalo, kumala) and with the remaining ten samples having total folate content that is < 50 μg/100g of sample wet wt range (swamp taro, wild yam, kumala, nama, mango, soursop, dawa, kavika, wi, duruka and ginger) and are therefore poor sources of folate. Literature data for the folate content for 14 of the food samples analysed was not available, however for those samples whose literature folate content was available, the analysed total folate contents were higher by

22 to up to 80%, except in the case of watercress which was slightly lower than the literature value.

Fig.3.6 Analysed total Folates of commonly consumed Fijian foods



From the data obtained from this study, it can be stated that the Fijian population has access to folate rich foods. The study by Winkels *et al*. (2007) in the Netherlands suggests that a diet composed of fruits and vegetables collectively rich in folates can efficiently meet the RDI of folate, it can therefore be concluded that this may be also the case for the Fijian population provided that the daily diets of the Fijian population are modeled around the traditional Fijian diet of staple foods with a green leafy dish coupled

with whichever fruit (that has been analysed) that is in season for snacking purposes. The staple foods which are also eaten in large quantities can also make a significant contribution.

The success of future folate-related studies will depend primarily on the existence of data on total folate content of all foods which are of high reliability (Gregory, 2001). Therefore further studies should be conducted to ensure that the total folate content of most if not all of the foods in Fiji and the Pacific are determined. The effect various cooking methods has on the folate content in foods should also be determined. Due to the wide variation in the value from previous values obtained in Fiji and elsewhere, and the values obtained in this study, additional analyses may need to be performed on these foods to better understand the range of values. The use of tetra-enzyme extraction procedure is also recommended when determining the total folate content of foods containing inherent or added fat. Tetra-enzyme extraction procedure is an extension of the tri-enzyme treatment where an additional enzyme, lipase, is included. The use of the tetra-enzyme extraction procedure has been shown to significantly increase the total folate content in foods containing fats (Vishnumohan, 2008).

This should then be followed by conducting an extensive study on the bioavailability of the folate as at present there are no available data on actual folate status of Fiji. Having access to folate rich foods does not necessarily equate to a high folate status of a population, which is why bioavailability studies are of great importance. These bioavailability studies will determine the effectiveness of the diet of a population, the bioavailability of different foods and the influence various cooking methods have on the

absorbance of folate in foods by the intestine, information that can eventually be used to determine the folate status of a population. Knowledge obtained from bioavailability studies will not only give a clearer indication of the folate status of the population but may also indicate the ideal cooking method to use in order to retain as much folate as possible in the food samples so the general public may be informed accordingly and foods with high bioavailability (with regards to folate absorption).

Lastly, the Institute of Applied Science, may also consider analysing the folate content of foods using the liquid chromatography-tandem mass spectrometry (LC-MS/MS). This procedure, like HPLC is able to differentiate between the various folate derivatives, however it is much more accurate and highly sensitive. Recent comparative studies have shown excellent agreement between the LC-MS/MS procedure (folate derivatives) and the MA procedure (total folate) (Fazili *et al*., 2007; Patring, 2007; Vishnumohan, 2008). IAS may develop this procedure if it considers conducting bioavailability studies, where the differentiation of the various forms of the vitamin is necessary. However for commercial analyses, the MA procedure may be sufficient which also has the added advantage in that it uses facilities that will be available 12 months a year. The LC-MS/MS procedure may only be considered should there be a demand from customers for the analysis of folate derivatives

Chapter 5

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Appendix 1

Reagents

1.1 Agar culture medium – *Lactobacillus* agar.

This was obtained from Difco Laboratories. It was prepared by dissolving 4.8 g of the agar in 100 mL of distilled water by heating on hot plate for 2 min. The molten agar was distributed into 10 mL MaCartney bottles and sterilized at 12^{o}C for 15 min. The agar was cooled to set at a slanting position, and stored at 4^{o}C.

1.2 MRS agar – *Lactobacillus* agar.

This was obtained from Difco Laboratories. It was prepared as indicated on the label. The agar was cooled to set at a slanting position, and stored at 4^{o}C.

1.3 Culture suspension – *Lactobacillus* broth AOAC.

It was prepared as written on the label. It is a broth so did not require to be cooled at a slanting position.

1.4 Folic acid *Lactobacillus casei* Medium.

It was prepared as written on the label, but no ascorbic acid was added as indicated on the label. The ascorbic acid added to the extraction and dilution buffer was deemed sufficient. This was prepared fresh on the day of use.

1.5 *Lactobacillus casei* culture subspecies Rhamnosus, ATCC 7469.

This was obtained from the Department of Immunology and Microbiology of the University of New South Wales, Sydney, Australia. Refer to section 2.2.2 and 2.2.3 for its preparation and optimization.

1.6 Phosphate ascorbate buffers:

1.6.1 Extraction buffer – 0.1N phosphate, 1.0% ascorbic acid, pH 6.1.

The following were measured correctly: 13.1 g KH_2PO_4, 17.42 g K_2HPO_4, and 10 g of ascorbic acid. This was dissolved in 400 mL of distilled water by stirring with a magnetic stirrer. This was quantitatively transferred to a 1 L volumetric flask and the volume made up with distilled water. The pH was adjusted to 6.1 using 0.1 M HCl. This was prepared fresh on the day of use.

1.6.2 Dilution buffer – 0.05N Phosphate, 0.15% ascorbic acid, and pH 6.1.

The following were measured correctly: 6.81 g KH_2PO_4, 8.71 g K_2HPO_4, and 1.5 g ascorbic acid. This was dissolved in 400 mL of distilled water by stirring with a magnetic stirrer. This was than quantitatively transferred to a 1 L volumetric flask with the volume made up with distilled water. The pH was also adjusted to 6.1 using 0.1 M HCl. This was prepared fresh on the day of use.

1.7 NaOH (0.01 M) in 20% ethanol.

NaOH pellets (0.4 g) was accurately weighed in an appropriate apparatus and dissolved in 400 mL distilled water. This was than transferred to a 1 L volumetric flask along with 200 mL of ethanol and again mixed well by stirring with a magnetic stirrer. The volume was made up with distilled water.

1.8 Folic acid (pteroyl glutamic acid or PGA).

The chemical was obtained from Sigma.

1.8.1 Stock solution (0.2 mg/mL)

This was prepared by accurately weighing out 100 mg of folic acid and transferring to a 500 mL volumetric flask. The final volume was made up by adding 0.01M NaOH in 20% ethanol (section 1.7). The solution was transferred to several 1 mL eppendorf tubes and stored at -80^{o}C. This could be stored for a period of 6 months.

1.8.2 Working standard solution (200ug/mL)

On the day of assay, an eppendorf tube of the stock solution (section 1.8.1) was thawed. 0.5 mL of the stock solution is transferred to 500 mL volumetric flask. The final volume is made up by using 0.01 M NaOH in 20% ethanol.

1.8.3 Assay standard solution (1 ng/mL)

This is prepared fresh on the day of assay. The working standard (0.5 mL) was pipetted into 100 mL volumetric flask. The volume was made up by using the dilution buffer (section 1.8.2).

1.9 Conjugase

There are various sources of conjugase which includes human plasma, rat serum, chicken pancreas and hog serum (Gregory, 1989; Arcot and Shrestha, 2005). However in this project, rat serum was used.

1.9.1 Rat serum

It was obtained from Sigma (R9759). The serum was thawed and transferred to a 1 ml eppendorf tubes at 0.5 mL volumes and stored at -20^{o}C. On the day of use, the appropriate number of the eppendorf tubes was thawed, depending on the amount of samples being analysed.

1.9.2 Human Plasma

A pint of frozen human plasma was obtained from the Blood Bank at the Colonial War Memorial Hospital, Suva. The plasma was re-distributed 5 mL volumes in vials and stored at -20^{o}C

1.9.2 α-amylase (20 mg/mL)

The α-Amylase (1.0 g) was accurately weighed into a 100 mL beaker. Distilled water (50 mL) was added to the beaker and stirred vigorously for 5 min to ensure thorough mixing. The solution was transferred to 50 mL centrifuge tube and centrifuged at 3000 rpm for 10 min. The supernatant was further filtered with glass wool. The filtrate was collected in a beaker, covered with parafilm and stored at 4ºC. This was prepared fresh on the day of use.

1.9.3 Protease (2mg/ml) from megazyme, subtilisin A and from B licheniformis

The protease solution (2 mL) was suspended in 50 mL of distilled water. The solution was stirred for 5 min, before being filtered through glass wool. The filtrate was collected in a beaker and covered with parafilm and stored at 4^{o}C.

1.10 Hydrochloric acid (0.1 M)

Hydrochloric acid with a concentration of 32% (9.8 mL) was accurately measured into a 1 L volumetric flask. Distilled water (400 mL) was added to the volumetric flask. The solution was mixed well for a few min by stirring with a magnetic stirrer. The volume was made up with distilled water.

1.11 Sterile solution (0.85 %)

NaCl (1.7 g) was accurately weighed and dissolved in 200 mL distilled water in a media bottle. The solution was autoclaved at 121ºC for 15 min. It was stored at 4^{o}C.

1.12 Glycerol (80%)

Glycerol (160 mL) and 40 mL of distilled water were transferred to a 500 mL media bottle and mixed well. The solution was autoclaved at 121^{o}C for 15 min. It was stored at 4^{o}C.

1.13 Standard Reference Material (SRM) 1846

It is a spray dried milk based formula prepared by NIST of USA.

Appendix 2

Equipment and Apparatus

2.1 Water bath maintaining a temperature of 37^{o}C – Laboratory Equipment, PTY Limited, LABEC.

2.2 Autoclave – flowing steam autoclave, ALP MCY – 40.

2.3 Heating plate with magnetic stirrer.

2.4 Plastic centrifuge tubes – 50 mL capacity.

2.5 pH meter – Hanna pH211.

2.6 Cold centrifuge – IEC Centre CL3R, refrigerated centrifuge, Thermo Electron Cooperation.

2.7 Spectrophotometer – Perkin Elmer, Lambda 3B UV/VIS Spectrophotometer.

2.8 Test tubes – 12 x 110 mm, Pyrex, England.

2.9 Eppendorf tubes – 1.5 mL capacity.

2.10 Test tube racks.

2.11 Vortex mixer.

2.12 Bunsen burner.

2.13 Automatic pipettes – 5 mL, 1 mL and 100 µL, Eppendorf.

2.14 Analytical balance – four places after decimal, Shimadzu AEX 200 g, serial # D419902030.

2.15 Hot air oven drier set at 105^{o}C – Contherm ovens, Model 2050 - 2400.

2.16 Incubator – set at 37^{o}C.

2.17 Media bottles – 500 mL, 250 mL, Shcotts, Germany.

2.18 Volumetric flasks – 100 mL, 250 mL, 500 mL, 1000 mL

2.19 Automatic water distillation apparatus.

Appendix 3

Table 3a Values obtained for the analysis of SRM 1846 for 4 assays.

No	Extraction/Analysis Date	Experimental Value (µg/100 g)	Certified Value (µg/100 g)
1	17/7/07	135	129 ± 28
2	4/8/07	154	
3	12/8/07	141	
4	9/11/07	142	
	Average	143	
	SD	7.9	
	%CV	5.7	

Table 3b Limit of detection (LOD)

Folic acid concentration (ng/mL)	**Absorbance after inoculation and incubation with *L. casei* (nm)**
0.1	0.251
5×10^{-2}	0.241
4×10^{-2}	0.227
3×10^{-2}	0.175
2×10^{-2}	0.152
1×10^{-2}	0.129
8×10^{-3}	0.016
6×10^{-3}	0.00
4×10^{-3}	0.00
2×10^{-3}	0.00
1×10^{-3}	0.00
5×10^{-4}	0.00

- Method detection limit = 8×10^{-3} (ng/mL).
- Limit of Reporting = MDL x 2
  $= 1.6 \times 10^{-2}$ (ng/mL)

(2002, IUPAC, Pure and Applied Chemistry 74, No. 5 pg 851).

Table 3c Repeatability Data

Extraction #	Calculated Value (µg/100 g)	Reference Value (µg/100 g)
1	38.34	22
2	44.06	
3	21.88	
4	29.33	
5	37.03	
6	27.81	
7	22.69	
8	40.31	
9	36.33	
10	37.61	
11	35.75	
12	21.91	
13	28.48	
14	16	
15	40.57	
16	46.92	
17	35.88	
18	35.57	
19	53.41	
20	41.09	
Mean	35.54	
SD	9.36	
% CV	27.1	

* Values collated as a result of analysing infant food, Cerelac by Nestle, twenty times.