The influence of formulation and processing on the retention of B vitamins in Asian noodles

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by

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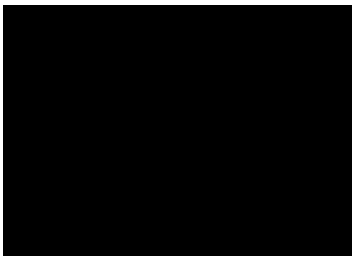
Declaration

I hereby declare that all work carried out in this project was performed while I was enrolled as a Ph.D. student in the School of Life Sciences and Technology, Victoria University of Technology, Werribee Campus. To the best of my knowledge, this work has not been submitted in whole or part for any other degree or diploma in any University and no material contained in this thesis has been previously written or published by another person, except where due reference is made in the text.



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Publications and presentations

Parts of the research reported in this thesis have been published and the details are as follows:

Journal article

Lan T. T. Bui and Darryl M. Small. 2003. The influence of formulation and processing on stability of thiamin in three styles of Asian noodles. Food Chemistry – accepted for publication.

Conference presentations

Four papers were presented at the 51st Australian Cereal Chemistry Conference held in Sydney in September 2001. The titles of the presentations are listed below. In addition, the page numbers for each correspond to the proceedings of the conference, edited by M Wootton, IL Batey and CW Wrigley, and published in 2002 by the Cereal Chemistry Division of the Royal Australian Chemical Institute, Melbourne, ISBN 1 876892 03 2.

The stability of B group vitamins during storage of Asian noodles, pages 228-231.

The influence of formulation and processing on stability of B group vitamins in Asian noodles, pages 232-236.

B group vitamins in Asian noodles – a study of commercial products, pages 217-220.

Methods for extraction and analysis of B group vitamins from flour and cereal based foods, pages 237-240.

Abstract

Foods prepared from wheat flour represent a potential source of B group vitamins in the diet. Although Asian noodles account for the end-use of at least twelve percent of all wheats produced globally, the contribution of these foods to dietary intakes of B vitamins is unknown. Other issues are the instability of vitamins and the analytical challenges of vitamin analysis resulting from the presence of vitamers in many foods. Accordingly the aims of this investigation were firstly to re-evaluate and validate procedures for extraction and measurement of B group vitamins in wheat flour and the three major styles of Asian noodles. Secondly, the stability of thiamin, riboflavin, folate and vitamin B-6 during processing of noodles has been investigated.

Initial studies of HPLC procedures which might allow simultaneous determination of all the B vitamins of interest indicated that no one approach to extraction and analysis provided reliable results. Accordingly the vitamins were investigated separately. In each case various alternative procedures were compared and these included a number of heating and enzymatic treatments for extraction as well as analysis methods based on microbiological growth, fluorimetry and HPLC. The procedures found to be most suitable were validated in terms of analyses of a reference sample, along with repeatability and recovery studies.

The three common vitamer forms of vitamin B-6 were found in flour although levels were relatively low. Fortification of flour resulted in Asian noodles with enhanced levels of the vitamin. In all three styles of noodle, losses occurred during dough mixing and also during cooking. The overall losses were in the range 57-65 percent of initial levels. The varying formulations and processes for each style had little impact on stability of added pyridoxine used for fortification.

Various published methods for extraction and analysis for riboflavin have been evaluated. Enzymatic digestion gave higher results for riboflavin than methods using acids and reverse phase chromatography was found to be effective with a solvent system including methanol and hexanesulphonic acid. When this was applied to white salted and yellow alkaline styles of Asian noodles stored under various conditions it was

observed that losses occurred particularly in fresh noodles. Moisture content, product pH and temperature appear to be the primary determinants of riboflavin retention. The stability of riboflavin varied markedly between the different styles with losses typically occurring at each step. Prior to cooking, losses of between 27 and 38 percent of the riboflavin were found in fortified noodles. During boiling to the optimum point, a further decline occurred resulting in overall losses of 52-71 percent. The incorporation of alkaline salts in the formulation appeared to be the primary factor influencing the extent of losses in the three styles of Asian noodles.

Folate was analysed by a microbiological method employing *Lactobacillus casei* in combination with enzymatic extraction procedures. The application of conjugase, α-amylase and protease was found to give the highest results for total folate contents of flour and noodle samples. Noodles were fortified with folic acid and up to forty percent of the added vitamin was lost for all three styles of noodle. The losses were greater during boiling in comparison with other steps in manufacture. Overall losses for each style were similar. The proportion of original folate levels present in the formulation which was lost during processing was the same for unfortified noodles and for varying levels of fortification up to amounts of half RDI per serving. It is concluded that all three styles of Asian noodles can provide effective vehicles for folate fortification.

A standard fluorometric procedure was selected and validated for the determination of thiamin in flours and noodle samples. A range of dried commercial Asian noodles was chosen to include white salted, yellow alkaline and instant styles of noodles and the thiamin contents measured before and after cooking. For most samples, the thiamin levels were relatively low and considerable losses occurred during cooking. Greater losses appeared to occur where higher pH was observed.

Thiamin losses during processing of white salted noodles in the laboratory were relatively low, whereas for yellow alkaline noodles decreases occurred at each step of preparation, including dough mixing and sheeting. During drying most of the loss in thiamin occurred in the first twenty-four hours. Upon extended storage, there was virtually no further decline in the thiamin level. Storage of fresh yellow alkaline noodles under different conditions including room, refrigeration and drying temperatures

showed only a minor impact on the overall decrease. For instant noodles, some losses occurred but these were lower than for yellow alkaline noodles. The boiling of dried noodles of all three styles resulted in significant losses of thiamin. Overall, as much as 97 percent of thiamin originally present in flour was lost during processing of yellow alkaline noodles, 68 percent for instant noodles and 44 percent for white salted noodles. It is concluded that the greater losses in instant noodles and particularly yellow alkaline noodles are due to the higher pH values of these products.

When formulations incorporated vitamins for fortification purposes, the stabilities of the various B group vitamins generally varied for the three different styles of noodles. Typically more than half of the added vitamin was lost upon cooking and overall losses were high. Folic acid was the most stable of the vitamins and these products represent a suitable vehicle for enhancing dietary intakes of this nutrient. Further investigation into the B group vitamins is warranted regarding ways to enhance stability, particularly where alkaline conditions contribute to losses of riboflavin and thiamin.

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Abbreviations

*a**	redness
AACC	American Association of Cereal Chemists
ANZ	Australia New Zealand
AOAC	Association of Official Analytical Chemists
*b**	yellowness
BRI	Bread Research Institute of Australia
FAO	Food and Agriculture Organisation
FDA	Food and Drug Administration (United States of America)
g	acceleration due to gravity
HPLC	High-performance liquid chromatography
ICC	International Association for Cereal Science and Technology
IFT	Institute of Food Technologists (Chicago)
IUPAC	International Union of Pure and Applied Chemistry
L	*Lactobacillus*
*L**	lightness value
n	the number of replicate analyses used in calculation of individual results
na	not applicable
NA	nicotinic acid
nd	not determined
NTA	nicotinamide
NHMRC	National Health and Medical Research Council (Australia)
PL	pyridoxal
PM	pyridoxamine
PN	pyridoxine
r^2	coefficient of determination for a regression curve or line
RDA	recommended dietary allowance
RDI	recommended daily intake
rpm	revolutions per minute

sd	standard deviation
TCA	trichloroacetic acid
UK	United Kingdom
US	United States of America
USDA ARS	US Department of Agriculture, Agricultural Research Service
uv	ultraviolet

Explanatory notes

The purpose of these notes is to briefly describe the approaches adopted during the preparation of this thesis. They relate to the nomenclature of vitamins and other chemical compounds, spelling, units of measurement, the expression of analytical results, as well as the referencing of literature sources:

1. In the naming of chemicals including the vitamins, the most recent recommendations of the International Union of Pure and Applied Chemistry have been followed (see Chapter 2).

2. The structures of vitamins presented in Chapter 2 have been drawn using CS ChemDraw Ultra® software (version 5.0) supplied by CambridgeSoft Corporation, Cambridge MA.

3. Where alternative spellings are in common use then the British rather than the American approach has been adopted in the text. Examples include the term colour (rather than color), words ending with –ise (rather than –ize) and some technical terms.

4. Generally, for the presentation of results SI units have been used, particularly for vitamin contents. It is noted that this differs from the approach generally used in food composition tables and much of the relevant scientific literature where nutrient data are expressed per 100 g of food. Accordingly, for comparison purposes some literature data was recalculated prior to presentation here.

5. Generally experimental data is presented on a dry weight (or dry matter) basis rather than a fresh weight (or as is) basis unless otherwise clearly specified. The reason that this approach was adopted has been to facilitate direct comparisons of results obtained at different processing stages during manufacture of Asian noodles. Again, some literature values have also been recalculated to facilitate comparisons.

6. The moisture contents and the time required for cooking to the optimum point were routinely measured for each noodle sample prepared and at different stages of processing. At different stages of this study, variations in the results were found, probably reflecting some minor differences in sizes of noodle strands obtained from the cutting rolls and also due to the variations in flour types and characteristics.

7. In the citation and listing of references and information sources, the current recommendations of the Institute of Food Technologists (IFT) for the Journal of Food Science (IFT 2002) have been applied throughout (see page 273).

Chapter 1

Introduction

The purpose of this chapter is to provide a very brief overview of the research program described in this thesis on the stability of B group vitamins during processing of Asian noodles. The project has been developed on the basis of the following issues:

- The B group of vitamins are essential to human health;
- There is considerable scientific evidence that at least some of the B vitamins are deficient in the diets of many individuals even in developed countries including Australia. It is likely that many more people are adversely affected in developing countries;
- A daily supply of these vitamins is required for health as a result of their water solubility and the excretion of amounts in excess of bodily requirements;
- The vitamins have varying stabilities and can be lost during processing of foods due to sensitivity to light, heat, oxidation, reactivity with food additives as well as different conditions of pH;
- Cereal-based foods represent a major source of the B group vitamins although losses do occur during milling as higher concentrations are found in the outer layers of these grains;
- Asian styles of noodles represent a major end use of wheat with an estimated proportion of more than twelve percent of total world wheat production used for these products. A number of distinct styles of noodles are popular in Asia and these include the traditional yellow alkaline types, the white salted styles and the newer instant noodle products;
- Consumer preferences also vary widely on a regional basis in the various countries where Asian noodles are popular;
- A range of ingredient formulations is used in noodle manufacture and these influence product quality particularly the colour and texture of the products. Furthermore, there are many variations in the specific processing steps applied during manufacture and drying as well as storage of Asian noodles;

- Currently, very little compositional data is available internationally on Asian noodles. In addition, there is virtually no published research into the factors influencing their nutritional quality, the significance of regional preferences and the impact of formulation and process variables.

Accordingly, this research proposal is based upon the hypothesis that for at least some styles of Asian noodles, considerable losses of the B vitamins may occur as a result of differences in formulation and the specific processes applied during manufacture and storage.

This project therefore seeks to investigate the influence of formulation and processing on the retention of B vitamins for various styles of Asian noodles.

Chapter 2

Background and literature review: the significance, sources and stability of the B group vitamins

The purpose of this chapter is to provide background and review the relevant scientific literature on the B group of vitamins. The areas covered are the chemical structures of the vitamins, their nutritional significance, deficiency symptoms and the adequacy of dietary intakes. In addition vitamin stability under various conditions encountered during food processing and fortification are reviewed.

2.1 B group vitamins as nutrients and food components

The vitamin B group is a complex of eight water-soluble vitamins which are thiamin, riboflavin, niacin, vitamin B-6, pantothenic acid, vitamin B-12, biotin and the folates. It is noted that the most recent recommendations of the International Union of Pure and Applied Chemistry (IUPAC) on nomenclature of vitamins (IUPAC 1966, 2002) have been used in this thesis.

The B group vitamins are necessary for the metabolism of carbohydrates, fats and proteins and are therefore essential for growth. They are involved in maintaining the health of the hair, skin, nerves, blood cells, immune system, hormone-producing glands and digestive system. As the B vitamins are water-soluble, excesses are excreted in the urine rather than being stored and a daily intake is necessary to maintain health (Robinson 1987; Reavley 1998).

Typically the major dietary sources include liver, kidney, whole grains, all seeds, nuts, dairy products, eggs, bran, wheat-germ, yeast and leafy green vegetables (Reavley 1998). Cereal grain products are a good source of five of these B vitamins: thiamin, riboflavin, niacin, vitamin B-6 and folic acid, and therefore this project has been specifically concerned with these.

2.1.1 Niacin

Niacin is a name used by nutritionists to include nicotinic acid (NA, Figure 2.1), its amide derivative nicotinamide (NTA, also called niacinamide), and other related compounds with this vitamin activity. In the past, niacin was known as vitamin B-3 and B-4, but these names are no longer used (Soothill 1996; Institute of Medicine 2000c).

Niacin is essential for the synthesis of enzymes that provide cells with energy through tissue respiration and carbohydrate, protein and fat metabolism. In these enzymes, niacin is involved in the form of the coenzymes nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate. Niacin participates in over 200 enzymatically catalysed reactions and is essential for healthy skin, tongue and digestive tract tissues and the formation of red blood cells (Reavley 1998).

Niacin in mature cereal grains exists largely as chemically bound forms of NA that are nutritionally unavailable, whereas in non-cereal foods it is present primarily as free NA. The niacin in meat is predominantly in the form of NTA which is also the form used for fortification of food products (Ball 1998). It is relatively soluble in water and in boiling alcohol, in solutions of alkaline hydroxides and carbonates, but insoluble in ether (Budavari 2001).

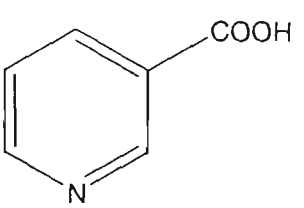


Figure 2.1 The structure of nicotinic acid

2.1.2 Vitamin B-6

There are various forms of vitamin B-6 in foods (Figure 2.2). The three primary forms are pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM), although various derivatives of these also occur naturally and have vitamin activity. Plant-derived foods

contain primarily PN, a significant proportion of which may be present as PN-glucoside or other conjugated forms which appear to be largely unavailable to humans (Vanderslice and others 1979; Gregory and Ink 1987; Ball 1998; Sampson and others 1996). Although there are exceptions, PL, as the phosphorylated form predominates in animal foods (Leklem 1991). The form most commonly used in fortified foods and supplements is PN.HCl (Soothill 1996; Institute of Medicine 2000f).

Vitamin B-6 in the form of pyridoxal 5'-phosphate, acts as the coenzyme of a series of enzymes that catalyse transamination, decarboxylation, deamination and desulphydration, and cleavage or synthesis of amino acids (Bailey 1991). Thus, it is involved in the manufacture of most protein-related compounds and plays a role in almost all bodily processes (Reavley 1998).

This is one of the vitamins that is rarely added to fortified foods in Australia, so fresh foods are the primary source (Soothill 1996). Vitamin B-6 is present in all natural unprocessed foods with yeast extract, wheat bran and liver containing particularly high concentrations. Other important sources include whole grain cereals, nuts, pulses, lean meat, fish, kidney, potatoes and other vegetables (Ball 1998). The B-6 vitamers are relatively soluble in water (Budavari 2001).

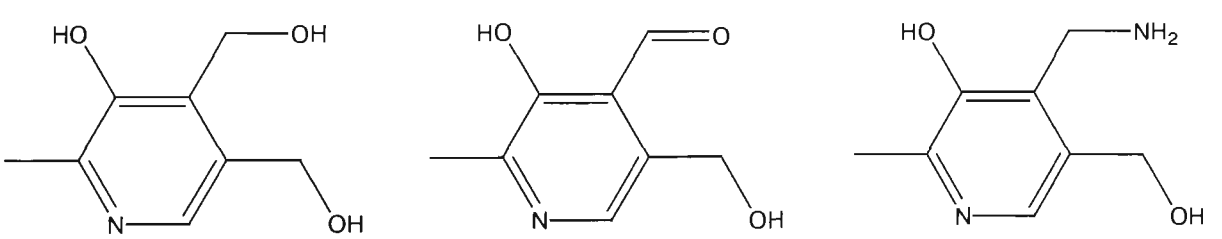


Figure 2.2 The structure of the most commonly occurring vitamers of B-6

2.1.3 Riboflavin

Pure riboflavin was first isolated from milk in 1933 by Kuhn, Gyori and Wagner-Jauregg, working at the Kaiser Wilhelm Institute for Medical Research in Heidelberg and it was chemically synthesised in 1934 (Soothill 1996). Among the various names

formerly used for this vitamin are vitamin B-2, vitamin G, ovoflavin, lactoflavin, lyochrome, uroflavin and hepatoflavin. In the United Kingdom (UK), the common spelling is riboflavine. The structure of riboflavin is 7,8-dimethyl-10-(1'-D-ribity) isoalloxazine and this is shown in Figure 2.3 (Cooperman and Lopez 1991).

Riboflavin is widely distributed in foods, and is almost exclusively bound to proteins in the form of flavin mononucleotide and flavin adenine dinucleotide (Cooperman and Lopez 1991; Combs 1992). Riboflavin functions as a component of these two flavin co-enzymes. It participates in oxidation-reduction reactions in energy production and in numerous metabolic pathways including the oxidation of glucose, certain amino acids and fatty acids; reactions with several intermediates of the Krebs cycle; conversion of PN to its active coenzyme and conversion of tryptophan to niacin (Mason 1995). Different crystal forms of riboflavin have varying solubilities in water. Riboflavin is slightly soluble in various non-polar solvents, but very soluble in dilute alkalis (with decomposition) (Budavari 2001).

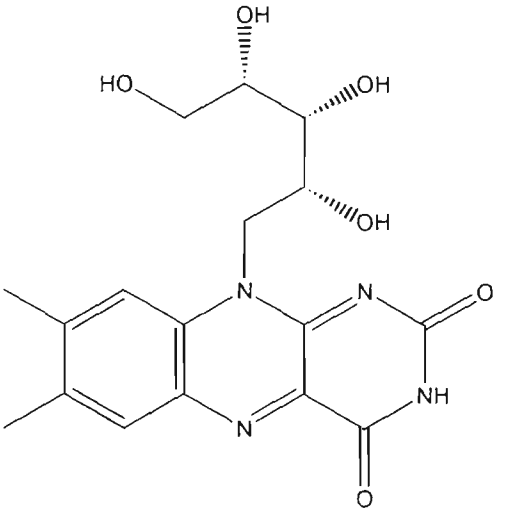


Figure 2.3 The structure of riboflavin

2.1.4 Folate

The term folate is used to encompass a large number of compounds with molecular structures based upon pteroic acid and having the same metabolic roles (Brody 1991; Gregory 1996; IUPAC 2002). The structure of pteroic acid is shown in Figure 2.4.

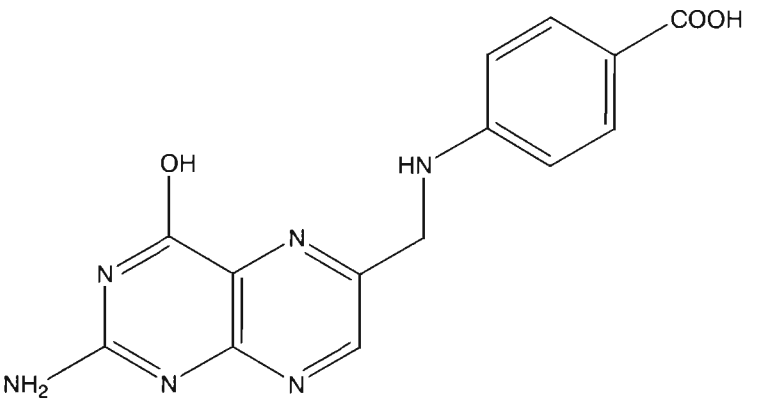


Figure 2.4 The structure of pteroic acid

The naturally occurring folate compounds in foods incorporate three types of molecular variation and these are:

- The chemical reduction of some of the nitrogen atoms in the ring system of the molecule;
- The presence of various substituent groups on the nitrogen atoms at positions 5 or 10, or, in some cases forming a bridge between the two of these; and
- The linking of one or more glutamate residues at the point where the free carboxylic acid occurs in the pteroic acid structure. The initial glutamate unit is joined by a peptide bond with additional units being joined through the γ carboxyl groups.

Folates are found in plants, and some animal tissues, largely in the form of conjugates with more than one molecule of glutamic acid and the various forms are demonstrated in Figure 2.5. The simplest naturally occurring folate is pteroyl glutamate and contains only one glutamate unit. This molecule is commonly referred to as folic acid and the structure is shown in Figure 2.6. Folic acid is the one form of folate which is

commercially available and permitted for use in fortifying food products (Bailey 1991; Food Standards Australia New Zealand (Food Standards ANZ) 2002).

It is noted that folic acid is generally thought of as a member of the B group of vitamins. However it does not demonstrate significant water solubility under conditions of neutral pH. It is reported to be more soluble in acetic acid, phenol, pyridine and solutions of alkaline hydroxides and carbonates as well as a limited number of organic solvents (Budavari 2001).

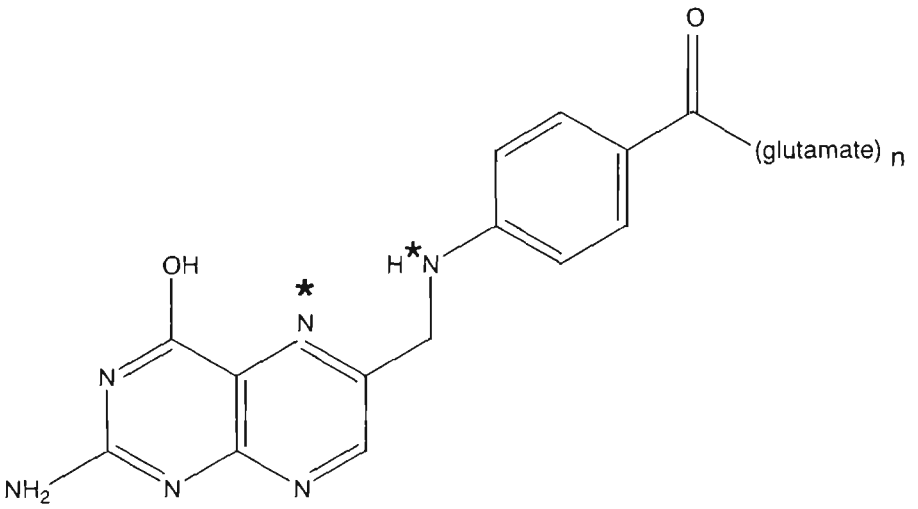


Figure 2.5 The basic chemical structure characteristic of folate compounds

Notes 1 The nitrogen atoms shown with asterisks are designated as 5 and 10 and are the points where substituents are linked in some folate compounds

2 When two or more glutamate units are present these form a chain where n may include up to seven or even more glutamates

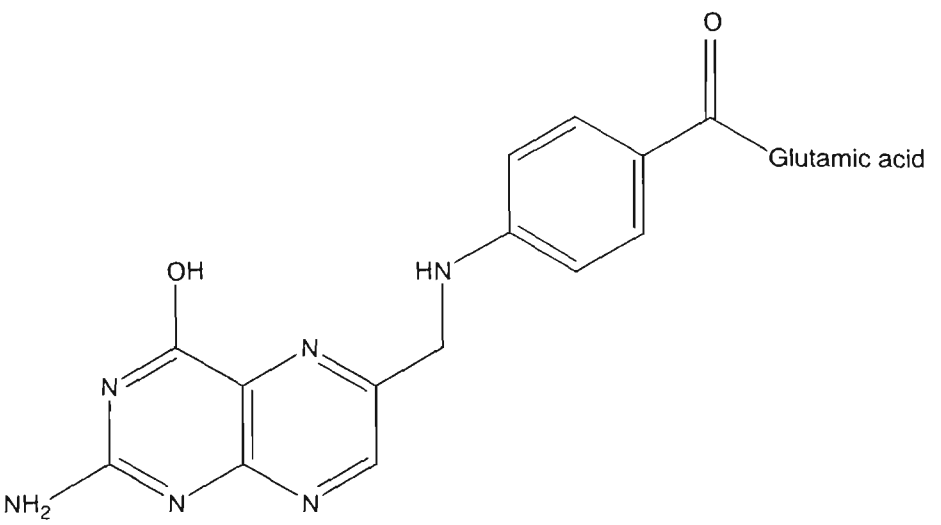


Figure 2.6 The structure of pteroyl glutamate (folic acid)

Folic acid is essential for the synthesis of deoxyribonucleic acid and ribonucleic acid, the genetic materials of cells, as well as for protein metabolism. As a part of its role in protein metabolism, it is involved in the conversion of homocysteine to methionine. High levels of homocysteine have been linked to an increased risk of cardiovascular disease (Lucock 2000). Folates are also essential for healthy pregnancy, red blood cells, bones, hair, nervous, digestive and immune systems (Reavley 1998) and may reduce the incidence of cataract formation in the eyes (Kuzniarz and others 2001). There is increasing evidence that folates play a role in protection against carcinogenesis and this issue has recently been reviewed (Kim 1999).

2.1.5 Thiamin

Thiamin was first identified as an essential nutrient in the human diet during 1921 (Guthrie 1989). Initially it was known as vitamin B-1 before the name thiamin was widely adopted. In some countries including the United States of America (US), the original spelling (thiamine) has been retained. In line with current IUPAC recommendations (IUPAC 1966) this vitamin is referred to as thiamin in this thesis.

Following the discovery of thiamin, the chemical structure was elucidated (Figure 2.7) and since then considerable knowledge has been gained on its role in the body, daily requirements and the symptoms associated with deficiency. The principal metabolic function involves the utilisation of energy. Specifically, thiamin is a coenzyme of many different enzymes (Wahlqvist 1997; Schellenberger 1998; Institute of Medicine 2000e). These include some key enzymes of carbohydrate metabolism - pyruvate and 2-oxo-glutarate dehydrogenases as well as transketolase. Thiamin is also believed to function in nerve conduction by activating the chloride ion channel in nerve cell membranes (Bender 1997). The enzymes which require thiamin for activity occur in all living organisms and hence it is present in a wide range of foods. However, in most foods the levels are very low (Briggs and Wahlqvist 1988). Typically the major dietary sources include pork and ham, cereal grain products, pulses and nuts (Guthrie 1989) although yeast and vegemite are also good sources (Wahlqvist 1997).

The thiamin molecule is known to occur in a number of molecular forms including the monophosphate, diphosphate (also commonly referred to as the pyrophosphate),

triphosphate as well as the free form (Ellefson 1985b; Gubler 1991; Gregory 1996). It is the diphosphate form of thiamin which acts as the coenzyme in carbohydrate metabolism, whereas the triphosphate functions in conduction of nerve impulses (Bender 1997). The various forms of thiamin are generally very soluble in water although the mononitrate is less soluble than the others (Budavari 2001). Most forms are partly soluble in alcohol and acetone but relatively insoluble in non-polar solvents (Gubler 1991).

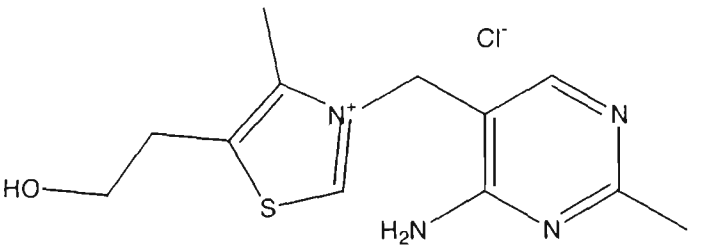


Figure 2.7 The structure of the thiamin molecule

2.2 The symptoms and effects of B vitamin deficiencies

There are varying symptoms of B vitamin deficiencies which have been recognised and these include:

2.2.1 Niacin deficiency

Deficiency of niacin results in the disease pellagra. This is typically characterised by two primary symptoms - firstly a sunburn-like rash in areas of the skin exposed to sunlight and, secondly, a depressive psychosis (Bender 1997). Other symptoms include weakness, loss of appetite, lethargy, a sore mouth and tongue, inflamed membranes in the intestinal tract and diarrhoea. In addition, niacin deficiency affects the nervous system with symptoms such as dementia, tremors, confusion and depression (Reavley 1998). People at risk of deficiency include those with absorption difficulties, alcoholics, the elderly who neglect their diet, some infants and pregnant women (Reavley 1998).

2.2.2 Vitamin B-6 deficiency

Severe vitamin B-6 deficiency results in seborrheic dermatitis in the areas of the nose, eyes and mouth, glossitis and abdominal distress. The deficiency may also lead to an incidence of kidney stones, and affects the brain and nervous systems (Bailey 1991). Low vitamin B-6 intakes are common among elderly people and may lead to increased risk of several disorders including heart disease. In a French study, published in 1997, researchers assessed elderly patients with infections during hospitalisation and found that vitamin B-6 levels were much lower than in healthy individuals (Reavley 1998). Deficiency is reported to be rare in Australia but groups at risk include infants (as the ratio of vitamin B-6 to protein may be low in human and bovine milks), alcoholics, women on oral contraceptives and those suffering from thyroid disease. In addition, certain medications such as isoniazid and penicillamine are known to inactivate vitamin B-6 (Wahlqvist 1997).

2.2.3 Riboflavin deficiency

Riboflavin deficiency in man may be due either to an inadequate dietary intake of the vitamin or to some conditioning factor that increases requirement or impairs absorption or utilisation. The signs of deficiency are more common during periods of physiological or pathological stress. They are more likely to appear when growth is rapid in childhood or during pregnancy and lactation (Goldsmith 1975). Deficiency symptoms usually include lesions of the lips and angles of the mouth, a fissured and magenta-coloured tongue, seborrhoeic follicular keratosis of the nose and forehead and dermatitis of the anogenital region (Ball 1998). Good sources of riboflavin include meat, dairy products and fortified grains (Reavley 1998). In Australia, there appears to be no evidence of riboflavin deficiency (Wahlqvist 1997).

2.2.4 Folate deficiency

Folate deficiency is thought to be the most common nutritional deficiency in the world, especially in developing countries. In Australia, between three and five percent of pregnant women are probably deficient and in developing countries the number is much higher (Soothill 1996). A deficiency in folate leads to lack of adequate nucleic acid

replication and consequent impaired cell division, especially in the haemopoietic tissue of the bone marrow and the epithelial cells of the gastrointestinal tract. In addition, a poor folate status in early pregnancy appears to be related to the occurrence of neural tube defects in the foetus (Ball 1994, 1998).

Diets low in vegetables, frequent alcohol and prescription drug use as well as the sensitivity of folate to light and heat contribute to this widespread deficiency. The elderly, alcoholics, smokers, psychiatric patients, people taking certain medications and women on the contraceptive pill may be at greatest risk of folate deficiency. Prolonged stress, viral infections and chronic liver disease are also risk factors (Soothill 1996; Reavley 1998).

2.2.5 Thiamin deficiency

Internationally, the occurrence of the different symptoms and the corresponding types of thiamin deficiency are as follows:

1. Beri-beri is primarily a problem in South-East Asia (Ensminger and others 1995; Bender 1997); this condition is associated with long-term deficiency and is more likely to occur where total food intake is relatively low and the carbohydrate consumption is higher.

2. Short term deficiency is commonly associated with excessive intakes of alcohol, particularly where little food is consumed; this can lead to enlargement of the heart and even heart failure; where binge drinking occurs, deficiency leads to acidosis and this may occur during periods as short as one week; these problems may be partly due to low intakes of thiamin, however, alcohol also impairs absorption and metabolism of thiamin (Bender 1997).

3. The Wernicke-Korsakoff syndrome (Clements 1986) which is characterised by mental confusion and deterioration of nerve function ultimately leading to coma. This disease particularly manifests itself in alcoholics who are prone to thiamin deficiency due to decreased food consumption. In addition, decreased liver function impairs the activation and utilisation of thiamin (Ball 1998).

The term sub-clinical deficiency has been used recently to describe the situation where an individual shows no clear physical symptoms of deficiency, but the thiamin intake is inadequate (Wahlqvist 1997). The condition is one of 'nutritional risk' and is difficult to measure. Sub-clinical deficiency is associated with a generally poor state of health due to the low thiamin consumption and overall metabolic efficiency is impaired.

2.3 Required levels of vitamin B intakes

The levels of B vitamins required in the diet have been determined and many countries have established reference or recommended values. The general term dietary reference value is used to include recommended dietary allowances (RDA) and recommended daily intakes (RDI) (Wenlock and Wiseman 1993). It is noted that in different countries the terminology adopted has varied, as has the underlying basis for establishment of the specific recommendations. Selected values for the B vitamins are presented in Table 2.1. These demonstrate that different intakes are required by particular groups of individuals depending upon age and gender. During pregnancy and lactation the requirements are also higher (Bender 1997).

2.4 Adequacy of vitamin intakes

It might generally be assumed that in developed countries including the US and Australia, dietary intakes of B group vitamins are in excess of reference values and some studies indicate this conclusion (Iyengar and others 2000). Whilst this may be generally true, serious doubt remains regarding the vitamin status of at least some people in these countries. Furthermore, even less data is available on the dietary status of the large majority of the world's population living in developing countries. Very recent studies into intakes of specific groups have confirmed that some individuals probably have inadequate intakes of particular vitamins. Examples include groups as diverse as North American Indians in the Yukon (Wein 1995) vegetarians and non-vegetarians in Taiwan (Huang and others 1999) and Costa Rican adolescents (Monge-Rojas 2001). From these studies folate intakes are of greatest concern.

Table 2.1 Dietary reference values for the B vitamins for selected countries

Vitamin	Country	Men	Women	Pregnancy	Lactation
Thiamin (mg)	US	1.2	1.1	1.4	1.5
	UK	1.0	0.8	0.9	1.0
	Australia	1.1	0.8	1.0	1.2
Riboflavin (mg)	US	1.3	1.1	1.4	1.6
	UK	1.3	1.1	1.4	1.6
	Australia	1.7	1.2	1.5	1.7
Niacin (mg)	US	16.0	14.0	18.0	17.0
	UK	17.0	13.0	13.0	15.0
	Australia	19.0	13.0	15.0	18.0
Vitamin B-6 (mg)	US	1.3	1.3	1.9	2.0
	(over 50)	1.7	1.5	—	—
	UK	1.4	1.6	—	—
	Australia	1.3 - 1.9	0.9 - 1.4	+ 0.1	+ 0.7 - 0.8
Pantothenic acid (mg)	US	5.0	5.0	—	—
	UK	—	—	—	—
	Australia	—	—	—	—
Vitamin B-12 (μg)	US	2.4	2.4	2.6	2.8
	UK	1.5	1.5	—	2.0
	Australia	2.0	2.0	3.0	2.5
Biotin (μg)	US	30	30	—	—
	UK	—	—	—	—
	Australia	—	—	—	—
Folate (μg)	US	400	400	600	500
	UK	200	200	300	260
	Australia	200	200	400	350

Note 1 Sources of data used were National Health and Medical Research Council (NHMRC) 1991; NHMRC 1995; Reavley 1998; Institute of Medicine 2000 a-f

2 These data are for adults and are the most recently published. In some cases they represent revisions of earlier published values

3 In some cases no recommendation has been made and this is indicated by no entry

2.4.1 Niacin intakes

The requirements for niacin are also related to energy intake. Since tryptophan obtained from protein can be converted to niacin in the body, nutritionists take both into account and refer to the number of niacin equivalents in food (Soothill 1996). For humans, approximately 60 mg of L-tryptophan yield 1 mg of niacin. One niacin equivalent is therefore equal to either 1 mg of available niacin or 60 mg of L-tryptophan (Ball 1998). The amount needed in the diet depends on the total energy consumed. In Australia, the RDI is 19 mg of niacin equivalent for young men and 13 mg for young women, with daily increments of 2 mg and 5 mg for pregnancy and lactation respectively (Soothill 1996).

2.4.2 Vitamin B-6 intakes

The intake values for vitamin B-6 have recently been revised. The tolerable upper intake limit has been set at 100 mg per day. The RDA for vitamin B-6 is based on protein intake. Those with high protein intakes may need higher levels of this vitamin (Reavley 1998). According to Pannemans and others (1994) age dependent differences also appear to be related to the protein intake needs for vitamin B-6. At a higher protein intake, the elderly need less of the vitamin than young adults. The recommendations for adults in the US and Australia are based on an allowance of 20 μg vitamin B-6/g protein, while those in the UK are based on an allowance of 15 μg/g protein. Individuals with higher alcohol consumption are also at risk of suffering vitamin B-6 deficiency (Soothill 1996).

2.4.3 Riboflavin intakes

In the US, the riboflavin allowances are also based on energy intakes. This leads to RDA values ranging from 0.4 mg/day for early infants to 1.7 mg/day for young adult males. However, for the elderly and others with a relatively low daily energy intake, a minimum of 1.2 mg/day is recommended to ensure tissue saturation (Ball 1998).

The Australian RDI is based on a requirement of 0.15 mg for every 1000 kJ of energy consumed. Women pregnant with or breastfeeding more than one baby have higher

riboflavin requirements (Reavley 1998). On the other hand, in the UK, recommendations for riboflavin intake are not based on energy requirement. In the latter case, the intake value is 1.3 mg/day for men and 1.1 mg/day for women, with daily increments of 0.3 mg and 0.5 mg for pregnancy and lactation respectively (Ball 1998).

2.4.4 Folate intakes

Much of the recent interest in folate has centred on the prevalence of neural tube defects. Studies have shown variations from regions of high occurrence, including Northern Ireland and parts of China (where rates of ≥ 4 per 1000 births have been reported), to low (< 1 per 1000) in the US and Japan. Intermediate rates (1-3 per 1000) are found in England, Wales, Hungary, Canada and Australia (Little and Elwood 1992).

A number of studies and trials have been conducted to explore the relationship between folate and neural tube defects, which included randomised controlled trials, non-randomised trials, cohort study and case control studies. In almost all these studies, folate was found to have protected against neural tube defects. The amount of supplemental folate which has been found to be effective in trials ranges from 360 μg to 4000 μg a day (NHMRC 1995).

Much of the published data on folate intakes has been questioned as methods for analysis have been re-evaluated. In a recent Dutch study of intakes it was concluded that up to half of the population does not meet existing RDI values (Konings and others 2001). Doubts concerning the adequacy of folate intakes in Switzerland have also been highlighted (Eichholzer 2003).

2.4.5 Thiamin intakes

Various approaches have been followed in an attempt to assess intake levels. One involves the assessment of the amounts of nutrients available nationally. Such data is available from the Australian Bureau of Statistics on a ten yearly basis (Rutishauser 1997). These average data range from 1.1 to 1.5 mg intake per head, indicating that, overall, the supply is adequate. In addition the calculated values show a general upward

trend in the decades since 1938-39. Similar data from the US indicates that an average of 2.2 mg of thiamin is available per head of population (Guthrie 1989).

Even though these values are in excess of minimum intake levels, separate survey data in the US indicates that significant proportions of the population consume less than the recommended levels of thiamin (Guthrie 1989; Iyengar and others 2000).

In Australia, doubts have also been expressed on the adequacy of intakes (Clements 1986). These concerns were first raised as long ago as 1941 and on numerous occasions since there has been both scientific and public debate surrounding thiamin. Surveys of dietary consumption of thiamin during 1973 showed results where up to 10 percent of households had intakes below minimum levels for good health (Clements 1986). More recently, in Australia the issue had been considered during the decade of the 1980s by expert committees of the NHMRC. Although definitive answers were not available, there was sufficient doubt regarding intakes that the decision was ultimately taken to introduce a requirement for fortification of a staple food (Lewis and others 2003).

In some countries, thiamin deficiency is a serious problem. In South East Asia, it is reported to be a leading cause of infant mortality as well as directly affecting many adults. It has been estimated that the average dietary intake of thiamin is below the level required to protect against beriberi (Guthrie 1989). In Australia, it is known that obvious deficiency symptoms are seen primarily among chronic alcoholics. However it is thought that thiamin is probably one of the most common vitamin deficiencies (Wahlqvist 1997).

2.5 Stability and factors influencing losses of the B group vitamins

Vitamins vary greatly in their stability characteristics (Table 2.2, Ryley and Kajda 1994; Gregory 1996; Kwok and others 1998). In addition different vitamins are unstable under different processing conditions particularly including frying (Fillion and Henry 1998) as well as storage. Losses may therefore occur and adversely impact on the nutritional value of food products.

Table 2.2 Factors influencing stability of B group vitamins

Vitamin	Neutral	Acid	Alkaline	Oxygen	Light	Heat
Thiamin	U	S	U	U	S	U
Riboflavin	S	S	U	S	U	U
Niacin	S	S	S	S	S	S
Vitamin B-6	S	S	S	S	U	U
Folate	U	U	U	U	U	U
Pantothenic acid	S	U	U	S	S	U
Vitamin B-12	S	S	S	U	U	S

Notes Based upon information from Gregory (1996)
S Stable (no significant destruction)
U Unstable (significant destruction)

A number of factors have been found to influence the amount of the B vitamins in foods (Gregory 1996). These include:

1. B vitamin molecules are relatively unstable compared to other vitamins and are subject to greater losses in food processing. Among the factors known to cause losses are heat, particular pH conditions, air, oxygen or light as well as the presence of reducing agents such as the sulphite ion in foods. These are summarised in Table 2.2.

2. The B vitamins are highly water-soluble, for example for thiamin, up to 100 g dissolves in 100 mL of water (Budavari 2001). Hence in the processing of some foods losses may occur by leaching during blanching and boiling (Selman 1994).

3. Considerable losses occur during storage of cereal grains (Pomeranz 1992), the milling of grain and the production of white flour; this is due to the high concentration of B vitamins in the bran layers and the germ. Hence the lower the flour extraction rate, the lower will be the vitamin content of a milled flour (Bauernfeind and DeRitter 1991; Gregory 1996).

2.5.1 Niacin stability

Niacin is widely distributed in nature. However, it generally occurs in a variety of forms that may be less available than free NA (Eys 1991). Niacin is present in uncooked foods mainly as nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate, but these nucleotides may undergo some degree of hydrolysis during cooking to yield NTA. The use of baking powder liberates much of the bound vitamin from cereal flours during baking.

In commercial processing, storage and domestic cooking of foods, niacin is generally stable. For example, there is no significant loss of niacin during milk processing. In the parboiling of rice, steaming prior to milling does not significantly increase the niacin content of polished rice as the NA in its bound form does not diffuse into the endosperm (Ball 1994, 1998). However, being water-soluble, some niacin is lost when frozen foods are thawed or when foods are boiled, but trytophan remains bound in the proteins until they have been digested (Soothill 1996).

2.5.2 Vitamin B-6 stability

It has been reported that there is relatively little data on factors affecting vitamin B-6 in foods (Gregory and Kirk 1981; Gregory and Ink 1985). However, the processes of heating, concentration and dehydration appear to affect both the quantity and form of vitamin B-6 present in food products.

Vitamin B-6 is relatively stable at low water activities (Evans and others 1981) and also under acidic conditions, but unstable under neutral and alkaline conditions, particularly when exposed to heat and light (Table 2.2). Of the several vitamers, PN is more stable than either PL or PM and therefore, PN.HCl is used for food fortification and in multivitamin supplements (Combs 1992). The form of vitamin B-6 found in animal products is more stable than those found in fruit and vegetables. However, long-term storage, canning, roasting or stewing of meat and other food processing techniques can destroy vitamin B-6. Cooking also reduces the vitamin B-6 content of food due to leaching into water (Reavley 1998).

2.5.3 Riboflavin stability

Milling of grain reduces the content of riboflavin because much of the vitamin is contained in the germ and bran. White flour milled to approximately 70 percent extraction has been reported to contain only about 35 percent of the riboflavin found in whole wheat. Although the milling of wheat does decrease the content of riboflavin, where it is permitted, enrichment may result in refined flour with a higher concentration of the vitamin than the whole wheat itself (Brooke 1968; Hunt 1975).

It has generally been reported that riboflavin is stable in foods at low water activities (Dennison and others 1997) and also when heated but will leach into cooking water (Reavley 1998). Under normal lighting, crystalline riboflavin is stable in dry form. In solution, vitamin activity is destroyed by exposure to ultraviolet (uv) radiation or visible light, the rate of destruction increasing with increases in either temperature or pH (Bailey 1991).

If protected from light, aqueous solutions of riboflavin in the pH range between 2 and 5 are heat-stable at temperatures of up to 120 °C. Alkaline conditions, including those produced by sodium hydrogen carbonate (baking soda) can destroy riboflavin (Reavley 1998). Above pH 7 the isoalloxazine ring is rapidly destroyed at elevated temperatures. Optimal stability is observed at pH 3.5-4.0. In the absence of light, riboflavin is stable towards oxygen and many oxidising agents (Ball 1998).

2.5.4 Folate stability

Folate vitamers differ widely with respect to their susceptibility to oxidative degradation, their thermal stability and the impact of pH (Chen and Cooper 1979; Paine-Wilson and Chen 1979; Gregory 1985). Most folates in foods and feedstuffs are easily oxidised and therefore are unstable. Folate activity is gradually destroyed during food processes including boiling and heating, and when foods are stored at room temperature for long periods (Malin 1977; Tung-Shan 1983; Hawkes and Villota 1986; Reavley 1998; Combs 1992; Melse-Boonstra and others 2002).

The most stable of the various folates at ambient and elevated temperatures is folic acid (Ball 1998). In aqueous solution folic acid is stable at 100°C for 10 hours in a pH range of 5.0-12.0 when protected from light, but becomes increasingly unstable as the pH decreases below 5.0. The stability of folic acid (and indeed all folates) is also reported to be greatly enhanced in the presence of antioxidants including ascorbic acid. In some recent studies it has been found that folate levels decline during processing of various grain-based foods although the extent of losses vary (Dang and others 2000; Arcot and others 2002b, 2002c).

2.5.5 Thiamin stability

Thiamin is unstable under a variety of conditions in foods (Table 2.2). In early studies it was established that heat causes losses in many foods (Farrer 1955). However, it is noted that thiamin is quite stable under some conditions and these include those of low water activity in combination with ambient temperatures as well as in acid pH conditions and direct lighting (Hebrero and others 1988; Gregory 1996). Chlorine present in water as hypochlorite ions and commonly used as a water treatment agent can cause cleavage of thiamin (Kimura and others 1990). Despite the instability at higher temperatures and pH values, it is also known that, in solutions with a pH less than 5, thiamin is stable at elevated temperatures and under oxidising conditions. In addition, food ingredients can cause degradation of thiamin; some of the molecules involved include tannins, haeme proteins as well as specific thiaminase enzymes (Bailey 1991).

2.6 Vitamin fortification strategies

Possible approaches to ensuring adequate intakes of vitamins include fortification and enrichment of foods. The term fortification generally refers to the addition of vitamins to restore the levels to those found prior to processing. Enrichment entails addition beyond levels originally present (Bender 1998).

In the decade between 1930 and 1940, the concept of fortification of staple foods was developed as a result of the discovery of certain vitamin deficiency diseases in the US. Scientific studies around this time showed that the dietary intakes of thiamin, riboflavin, niacin and iron were below desirable levels in the diet and therefore deficiency diseases including rickets, beriberi, and pellagra occurred. As a result, the Standard of Identity

for enrichment of flour with thiamin, niacin, riboflavin and iron was established in 1942 by the US Food and Drug Administration (FDA) (Watson 1981; Iannarone 1991). In the following year enrichment of all bread and rolls was introduced. Since then considerable practical knowledge on fortification has been developed in the US and the approach is feasible for many different products (Borenstein 1971; Steele 1976; Colman 1982; Bauernfeind and DeRitter 1991; Giese 1995; Brady 1996). In some applications problems may be encountered where storage involves higher temperatures and humidity (Bauernfeind and DeRitter 1991; Borenstein and others 1991). In January 1998, a new legal requirement was introduced in the US so that commercial grain products are now also enriched with folic acid to a minimum level of 140 μg/ 100 g (Ranum 1996; Reavley 1998).

Whilst fortification with various nutrients has been mandatory in the US for many years (Berner and others 2001), it is noted that doubt remains regarding adequacy of US vitamin intakes (Guthrie 1989). Furthermore, relatively few other countries practice fortification to the extent found in the US. Fortification of flour in the UK has primarily focused on mineral nutrients (Elton 1975). In Australia and New Zealand there has been considerable hesitation shown towards expanding legal approval of fortification (Becker 1993; Nordmark 1999). Mandatory addition of thiamin to all breadmaking flours was introduced to Australia in 1990 (Bread Research Institute of Australia (BRI) 1989a; Food Standards ANZ 2002; Lewis and others 2003). It is also noted that the fortification of flours with thiamin remains an issue of controversy. More recently optional fortification with folic acid has been permitted in Australia for cereal-based foods (NHMRC 1995; FDA 1996).

In relation to folate fortification there has been considerable international interest and controversy in recent years particularly concerning an appropriate level of addition (Walter 1994; Crane and others 1995; Neuhouser and Beresford 2001; Wald and others 2001; Walter 2001; Wright and others 2001; Oakley 2002). On the one hand some studies indicate that fortification has effectively enhanced intakes and also blood folate levels (Lawrence and others 1999; Lewis and others 1999). However cautionary warnings about fortification to high levels have also been sounded due to safety concerns regarding intakes in excess of requirements (Crane and others 1995; Walter

2001; Wright and others 2001). Recently the need for ongoing monitoring of the effects of folate fortification has been highlighted (Rader and Yetley 2002).

Fortification, as traditionally practiced, has a possible limitation in that added vitamins may also be lost during processing due to a lack of stability. A new approach which may offer a solution to these problems is encapsulation. This entails the coating or entrapment of an ingredient using a protective material. The technology of microencapsulation was introduced around 30 years ago and has been particularly applied to pharmaceutical materials (Park and others 1984) and more recently for the introduction of flavourings into foods (Versic 1988; Brazel 1999; Gibbs and others 1999; Qi and Xu 1999; Augustin and others 2001). The potential of this approach to fortification of foods with vitamins has not been thoroughly documented in the scientific literature.

Chapter 3

Background and literature review: the processing of Asian noodles and their global significance as a food source

The purpose of this chapter is to provide background and review current knowledge on the utilisation of wheat flour for manufacture of Asian noodles. The areas covered include a comparison of durum pasta products and Asian noodles, the international significance of Asian noodles, the different styles of these products, the ingredients and processes applied as well as relevant data on the retention of the B vitamins in flour-based foods.

3.1 Cereal grains and their classification

The cereals are members of the grass family and are cultivated primarily for human food, for livestock feed and as a source of industrial starch. The cereal grains are wheat, rice, corn (also commonly referred to as maize), rye, oats, barley, sorghum, millets and triticale. Among these, wheat, rice and maize are the most important cereal grains produced, consumed and traded in the world. In recent years global production of wheat has been approximately 575 million tonnes annually (Food and Agriculture Organisation (FAO) 2002). Two different species of wheat represent the bulk of international production and trade (Cornell and Hoveling 1998). These are bread wheats (*Triticum aestivum*) that are used for production of many food products and durum wheat (*Triticum durum*) which is used for manufacture a wide variety of pasta products (Hoseney 1994). Durum comprises a relatively small proportion of all wheat grown with global production estimated at 28.5 million tonnes in 1999 (Kill 2001).

Normally, bread wheat is classified into two broad categories, hard and soft wheat, based on agronomic and end-use attributes. Hard wheat flours have high protein content (approx 12-15 percent) and are suitable for products requiring stronger structure, such as breads and some types of noodles. Soft wheat flours contain approximately 8-12 percent protein and are suitable for products requiring minimal structure such as cakes and biscuits. In addition, there are flours having a combination of these quality

characteristics, and the wheats are described as having semi-hard grain characteristics. These are used in unleavened breads as well as Asian steamed bread and certain noodles (Bushuk and Rasper 1994).

3.2 Cereal-based foods as sources of B vitamins

Cereal grains have long been regarded as valuable sources of essential nutrients. They provide energy, protein, minerals and vitamins in the human diet (Kent and Evers 1993; Ranhotra 1994; Fujino and others 1996; Cornell and Hoveling 1998). Cereal grain foods are typically regarded as among the best sources of the B vitamins, even in the absence of fortification (Keagy and others 1980; Briggs and Wahlqvist 1988). The relative contribution and importance of cereal grains to intake is shown in Table 3.1. This data is based on US studies. Comparable data is less readily available for other countries although it is likely that cereals represent a primary source of B vitamins for the majority of the world's population.

Table 3.1 Contribution of cereal products to B vitamin intakes in the North American food supply

Niacin	Vitamin B-6	Thiamin	Folate	Riboflavin
28	10	43	11	23

Notes 1 Expressed as the proportion (percent) of total intake derived from cereal foods
2 Data tabulated from Guthrie (1989)

It is possible that losses during processing and storage of cereal foods limit the contribution of these foods to dietary intakes. There have been some studies in this area and there is evidence that B vitamins are lost. Levels of the vitamins are high in the aleurone layer and scutellum portion of the germ but the flour milling process removes the bran and germ where these vitamins are found in the greatest abundance (Kent-Jones and Amos 1967; Saxelby and Brown 1980; Simmonds 1989; Stenvert 1995; Forder 1997). In addition, it is generally recognised that during the baking process approximately 20-30 percent of thiamin is lost and greater destruction occurs in the crust than in the crumb (BRI 1989b). Finnish rye breads have also been studied recently and the result showed quite variable losses during baking (53-76 percent) (Hägg and

Kumpulainen 1994). The factors influencing nutrient retention during extrusion processing have been reported (Asp and Bjorck 1989) and the data indicates that elevated temperatures impact adversely on thiamin levels.

3.3 A brief comparison of pasta and Asian noodle products

Prior to providing a review of Asian noodles and their likely contribution to dietary intakes of essential nutrients, it is necessary to clearly describe and differentiate durum wheat products and Asian noodles. It is possible that the two groups of products had a common origin and in some parts of the world the term noodles is applied to pasta products. It is noted that there is the potential for confusion due to the use of the name noodle to refer to a variety of products. For example the term has been used in US Standards of Identity to include pasta, prepared from durum wheat and containing egg (Hahn 1993; FDA 2004). Whilst there are apparent similarities between durum and Asian noodle products, they are quite different in a number of ways and a comparative summary is presented in Table 3.2. This demonstrates that the two groups of products can be clearly differentiated in relation to the wheat species, the ingredient formulations as well as the processing methods applied. In this thesis the term noodles is used only in reference to Asian noodles.

There has been considerable research into the factors affecting consumer appeal and other aspects of pasta processing (Asp and Bjorck 1989; Kill 2001). There is also information available on the nutritional profile of durum-based products (Kill 2001). Various studies have demonstrated that there are significant but quite variable losses in selected B group vitamins during cooking of durum-based products (Dexter and others 1982; Douglass and Matthews 1982; Ranhotra and others 1983, 1985, 1986). Very little data has been presented on the extent of losses during manufacture of pasta products. In addition, the relationship of the findings on durum products to those which might be found for Asian noodle products has not been investigated. However it cannot be assumed that similar results may apply due to the significant differences between these products (Table 3.2).

Table 3.2 A comparison of the ingredients and methods used during processing of durum pasta and Asian noodles

	Pasta products	Asian noodles
Species of wheat utilised	*Triticum durum* commonly referred to as durum wheat	*Triticum aestivum* also known as breadwheat
Desired grain harness	very hard	medium
Ploidy of wheat	4N	6N
Primary raw ingredient of dough	semolina	flour
Granularity of primary ingredient	relatively coarse	very fine desirable
Other ingredients	egg (optional)	common salt and/or alkaline salts (many other optional ingredients)
Preferred colour of product	yellow	depends on style: consumer preferences range from white to yellow
Dough preparation	mixing of a crumbly dough	mixing of a crumbly dough
Basic processes applied to dough	extrusion typically applied in commercial production; sheeting and cutting may be used for home and small scale operations	repeated sheeting, then resting followed by further sheeting and cutting into strands using cutting rolls
Typical shapes of product	various including strands (spaghetti, fettuccini), macaroni, shells, bows and sheets (lasagne)	commonly strands: cross sections typically square or rectangular
Typical cooking times	typically ten minutes or longer	Usually less than 10 minutes; as short as 2 minutes where processing includes steaming or frying

Note Information based on various sources, particularly Hoseney 1994; McKean 1999; Kill 2001

In the context of the current study on the vitamin contents of flours and Asian noodle products, the purpose is now to provide background on their importance, to describe processes for their manufacture and to review existing knowledge and recent research in the area.

3.4 Origins and development of Asian noodles

Noodles are staple foods in many Asian countries (Miskelly 1993). Historically, Asian noodles originated in northern China, then they were introduced to other Asian countries by traders, seafarers and migrants. It is known that the art of noodlemaking has been developing for more than 2000 years. Early records show that the technology was already remarkably well developed during the Han dynasty, 206BC-220AD. At this time the products were referred to as 'long life noodles' and their use was reserved for special occasions, particularly birthday celebrations. However during the Sung dynasty, 960-1279AD, noodles became more widely consumed on an everyday basis and storage was facilitated by the introduction of dried noodles. In addition, a great variety of noodle toppings and tastes had been evolving along with the art and technology of noodlemaking (Huang 1996a, 1996b).

Traditionally noodles were made by hand until the development of a noodlemaking machine in Japan during 1884, and this revolutionised the manufacture of these products. At the beginning of the twentieth-century, yellow alkaline style noodles were introduced to Yokohama and became popular throughout Japan. In 1964, instant style noodles were first manufactured commercially. More recently further innovations have also been applied to improve noodle quality and satisfy consumer demands. These include the use of freezing to enhance the taste of boiled noodles (1974); freeze-drying for instant noodles (1977) and precooked noodles having long shelf life (1988) (Nagao 1996).

3.5 Importance of noodles internationally

Asian noodle products have become a major source of enjoyment and nutrition. In describing these foods it is noted that flours other than wheat flour can be used and that in some cases noodles are made from composite flours. For example, in Thailand,

traditionally the most widely consumed styles of noodles are made from rice flour (Ohtsubo 1998; Luh 1999), in Japan buckwheat noodles containing both wheat and buckwheat are popular (Nagao 1995a, 1995b) and in a number of countries a style of translucent noodle is made from mungbean starch (Singhakul and Jindal 1990). However, overwhelmingly the major ingredient used in manufacture of Asian noodles is wheat flour. The significance of wheat is demonstrated by the extent of utilisation in Asian countries (Table 3.3). These data show that annually, the total amount of wheat used for noodles in these countries is approximately 70 million tonnes, representing in excess of 12 percent of total world wheat production (FAO 2002; FAO 2004).

Noodles are popular in each of the countries described although total consumption in China is particularly high because of the large population. It is also significant that wheat consumption continues to expand in Asian countries with this trend predicted to continue over coming decades (Nagao 1998; Oleson 1998). The consumption of noodles, particularly instant noodles has also been increasing very rapidly in recent years (Anon 1996). It is noted that the data presented in Table 3.3 are based upon estimates made in 1989. Information published more recently (Hou 2001) indicates that the proportions of wheat flour used in noodle manufacture in most of the countries remain at similar levels. In the cases of Thailand and the Philippines there has been a trend to greater use of flour for noodle production. In addition, noodles are now popular in many other countries including western nations. For Australia, the consumption has been increasing as Asian cuisines have been adopted during the past decade (McKean 1999).

As noodlemaking has spread, a range of different noodle styles have developed. Cultural heritage influences dietary habits and preferences (Ang 1999; Newman 1999) and regional preferences even within one country have resulted in a wide diversity of noodle products becoming available (Nagao 1996).

Table 3.3 Estimated values for the annual consumption of wheat for various Asian countries and the proportions used for noodle production

Country	Annual wheat consumption (million tonnes)	Proportion used for noodle production (percent)
China	107	50-60
Indonesia	1.9	45
Japan	6.3	36
Malaysia	0.84	45
Singapore	0.44	40
South Korea	2.2	40
Taiwan	0.9	36
Thailand	0.37	13
The Philippines	1.6	14
Total wheat consumption in Asia	**121.6 million tonnes**	
Wheat used for noodle production in Asia	**69.4 million tonnes**	
World wheat production	**546.6 million tonnes**	
Proportion of world wheat production used for noodle production in Asia	**12.7 percent**	

Notes
1 All estimates apply to the same crop year (1989)
2 World wheat production data is from FAO (2002) and includes all species of wheat
3 Data for individual countries (other than consumption for China) was tabulated from Nagao (1995b)
4 Consumption for China was estimated from FAO production and import values (FAO 2004)

3.6 Classification of noodles

Asian noodles have generally been classified on the basis of the major ingredients as well as the manufacturing method (Miskelly 1993). Primarily, noodles are made from flour, water and either common salt (sodium chloride) or alkaline salts including sodium

carbonate. In the marketplace, wheat flour noodles are sold in the form of uncooked wet, dried, boiled, steamed, frozen boiled and instant noodles (Nagao 1996).

Three main styles have been recognised in the scientific literature and these are white salted, yellow alkaline and instant noodles. These have been studied in this project as they have different production requirements and different quality attributes as well as varying consumer preferences. The characteristics of each style are briefly described as follows.

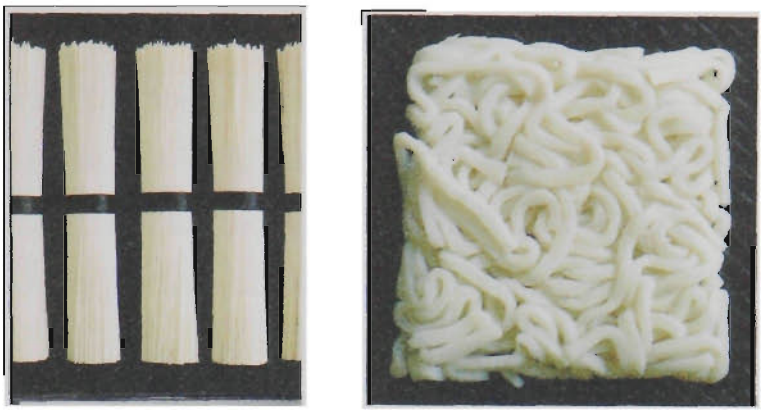
3.7 White salted noodles

Soft and elastic textures as well as a uniform white appearance are the traditional characteristics of white salted noodles. They are manufactured in many countries and have universal popularity. The pH of these noodles is typically around 6.2 (Moss and others 1987). Some examples are depicted in Figure 3.1.

Considerable attention has been focused on the white salted noodles originating in Japan. For these, medium strength flour with 9.0-9.5 percent protein is suitable (Simmonds 1989). There are four sizes of noodle strands: very thin (somen), thin (hiya-mugi), standard (udon) and flat noodles (hira-men) (Nagao 1996). In the marketplace, these noodles are sold in a variety of forms including uncooked wet noodles (nama-men), dried noodles (kan-men), boiled noodles (yude-men), steamed noodles (mushi-men), frozen boiled noodles and instant noodles.

3.8 Yellow alkaline noodles

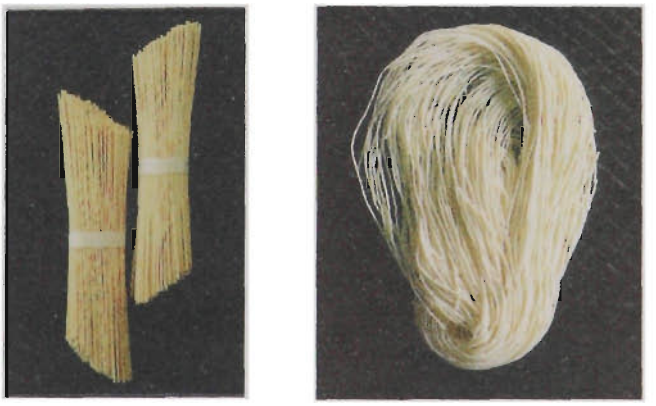
These noodles have a bright, clear yellow colour, with a firm, chewy texture and a smooth surface. The degree of yellowness depends upon regional preferences. It is noted that the terms Chinese noodles and yellow alkaline noodles tend to be used interchangeably in the literature. However in this thesis, the term yellow alkaline has been adopted because the noodles encompassed are popular in most countries in Asia and wherever Asian noodles are consumed. Some of the more common forms of yellow noodles are presented in Figure 3.2.



Somen Udon

Figure 3.1 Some common forms of white noodles
Source: Durack 1998

Shanghai Hokkien E-fu



Ramen Fresh

Figure 3.2 Some common forms of yellow noodles
Source: Durack 1998

Different types of noodles vary according to the method of processing and the ingredients. Medium or hard wheats that contain approximately 11 to 13 percent protein or higher are used to produce yellow alkaline style noodles (Wrigley 1994). In Australia, the Prime Hard wheats from Queensland and northern New South Wales are suitable for this purpose as they provide a semi-strong flour when mixed with water and alkaline salts (Simmonds 1989). It has been suggested that the alkaline conditions (pH 9-11) in the product inhibit enzyme activity and cause the flour pigments, flavonoids to change to a yellow colour. This results in the desirable bright yellow colour and products having favourable strength and palatability (Lorenz and others 1994).

There are different forms of yellow alkaline style noodles including: fresh noodles (Cantonese noodles) having a moisture content of 32-35 percent; wet or boiled noodles (Hokkien style) about 52 percent moisture; steamed and dried noodles approximately 10 percent moisture; as well as the newer steamed and fried noodles (instant noodles) having about 8 percent moisture content (Simmonds 1989).

3.9 Instant noodles

Instant noodles are typically produced by a fully automated process (Kim 1996a; Kubomura 1998). This involves steaming and deep-frying prior to packaging in either a polyethylene bag or cup. During processing, the noodles are cut, formed into a waved arrangement, precooked with steam, formed into individual servings, and then deep-fried. Hence, they are characterised by relatively low moisture contents (typically 5 percent), but high fat (20 percent) levels. The fat content results in a taste distinct from that of other noodles (Hoseney 1994) but renders the food susceptible to oxidative rancidity thereby limiting the shelf life to 5-6 months (Kim 1996b).

3.10 Basic processing of wheat flour noodles

In the manufacture of the various styles of Asian noodles from wheat flour the same preliminary processing steps are applied (Huang 1996b; Corke and Bhattacharya 1999). The flour and all other ingredients are mixed together to distribute the water uniformly and to produce a crumbly consistency. The mixers used commercially are typically of horizontal design with two parallel shafts to which are attached short beater arms that

facilitate distribution of the water but without significant development of the gluten dough matrix which typifies a bread dough. The resulting crumbly material may be rested or mixed for longer periods at low speeds prior to the next stage of processing. This entails the compression of the crumbly material in order to produce a dough in which the crumbs combine to form a single piece. The equipment used to make the dough piece consists of a set of two metal rolls which have a suitable gap so that as the mixture is passed between the rolls compression occurs and a dough sheet is formed. Development of the dough occurs during a series of subsequent steps in which the dough sheet is passed through further sets of rollers. These are arranged so that the aperture of each successive set of rolls is smaller. The number of sets of sheeting rolls is typically six or seven. An example of a commercial noodle plant is shown in Figure 3.3, demonstrating how a dough sheet is processed. The resulting dough is a sheet having a thickness appropriate to the style of noodle and the preferences of the consumers. The sheets are then cut into long strands by passing them through one set of rolls which are designed as cutting rolls. A closer view of these rolls is presented in Figure 3.4.

Following these primary steps in noodle processing there are many variations possible and these are summarised in Figure 3.5. The cut strands may be sold without further processing as fresh noodles. Alternatively drying and cooking processes might be applied in various combinations. This partly explains the diverse range of noodle products which are available to consumers.

Another factor which provides an even greater range of options is the use of different combinations of ingredients. Thus, for example, white salted noodles are prepared by mixing wheat flour, common salt and water to produce a white colour. On the other hand yellow alkaline noodles are made using alkaline salt (kansui) which softens and moistens the noodles, giving the dough a yellowish colour and enhancing viscosity, thereby allowing the dough to mix with an appropriate amount of water (Nissin Foods 2003). Instant noodles are typically precooked by steaming followed by the frying step. The resulting noodle products have a variety of colour, texture and flavour characteristics.

Figure 3.3 A commercial noodle processing plant in Asia showing a dough sheet passing through successive sets of rolls followed by a cutting step and steaming prior to packaging

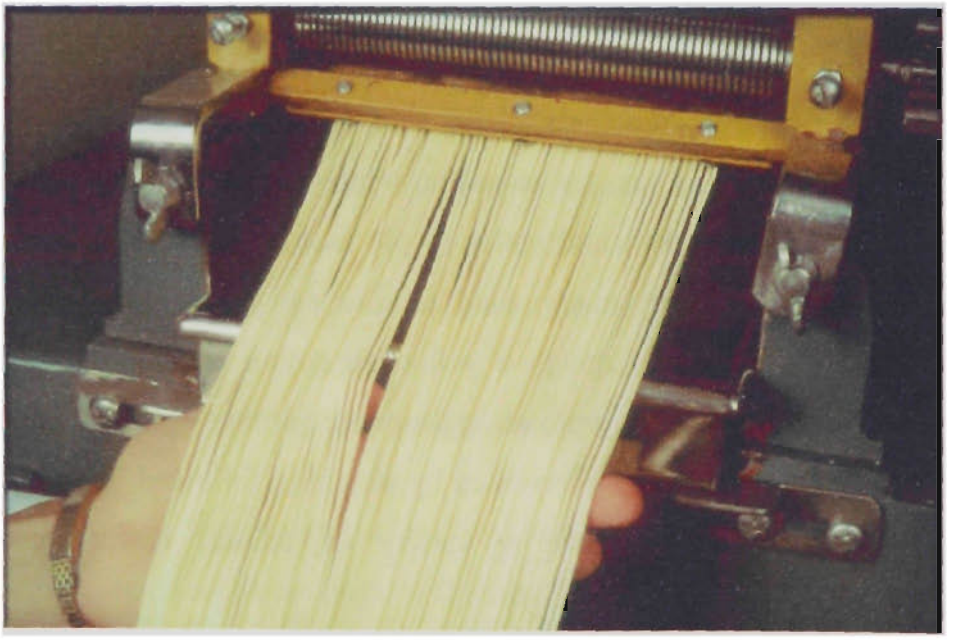


Figure 3.4 The use of a set of cutting rolls to form noodle strands from a dough sheet using laboratory scale processing equipment

3.11 Function of ingredients in noodle-making

The basic ingredients for noodle manufacture are wheat flour, salt, and water. Flour is the most important ingredient as it gives the dough strength for processing and the desired colour to the product (Nagao 1995a, 1995b, 1996). The functions of the main ingredients are now briefly described.

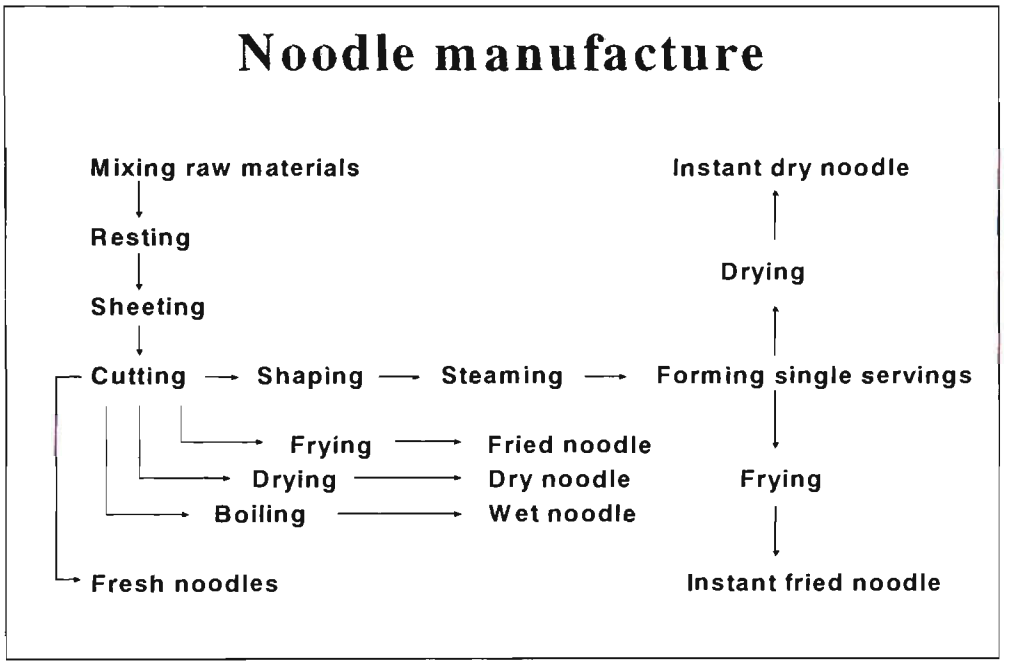


Figure 3.5 Processes used in the manufacture of different forms of Asian noodles
Note Source of information was Hoseney 1994

Flour makes up 95-98 percent of noodle solid (Hoseney 1994). Selection of the wheat flour depends on the type of product in order to adapt the quality aspects for the consumer. Generally, the flour should have a fine granularity, low ash, good colour, reasonable degree of extensibility and rapid water absorption in a short mixing time (Simmonds 1989). Desirable flour characteristics for particular noodles have been described:

1. In Japan, flours of 0.33-0.45 percent ash, bright colour and protein content of 8-10 percent are used for white salted noodles and the Australian Standard White class of wheat is reported to be particularly suitable (Nagao 1996).

2. Yellow alkaline noodles require flour with very bright colour, 0.33-0.38 percent ash and 10.5-12 percent protein content. Hard wheat is suitable for yellow alkaline noodle flours (Nagao 1996).

Salt is added to noodles in small amounts either in the form of NaCl (white salted noodles) or as alkaline salts (yellow alkaline noodles). The alkaline salts are often referred to as lye water or kansui (also written as kan swi) and are most commonly mixtures of sodium and potassium carbonates (Moss and others 1986; Miskelly 1996, 1998). Salts have a variety of important functions in the processing of noodles. They not only enhance flavour and strengthen the gluten network but are also thought to facilitate moisture diffusion, inactivate enzymes and inhibit dough fermentation (Nagao 1996). Beside these functions, Moss (1984) also hypothesised that the addition of kansui confers a distinctive flavour as well as producing a natural yellow colour in the noodle by detaching the flavones from starch.

Water: usually the amount of water added is limited (less than 35 percent of flour weight) therefore the mixing stage forms stiff and crumbly spheres. The advantage of less water in noodlemaking is to shorten the subsequent final drying or frying stage, thus saving energy as there is less water to remove (Kruger and others 1996).

The potential of adding other flours has been evaluated for white salted noodles (Jeffers and others 1979). A variety of other ingredients are now available to the processor formulating Asian noodle products and these include additives particularly colours and stabilisers. The recent developments in this area have been reviewed by Miskelly (1998).

3.12 Current status of research on Asian noodles

Australian researchers pioneered scientific research on Asian noodles (Moss 1971) particularly for the Japanese white salted (Udon) type of product. Since that time considerable research has been undertaken and has led to the publication of a monograph reviewing noodle and pasta technology (Kruger and others 1996) as well as comprehensive reviews (Corke and Bhattacharya 1999; Hou 2001). Much of the literature has focused on the identification of the flour characteristics which should be

chosen to give the best quality of noodle products (Crosbie and others 1998; Janto and others 1998; Seib and others 2000). These studies have included white salted styles of noodles (Oda and others 1980; Hatcher and others 2002) and yellow alkaline noodles (Moss 1982; Miskelly and Moss 1985; Shelke and others 1990; Kruger and others 1996; Ross and others 1997). Relatively little has been published on the instant types of noodles which are now widely available (Kim 1996a, 1996b; Kubomura 1998).

Some research has specifically focused on the development of procedures which might allow agronomists and wheat breeders to conveniently select new genotypic varieties for particular noodle end uses (Crosbie 1991; Baik and others 1994a; Azudin 1998; Morris 1998). In this context, the significance of variations in the encoding for granule bound starch synthase for noodle making have been reported (Briney and others 1998; Epstein and others 2002). Factors influencing noodle colour have also been studied from the perspectives of genotypic influences (Miskelly 1984; Kruger and others 1992), the role of enzymes (Orth and Moss 1987; Baik and others 1994b) and the effect of alkaline salts used in yellow alkaline noodles (Moss and others 1986, 1987; Miskelly 1996; Morris and others 2000). The issue of grain sprouting adversely impacting on noodle quality has been reported (Edwards and others 1989; Hatcher and Symons 2000). In addition, the significance of starch and other flour components as determinants of product texture have been the subject of various studies focusing on white salted (Toyokawa and others 1989a, 1989b) and yellow alkaline noodles (Seib and others 2000).

Newer processing approaches are under development including vacuum mixing, freezing and long life technologies. Recent research carried out in these areas in Taiwan (Wu and others 1998) and elsewhere (Nagao 1998) has been now been summarised. In addition the importance of appropriate drying conditions for white salted noodles has been reported (Inazu and others 2003).

The significance of Asian noodles as a major end use for Australian wheat exports has been highlighted (Wrigley 1994). In addition, the importance of supplying grain having characteristics appropriate to the specific end use has been emphasised in relation to the breeding of new genotypes and the application of suitable quality control measures to the receival, transport, selection and shipment of wheat cargoes to meet the demands of

processors (Wrigley 1994). One aspect of Asian noodles which appears to have received scant attention is that of composition and nutritional value.

3.13 Current knowledge on the nutritional value of Asian noodles

At this time there is relatively little data available on the nutritional value of any Asian noodle products (English and others 1990; Holland and others 1991; Ang 1999; US Department of Agriculture, Agricultural Research Service (USDA ARS) 2002) or the contributions they make to dietary intakes of essential nutrients. There have been some studies of the nutrient status of durum-based pasta products in the US. However it is not clear whether similar patterns might be expected for Asian noodles. There are significant differences in the ingredients and processing between durum products and Asian noodles. Currently there is no data on Asian noodles indicating to what extent either naturally occurring or added vitamins used in fortifying foods might be retained during the processing and storage of these widely consumed products. In addition there are considerable variations in the manufacturing processes for different styles of Asian noodles which may be of significance. On this basis, the overall purpose of the current project has been to study the B group vitamins in Asian noodle products.

Chapter 4

Background and literature review: procedures for the analysis of the B group vitamins

The purpose of this chapter is to provide a brief background on the methods available for measurement of the B group vitamins. In addition the specific challenges associated with vitamin analysis are reviewed.

4.1 Review of methods for analysis of B group vitamins

Since the identification of vitamin deficiencies and the subsequent discovery of the corresponding vitamin compounds many different analytical methods have been employed. The range of analytical procedures has been reviewed extensively (Brubacher and others 1985a; Ellefson 1985a, 1985b; Finglas and Faulks 1987; Hebrero and others 1988; Hasselmann and others 1989; Jeon and Ikin 1995; McGown and Mithipatikom 1997). Procedures for determination of vitamins have evolved from approaches using laboratory animals, through microbiological, chemical, spectrophotometric and fluorimetric methods to relatively sophisticated instrumental techniques including liquid and gas chromatography as well as capillary electrophoresis.

A number of traditional methods continue to find application and remain as reference procedures. These include the standard methods of the Association of Official Analytical Chemists (AOAC), the American Association of Cereal Chemists (AACC) and International Association of Cereal Science and Technology (ICC). Some of these have also been published in the reference monograph Methods of Vitamin Assay (Augustin and others 1985). Although vitamin analyses have been thoroughly researched, this remains an area of intense activity. Some of the reasons for this include the relatively low levels of these compounds in many foods, the requirement for monitoring of food fortification levels, increasing demands for nutrition labeling and the interest in development of comprehensive food composition tables and databases. The increasing availability of analytical instrumentation has also had a significant impact. For example in the past decade many researchers have investigated the application of

capillary electrophoresis to the analysis of vitamins (Ong and others 1991; Jegle 1993; Lindeberg 1996; Ward and Trenerry 1997; Frazier and others 1999).

4.2 Procedures for simultaneous determination of a number of B group vitamins

Traditionally, the quantitation of a series of vitamins has required the separate extraction and analysis of each individual vitamin. Typically these methods are time consuming and involve pre-treatment of the sample to eliminate interferences from the matrices. However, recently procedures have been published which appear to allow for the rapid, simultaneous separation and quantification of a number of water-soluble vitamins. These have been based upon capillary electrophoresis and high-performance liquid chromatography (HPLC) (Lavigne and others 1987; Nishi and others 1989; Rizzolo and Polesello 1992; Jegle 1993; Huopalahti and Sunell 1993; Dinelli and Bonetti 1994; Frazier and others 1999; Ivanovic and others 1999; Moreno and Salvado 2000). Among the advantages claimed for the procedures are the levels of selectivity, automation and reproducibility, economy, as well as a requirement for relatively small amounts of sample for analysis (Lam and others 1984; Amin and Reusch 1987a, 1987b; Papadoyannis and others 1997; Frazier and others 1999).

4.3 Procedures for analysis of niacin

A variety of analytical techniques have been used for determination of niacin and these include: the microbiological method based on the growth response of *Lactobacillus* (hereafter abbreviated as *L*) *plantarum* (Niekerk and others 1984) and the AOAC colorimetric method. The latter utilises the König reaction in which NA reacts with cyanogen bromide to form a coloured complex (Eitenmiller and DeSouza 1985; AOAC 1990g; Eys 1991; Ball 1998). The microbiological assay is not well suited for occasional assay because the test organism requires frequent attention to maintain continuity in the assay. On the other hand the colorimetric method uses hazardous reagents and is less sensitive than microbiological methods thereby limiting its application for foods containing low levels of niacin (Eitenmiller and DeSouza 1985).

The problems inherent in existing methods have led investigators to develop alternative methods using HPLC (Chase and others 1993a; Vidal-Valverde and Reche 1991;

Krishnan and others 1999; LaCroix and others 1999; Lahély and others 1999) and capillary electrophoresis (Ward and others 1996; Ward and Trenerry 1997; Windahl and others 1998). To overcome poor resolution on HPLC, extensive extract purification methods are required. Most HPLC methods involve ion exchange or reversed-phase chromatography. In addition, ion pair reagents can be used to enhance resolution of niacin from interfering peaks (Eitenmiller and Landen 1995). The application of capillary electrophoresis offers potential for quantitating total niacin in many different types of sample matrices (Eitenmiller and Landen 1995).

4.4 Procedures for analysis of vitamin B-6

Vitamin B-6 analysis in foods is a difficult analytical problem because it occurs firstly at relatively low levels and secondly in complex organic matrices. A variety of different methods have been developed for determination of vitamin B-6 and these include: spectrophotometric (Srividya and Balasubramanian 1997), chemical (Dakshinamurti and Chauhan 1981), microbiological (Atkin and others 1943; Storvick and others 1964; Toepfer and Polansky 1970), HPLC (Wong 1978; Vanderslice and others 1984) as well as gas chromatography - mass spectrometry methods (Lim and others 1982; Careri and others 1996). The approaches applied most widely for food products have been microbiological and HPLC methods (Wong 1978; Gregory and Kirk 1978; Vanderslice and others 1979; Morita and Mizuno 1980; Gregory and others 1981; Brubacher and others 1985b; AOAC 1990e; Schoonhoven and others 1994; Bergaentzle and others 1995; Ollilainen 1999).

The extraction of B-6 for microbiological assay procedures relies on autoclaving of homogenised samples in the presence of HCl. The acidic conditions result in hydrolysis of both phosphate esters and glycoside conjugates, thus resulting in an overestimation of the biologically available vitamin B-6 content of foods (Ubbink 1992). Another problem reported with the microbiological assay is the variable growth responses to different vitamers (Ollilainen 1999).

More recently, the use of HPLC coupled with fluorimetric detection has enabled the development of specific and sensitive methods for the determination of B-6 as the preferred method for routine food analysis. Accordingly, HPLC analysis was proposed

as a method for adoption by the European Community during a comprehensive review of vitamin analysis procedures (Brubacher and others 1985b).

4.5 Procedures for analysis of riboflavin

The range of methods for the analysis of riboflavin has expanded rapidly since 1931 when the early rat growth assay was developed. Unlike the other vitamins, the riboflavin molecule is naturally fluorescent allowing direct fluorimetry without the need for further chemical reaction. Some of the more widely used methods of measurement are fluorimetry (including AACC 1994d; AOAC 1990f, 1990h; Ball 1994; Eitenmiller and Landen 1995; Russell and Vanderslice 1992), microbiological assay with *L casei* (Barton-Wright 1945; AOAC 1990b; Rougereau and others 1997) and HPLC methods with either fluorimetric or spectrophotometric detection (Brubacher and others 1985c; Wimalasiri and Wills 1985; Finglas and Faulks 1987; Chase and others 1993b; Sims and Shoemaker 1993; Russell and others 1998).

Typically HPLC procedures are reported to offer the advantage of reliability. In addition, they can be carried out rapidly, economically and consequently are applicable to routine determination of riboflavin (Ellefson 1985a). Precision is known to be generally very good for these analytical procedures. HPLC methods have been widely applied in determining this vitamin in many foods including fruits and vegetables as well as dairy and cereal-based products (Finglas and Faulks 1987).

One issue reported to be of particular significance for the analysis of riboflavin is the extraction procedure. Typically this requires that the food matrix be broken down and for this purpose commercial enzyme preparations are widely used (Brubacher and others 1985c). These have been found to be effective even for sausages which present difficulties in analysis (Valls and others 1999).

4.6 Procedures for analysis of folate

There are many different methods for folate analysis including bioassays, microbiological, HPLC, ligand-binding and radio-immunoassay procedures (Eitenmiller and Landen 1995). Recently a procedure based upon enzyme protein binding has been introduced and evaluated (Arcot and others 2002a).

The concentration of folates is low in most foods. In addition they are reported to be relatively unstable due to factors including heat, pH, oxygen and endogenous enzymes which partially hydrolyse some forms of the vitamin, which limit the usefulness of extraction procedures and applicability of any separation methods. The need for a sensitive detection technique has also been emphasised (Hawkes and Villota 1989). Although the minimum analysis time to obtain a result for microbiological assay is typically five days compared to one day for HPLC analysis, many laboratories internationally prefer the use of the microbiological assay for determination of folates naturally present in foods, as well as folic acid added for fortification purposes (NHMRC 1995). A variety of different bacteria have been utilised in the assay of folate (Hawkes and Villota 1989). Recently *L casei* ATCC 7469 has been preferred because it responds to mono, di and tri-glutamate forms of folate which are the most common products after conjugase treatment of higher glutamate forms (Tamura and others 1972; Phillips and Wright 1982 and 1983; Hawkes and Villota 1989).

In the context of folate analysis it is necessary to briefly describe the enzyme generally referred to as conjugase in the literature. This enzyme is commonly used to remove some or all of the glutamyl residues attached to the pteroic acid based part of the molecule during analysis. The enzyme appears to be relatively specific and removes γ linked glutamyl units (compare with Figure 2.5 and Section 2.1.4). It is currently ascribed the number EC 3.4.19.9 (Nomenclature Committee of the International Union of Biochemistry and Molecular Biology 2004). Some confusion as to classification and naming appears in the literature as the most widely used name of folate conjugase is commonly shortened simply to conjugase (for example: Angyal 1996; Tamura 1998; DeVries and others 2001). As the reaction catalysed is one of deconjugation, the term deconjugase is also used to refer to the same enzyme (for example: Vahteristo and others 1996; Shrestha and others 2000; Osseyi and others 2001). In this thesis the name conjugase is adopted for simplicity.

Microbiological analysis for folate is applicable to most food matrices and vitamin supplements. The minimum detectable level on a wet basis is 0.25 μg/100 g expressed as folic acid. This detection level assumes that the lowest practical sample dilution factor is 1:100 (Eitenmiller and Landen 1999). Muller and others concluded that HPLC

is not the most appropriate technique for determining folates in cereal grain food products because the presence of many interfering compounds results in misinterpretations. According to Gregory (1984), in the study of analysis of naturally occurring folates in selected foods, the folate content determined by microbiological assay was frequently greater than that determined by HPLC analysis of the same extract.

The AOAC microbiological method has been used over a long period of time but has been the subject of intensive study particularly during the past five years. There have been various reasons for this including the increasing evidence that folate has broader nutritional and health implications. The move to fortification of foods with folate and the need to monitor compliance with regulatory requirements has also prompted some of the research in this area. Among the issues that have been addressed are:

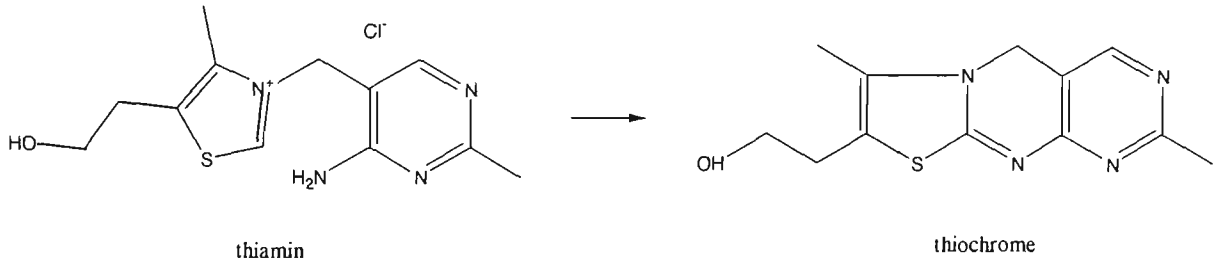
- The effectiveness of various conjugase treatments (Rader and others 1998).
- Procedures to enhance the use of the bacterial cultures by cryoprotective treatments and storage to maintain constant growth-response curves in day-to-day assay (Grossowicz and others 1981; Wilson and Horne 1982; Tamura 1990; Shrestha and others 2000).
- The release of folates from food matrices by various enzymatic treatments (Martin and others 1990; Johnston and others 2002 a and b).
- The impact of various pH conditions during assay (Kirsch and Chen 1984; Aiso and Tamura 1998).

Despite the widespread use of the microbiological method there have been a number of studies of folate analysis by HPLC (Lucock and others 1995; Vahteristo and others 1996; Osseyi and others 1998; Finglas and others 1999).

4.7 Procedures for analysis of thiamin

The analysis of thiamin levels in foods has traditionally been based upon the oxidation of free thiamin to thiochrome (Figure 4.1) (Gregory 1996). This is achieved using a

ferricyanide reagent and the product has enhanced fluorescence characteristics which facilitate the measurement of small amounts of the vitamin.



thiamin thiochrome

Figure 4.1 Oxidation of thiamin to thiochrome (Gregory 1996)

Generally thiochrome has been measured with a spectrofluorimeter. Various procedures based upon this approach have been validated and accepted internationally as standard methods by the AOAC, AACC and ICC (AOAC 1990d; AACC 1994a; ICC 1990).

More recently various methods using high performance liquid chromatography (HPLC) have been reported. These have been applied to a range of food products, using various procedures and column types. Some earlier HPLC procedures for thiamin and other water-soluble vitamins have been reviewed (Polesello and Rizzolo 1986, 1990; Finglas and Faulks 1987). At that time the reviewers concluded that further studies were required in order to establish validated methods that could be applied readily to a wide range of foods.

Many researchers have studied the analysis of thiamin by HPLC (Kamman and others 1980; Wimalasiri and Wills 1985; Hebrero and others 1988; Hasselmann and others 1989; Laschi-Loquerie and others 1991; Ollilainen and others 1993; Hägg and Kumpulainen 1994). These have generally involved pre-column derivatisation of thiamin to thiochrome followed by chromatography on reversed-phase columns (usually either C-14 or C-18). There have been some differences in fluorescence excitation and emission wavelengths utilised in the various studies.

It has been reported that enhanced recoveries of added thiamin have been achieved with HPLC methods in comparison with the standard fluorimetric methods (Wills and others 1985). However, in a more recent comparative study, no statistical difference was found between the results of the AOAC fluorimetric procedure and an HPLC method (Abdel-

Kader 1992). The suitability of available vitamin analysis procedures was assessed for regulatory purposes in the European Union (Brubacher and others 1985a). It was concluded that the fluorimetric procedure for thiamin offered the most reliable and widely applicable method available.

4.8 Other challenges in the analysis of B group vitamins in foods

In reviewing the literature on vitamin analysis, it is clear that at least four other challenges confront the analyst. These are:

- The existence of multiple forms of some of the vitamins. The different molecular forms of one vitamin are referred to as vitamers and these may vary in solubility, stability, vitamin activity and ease of measurement by particular analytical procedures.

- The presence of vitamin molecules in tissues and foods in forms where they are covalently linked to other components including proteins. For example riboflavin generally occurs as protein-bound flavin adenine dinucleotide and flavin mononucleotide (Cooperman and Lopez 1991; Combs 1992).

- The difficulty of extracting all of the vitamin due to physical entrapment within the matrix of some foods.

- The instability of vitamins under some of the conditions which might be employed during extraction and assay.

Prior to any quantitative measurement, it is necessary to extract the B group vitamin from the food. The levels of the vitamins are often very low therefore different extraction techniques may be required depending on the material being analysed. This typically involves removal of unwanted protein as well as non-protein material that would interfere with the final determination. For example, hydrolysis by either enzymatic catalysis or using an acidic solution results in release of the compound of interest from the matrix (Hägg 1994). In addition, the application of solid phase extraction may also enhance sample preparation (Papadoyannis and others 1997). Over recent years the significance of thorough sample preparation has been investigated for

riboflavin (Lambert and DeLeenheer 1992) as well as folate (Tamura and others 1997; Rader and others 1998; Kariluoto and others 2001).

In the case of vitamin B-6 the distributions of the vitamers as well as their stability varies (Toepfer and Lehmann 1961; Gregory and others 1981). It has been reported that foods derived from plants contain primarily PN, a significant proportion of which may be present as PN-glucoside or other conjugated forms which appear to be largely unavailable to humans (Vanderslice and others 1979; Ball 1998).

Due to the very different molecular structures of each of the water-soluble vitamins, they show varying susceptibilities to oxidation as well as degradation by exposure to alkaline conditions, air, light and heat. Therefore specific procedures have been developed for each vitamin (Lambert and DeLeenheer 1992).

On the basis of the literature reviewed here there is a large range of approaches available for analysis of the B group vitamins. It also appears that although flour- and grain-based products have been studied, the specific challenges of vitamin analysis in Asian noodle products warrant evaluation in this study.

Chapter 5

Summary of background and description of the project aims

The purpose of this chapter is to summarise the context in which this project has been developed and to describe the aims of the project.

5.1 Summary of current situation and significance of the project

Almost one third of all wheat produced globally is used for Asian noodle production. In addition, it has been estimated that a similar proportion of the wheat exported by Australia is used for noodles (Wrigley 1994) as these products are a staple food in many Asian countries. In addition, the consumption is increasing in Australia due to the popularity of Asian cuisines.

During the early twentieth century, nutrient deficiency was identified as a major public health problem. The adequacy of B vitamins including thiamin, riboflavin, niacin, vitamin B-6 and folate in the diet remains a current concern and has been the subject of research and attention in recent years. These vitamins have been measured in a wide range of foods including beverages, meats, cereal products and bread (Ottaway 1993). However, there have been no previous studies of these vitamins in Asian noodles, therefore this project was developed to extend our knowledge in this area.

5.2 Hypothesis

This project has been based upon the hypothesis that for different styles of Asian noodles, losses of the B vitamins may vary significantly as a result of the formulations used and the specific processes applied during manufacture and storage.

5.3 Project aims

The aims of this project are:

1 To evaluate methods for measurement of B group vitamins in Asian noodle products: This preliminary phase is essential as there are many published procedures including newer methods based upon HPLC. These are to be evaluated for their suitability for wheat flours and grain-based foods. Two other significant issues are:

a) The need to consider the likely presence and analysis of different molecular forms of the vitamins in these foods; and

b) The difficulty in efficiently extracting vitamins from food matrices.

For this latter purpose, various enzymatic treatments have been described in the recent scientific literature and these are evaluated. In addition, internationally accepted standard methods (AOAC, AACC and ICC) are evaluated along with newer chromatographic procedures.

2 To analyse commercial Asian noodles: a range of products and flours are analysed for vitamin B group contents, as a basis for selecting specific styles of noodle for further investigation. Noodles are studied for vitamin B group content and also for the pH characteristics in relation to the ingredients and style of noodles.

3 To investigate the factors impacting on vitamin stability in Asian noodles: Different styles of noodles are prepared on a laboratory scale and influence of ingredients and process variables on the contents of B group vitamin studied. For this work, pH and other variables are monitored through the steps from dough mixing, sheeting, and cutting as well as drying, cooking, steaming and frying. The analytical data are compared with those obtained for commercial samples in order to ensure the applicability of results to the commercial situation. In addition to assessing the stability of the vitamins naturally present in the flours, further studies are designed to evaluate the feasibility of fortification of the various noodles styles.

4 To investigate vitamin stability during storage of noodles: Noodles are commonly stored in both the fresh and dried forms and so the impact of storage conditions on the different B group vitamins are investigated. The influence of storage time and conditions have been monitored by measurement of contents of B group vitamins in a range of Asian noodles during storage. This includes both short-term studies of fresh noodles as well as longer term storage of dried products. Among the factors considered are the temperature, pH and product moisture levels.

5 To study the effect of fortifying noodle products with B group vitamins and strategies for enhancing stability: Asian noodles may represent an appropriate food for routine fortification in order to ensure the adequacy of intakes of the individual B group vitamins. The suitability of the various styles of noodles for this purpose has also been investigated.

Chapter 6

Materials and methods

The purpose of this chapter is to describe the chemicals, reagents, equipment and methods used during this study. This encompasses procedures applied in the sampling and preparation of noodles, methods for fortification with B group vitamins, extraction procedures and analytical methods, along with details of calculations for individual B group vitamins.

6.1 Materials

The chemicals including enzyme preparations and vitamins used in product formulations and analytical procedures were of analytical grade or of the highest purity available, unless otherwise specified. Vitamins were used in the current study as chemical standards for analytical purposes as well as for spiking and fortification purposes. In addition a reference sample was used for method validation and in the assessment of sample extraction procedures. The details of the vitamins and reference used are presented in Table 6.1. The specifications supplied with the reference sample are shown in Table 6.2. Details for other chemicals used in the study are presented in Table 6.3.

In this study water was used in a wide variety of procedures including the preparation of solutions of standards, reagents and all HPLC solvents. In addition, water was used in the preparation of extracts for analysis of vitamin contents and the measurement of pH values, for the preparation of noodle samples in the laboratory and for cooking of both commercial and laboratory noodles. In all cases the water used was of MilliQ grade and this was freshly prepared in the laboratory as required. The same water type was used at all times in the final rinsing stages during the washing of all laboratory glassware and utensils. Tap water was not used at any stage of this study. The term water is used in this thesis to specify MilliQ water.

The cultures of *L casei* subsp. Rhamnosus (ATCC 7469) used in this study were obtained from three different sources: Ms Lata Masih, University of Melbourne, Gilbert Chandler College, Werribee; from the culture resources of Victoria University, School of Life Sciences & Technology (originally sourced from the Australian Starter Culture Research Center, Werribee, type ASCC 1521 ≅ ATCC 7469); and from the Department of Microbiology, the University of New South Wales, Sydney. The three cultures were compared in preliminary evaluations and found to give similar results. The culture selected for routine use was that obtained from Sydney.

The details of commercial flours used together with the respective suppliers are listed in Table 6.4. Commercial noodles analysed in this study were purchased from various retail outlets in Melbourne. Based upon the appearance of the products as well as the information provided on the packages, the samples were classed as belonging to one the three styles of noodles. In some cases it was not possible to clearly categorise the samples and these are designated as 'miscellaneous'. The basis for the designation of samples is described further in Section 10.9 (Chapter 10). Details of the samples are presented in Tables 6.5–6.8. Each commercial flour and noodle sample was analysed prior to the use by date provided on the packaging of the product.

Table 6.1 Sources and details of vitamins and the vitamin reference sample

Vitamin/sample	Description	Supplier
Thiamin hydrochloride	T4625, 49H0392	Sigma Chemical Co, USA
Riboflavin	R-4500, 97H0433	Sigma Chemical Co, USA
PM dihydrochloride	P-9380, 19H0771	Sigma Chemical Co, USA
PL hydrochloride	P-9130, 39H0693	Sigma Chemical Co, USA
PN hydrochloride	P-9755, 129H0788	Sigma Chemical Co, USA
NA	N-4136, 85 H0271	Sigma Chemical Co, USA
NTA	N-3376, 128H1168	Sigma Chemical Co, USA
Folic acid	4-7866, LA 98285	Sigma Chemical Co, USA
AACC reference sample	Cereal sample from the Vitamin, Mineral and Proximate Check (VMP-3) Sample number VMA 399	AACC, St Paul, MN, USA

Note Description presented as product number, batch or lot number

Table 6.2 Specifications for vitamins in the AACC reference sample

Vitamin	Mean (mg/kg)	Number of analyses	Standard deviation	Coefficient of variability (percent)	Range of vitamin contents (mg/kg)
Thiamin	69.3	14	7.0	10.1	60.6 – 82.8
Riboflavin	59.7	16	7.9	13.2	48.9 – 76.0
Vitamin B-6	85.0	11	9.4	11.1	73.0 – 100.5
Folic acid	13.95	9	1.43	10.3	11.60 – 16.20

Note The values in this table are those provided with the sample and represent the results obtained by the laboratories which originally participated in the check sample collaborative survey run by AACC. The original data have been recalculated (to units of /kg) where necessary.

Table 6.3 Details of chemicals and suppliers

Supplier	Chemicals
Sigma Chemical Co, USA	α-Amylase (A-3176, 11K1563), Ammonium hydroxide (A-6899, 118H4355), Bromophenol blue (2383, B82), Glutathione (G-4626, 88H7440), Hydrochloric acid (101256J, K26945452), L-Ascorbic acid (A-7630, 110K1256), Potassium ferricyanide (P-8131, 54H3488), Quinine (Q-125063H0765), Trichloroacetic acid (T-6399, 39H0412)
BDH Laboratory Supplies, England	1-Hexanesulphonic acid sodium salt (152793M, K28238256 045), 1-Octanesulphonic acid sodium salt (152803U, K26857058), 2-methyl-1-propanol (100625K, H728341-920), Acetic acid (100015N, K27574117012), Acetonitrile (152856K, 034210 221), di-Sodium hydrogen orthophosphate (30158.5000, 22160), Ferrous sulphate (101124V, TA711641511), Formic acid (101156G, K25587943), Glycerol (101118.4k, 27685), MeOH (15250, L050502), n-Hexane (103876Q, I870778919), Octan-1-ol (294084 B), Orthophosphoric acid (153153N, K29299573128), Papain (390303G, F389661883), Perchloric acid 60% (10175, 249C235675), Potassium chloride (10198.7X, 30523), Potassium hydroxide (10210.5000, 21336), Sodium acetate (010236.5000, 20876), Sodium chloride (10241.AP, 26194), Sodium dihydrogen orthophosphate (30132, 18560), Sulphuric acid (303246E, Z50960792), Toluene (010284.2500, 20985), Triethylamine (153293B, L456341 028)
Ajax Chemicals, Melbourne	Sodium hydroxide (10252.7R, 24444), Glasswool (1755, 70147712), Sodium carbonate (1225, 216302)
Solvay Biosciences, Melbourne	Clarase concentrate (6/1995)
Megazyme,	Protease (Subtilisin A) (10401)
Fluka Chemie Gmbh, Germany	Clara-diastase (86959, 9001-11-0), Acid phosphatase (85433020-16/28, 108227)
EM Science, USA	*iso*-propyl alcohol (PX 1838-1, UN 1219)
CSR, Melbourne	Ethanol (UN 1170)
Difco Laboratories Detroit, Michigan, USA	Lactobacilli broth AOAC (290110, 141949), Folic acid casei medium (282210, 1290000), Chicken pancreas (245910, 140021XB)

Note Description presented as chemical name (product number, batch or lot number)

Table 6.4 Description of flour samples used for this study

Flour	Description	Supplier
Continental Farina	Specialty flour Packed on 27.4.1999, code 14199	Weston Milling, North Melbourne, Victoria
Maximus	Strong bakers flour Packed on 27.4.1999, code 11564	Weston Milling, North Melbourne, Victoria
Milano	Pizza flour Packed on 01.9.97, code 60594	Weston Milling, North Melbourne, Victoria
McAlpine	Wholemeal self-raising flour MC 317:08	White Wings Foods, New South Wales
Defiance	Plain flour code 11:27	Defiance, Queensland
P-farina	Extra-white flour Packed on 16.1.01, code 1474D	Allied Defiance, New South Wales
Laboratory millings	Various grades of wheat from different parts of Australia and US Hard Red Winter	Agrifood Technology, Werribee

Table 6.5 Description of samples of commercial white salted noodles

Brand	Ingredients (as listed)	Country of origin
AOI	Wheat flour, salt, water	Japan
Bea suey somen	None declared	Taiwan
Dragons	Wheat flour, salt, water	China
Enriched	Flour, starch, salt	Taiwan
Jona	Organic wheat flour, salt, water	Australia
Shanghai	Wheat flour, salt, water	China
Thin Kanto	Wheat flour, salt	Taiwan
Tomoshiraga somen	Wheat flour, salt, water	Taiwan
Trident	Wheat flour, tapioca starch, mineral salts (339, 340), emulsifying salt (450), water added	Australia

Table 6.6 Description of samples of commercial yellow alkaline noodles

Brand	Ingredients (as listed)	Country of origin
Fantastic noodles	Wheat flour, water, salt	China
Gold star	Egg, plain flour, water, natural colouring, salt, alkaline water	Australia
Golden horse	Wheat flour, salt, water	Viet Nam
Longevity	Wheat flour, lye water, corn starch & water	Hong Kong
Mai Hong	Wheat flour, egg, salt, potassium carbonate, sodium carbonate, starch, potassium sorbate and/or Calcium propionate, tartrazine, sunset yellow, ascorbic acid, water added	Australia
Mi ga	Wheat flour, egg, water, lye, vegetable oil	Viet Nam
Nouilles Chinois	Wheat flour, water, salt	Thailand
Song Long	Not declared	Viet Nam
Taings	Australian wheat flour, water, lye water, canola oil, salt, food colour (102, 110)	Australia
Vegetarian	Wheat flour, vegetable oil, water ash	Viet Nam

Table 6.7 Description of samples of commercial instant noodles

Brand	Ingredients (as listed)	Country of origin
Heinz	Wheat flour, starch (potato, tapioca), salt, mineral salts (450, 501, 500)	Indonesia
Indomie	Wheat flour, edible vegetable oil, salt, potassium carbonate, sodium polyphosphate, natural gum, sodium carbonate, and tartrazine Cl 19140	Indonesia
Maggi	Wheat flour, vegetable oil, salt, mineral salts (450, 501, 500), vegetable gum (412), colour (101)	Australia

Table 6.7 Description of samples of commercial instant noodles (continued)

Brand	Ingredients (as listed)	Country of origin
Mama	Wheat flour, palm oil, salt 0.1 %, sugar 1.2 %	Thailand
Ma Ma Mien	Wheat flour, starch, vegetable oil, salt	Taiwan
Marketta	Wheat flour, salts, water	China
Nisin Demae Ramen	Wheat flour, refined vegetable oil, salt, potassium carbonate, tocopherols	Hong Kong
Nissin JAS	Wheat flour, potato starch, palm oil, salt, sodium carbonate, calcium, pepper, monosodium glutamate, chicken extract	Japan
Pancit Canton with Kalamansi	Enriched flour (Wheat flour, niacin, reduce iron, thiamin mononitrate, riboflavin), palm oil, salt, guar gum, sodium carbonate, whole egg powder, potassium carbonate, FD&C yellow no. 5 (tartrazine), sodium polyphosphate, FD&C yellow no.6, BHA, BHT	Philippines
Pancit Canton	Wheat flour, palm oil, salt, guar gum, sodium carbonate, potassium carbonate, whole egg powder, BHA, BHT, FD&C yellow no. 5 (tartrazine), FD&C yellow no.6	Philippines
Sapporo Ichiban	Enriched wheat flour (flour, niacin, iron, thiamin mononitrate, riboflavin), partially hydrogenate palm oil, alginic acid, sodium carbonate, sugar, monosodium glutamate and potassium carbonate	USA
Trident	Wheat flour, palm oil, salt	Thailand
Trident Authentic	Wheat flour, palm oil, salt, mineral salts: 500-501	Australia
Wai Wai	Wheat flour, palm oil, salt, chili	Thailand

Table 6.8 Description of samples of miscellaneous noodles

Brand	Ingredients (as listed)	Country of origin
Fantastic	Wheat flour, salt, water added	China
Koyamaseimen	Wheat flour, salt	Japan
Kantong	Wheat flour, modified starch, salt, gluten, potassium carbonate, natural plant extract, water added	China
Taings udon	Wheat flour, salt, water, canola oil	Australia
Wokka	Wheat flour, starch, rapeseed oil, salt, egg flavouring	Korea

6.2 Apparatus and auxiliary equipment

The items of equipment used, together with the details of manufacturers and model numbers are presented in Table 6.9. The HPLC instrumentation is described in Table 6.10 and the columns and ancillary items used for HPLC are detailed in Table 6.11.

Table 6.9 Description of equipment and instrumentation

Equipment	Manufacturer/supplier	Model No
Water bath (thermostatically controlled)	Thermoline Scientific Instruments Pty Ltd, Melbourne	BTC 9090
Minolta Chroma Meter	Minolta Camera Co Ltd, Japan	CR 300
Sonicator (bath type)	Unisonics Pty Ltd, Sydney, Australia	Type Fx 14PH Serial no. 469
Cary Spectrophotometer (uv-visible)	Varian Australia Pty Ltd, Melbourne	1E
Ultra-Turrax homogeniser	Janke and Kunkel, Stanfen, Germany	T 25
Kenwood mixer	Kenwood Ltd, Britain	KM210, Serial no. 0309397
Noodle maker	Domestic 'spaghetti machine' Imperia, Italy	MOD 150, design no. 1048534
Cutting attachment for noodle maker	Imperia, Italy	MOD 150
Kambrook deep fryer	Kambrook distributing Pty Ltd. Australia	KD 53
Sieve (500 μm)	Endecotts Ltd. London, England	Part No. 667924, 737176
Autoclave	Getinge Australia Pty. Ltd. Bulimba Brisbane	Unit No. 2206.00
Steam bath	Labec, Laboratory Equipment Pty. Ltd. Sydney	Serial No. G240
Oven	Memmert, GmbH, Germany	Type: UML 500, F No: 891319, NIN 12880-KI
Centrifuge	Beckman Instrument, Inc, Germany	GS-15R, 360904, series GYD 95H13.
Spectrofluorimeter	Perkin-Elmer Corp, UK	LS 50, Serial No. 26309.
Grinder	Philips, China.	HR 2185
Cuvettes	Kartell Pacific Ltd, Singapore	PMMA 1941, PN 1961

Table 6.10 Description of HPLC system components

Equipment	Manufacturer	Model No
Variable wavelength detector (uv-visible)	Varian Australia Pty Ltd, Melbourne	9050
Fluorescence detector	Varian Australia Pty Ltd, Melbourne	9070
Polychrome	Varian Australia Pty Ltd, Melbourne	9065
Auto Sampler	Varian Australia Pty Ltd, Melbourne	9100
Solvent Delivery System	Varian Australia Pty Ltd, Melbourne	9012
Data handling system	Varian Australia Pty Ltd, Melbourne	Star Chromatography software, Star WS 5.31
SSI 505 LC column oven	BioRad Laboratories, Melbourne	AA4910282

Table 6.11 Description of columns and ancillary items used in HPLC analysis of vitamins

Item	Manufacturer	Part No
Waters Spherisorb ODS 2, 5μm, 250 × 4.6 mm	Waters Australia Pty Ltd, NSW	SN: 00061703.1, Lot No.126
Allsphere ODS 2, 5μm, 250 × 4.6 mm	Alltech Associates (Aust) Pty Ltd, Melbourne	SN: 97120273 Lot No. 26/045
Waters-Resolve C-18, 5μm (3.9 × 150 mm)	Waters Australia Pty Ltd, NSW	85711, T 31721
Phenomenex ODS 3, 5μm (250 mm×4.6)	Australian Chromatography Co, Melbourne	SN: 169297, P No. 006-4097-EO
Guard column Allsphere ODS 2 C-18 5 μm, 7.5 × 4.6 mm	Alltech Associates (Aust) Pty Ltd, Melbourne	96403, H27
Reversed-phase test mix	Alltech Associates (Aust) Pty Ltd, Melbourne	PN: 1895, L16
C-18, SAX, Silica cartridge	Alltech Associates (Aust) Pty Ltd, Melbourne	500mg size
Solid phase extraction: OASIS HLB, OASIS MCX, OASIS MAX, Sep-pak plus	Waters Australia Pty Ltd, NSW	500mg size
Advantec filter paper	Toyo Roshi Kaisha, Ltd. Japan.	Type 5C Lot B0810111
Filter unit, FP	Schleicher & Schuell, Germany	Point 2 (0.2 μm) and point 4 (0.4 μm)
Nylon membrane filters Cellulose acetate filter	Alltech, Australia	0.45 μm 0.2 μm

6.3 Laboratory procedures for manufacture and processing of Asian noodles

6.3.1 Description of flour samples used in preparation of particular types of noodle samples in the laboratory

A series of flour samples having different characteristics and suited to different end uses were purchased from retail stores, P-farina (from Goodman Fielder); Continental Farina and Maximus (from Weston Milling), all located in Melbourne (compare with Table 6.4). These were used for the testing of various vitamins as well as for the preparation of laboratory noodles. In most phases of the research reported here P-farina was used for preparation of noodles in the laboratory. In the case of noodles made for studies of thiamin stability, Continental Farina was used for white salted noodles and Maximus for yellow alkaline and instant noodles.

In studies of noodles prepared in the laboratory, a number of batches of the same noodle style were made and the samples of these batches were analysed at least in triplicate. The averages of the results from the analysed data were calculated and are presented in this report, representing the value of each batch of noodles prepared.

6.3.2 General procedures applied in the preparation of noodle samples

Noodle samples were prepared using procedures based on those described by Moss and others (1987). It is specifically noted here that all steps in the preparation of noodles were carried out in subdued lighting conditions in order to minimise the potential impact of light on vitamin retention. In general, the noodle making methods for the three styles have four common steps: mixing, sheeting, cutting and drying. However, for instant noodles, after cutting, steaming and frying steps were also required. The preparation of each of the three styles of noodles are described in detail as follows:

6.3.3 Preparation of white salted noodles

Ingredients used for white salted noodles

White salted noodles were prepared from either P-farina or Continental Farina as these had processing characteristics generally suitable for this style of noodles. It is noted that these were not specifically designed to be white salted noodles of the Japanese Udon

type which require high swelling starch properties. The basic ingredients used for making all white salted noodles were: 300.0 g flour, 96.0 g water and 9.0 g common salt. In some instances, one or more vitamins were also incorporated into the formulation as described in the results section.

Method for white salted noodles

Mixing: The salt was first dissolved in the water and this solution was added to the flour over a period of 30 s in a Kenwood mixer set on speed one. Timing of mixing then began when all the liquid had been added. The mixer was set at the lowest setting (speed 1) for 1 min then it was stopped so that the dough material adhering to the bowl and beater could be scraped down. After that, the speed of the mixer was increased smoothly to setting 4 and allowed to mix for a further 4 min. After a total of 5 min mixing (1 plus 4 min), the resultant dough had a crumbly consistency similar to that of moist breadcrumbs.

Rolling: The dough was first formed into a dough sheet by a process of folding and passing the crumbly dough through the rollers of the noodle machine several times. For this combining step the rollers were set at the maximum gap available, corresponding to 2.7 mm. Typically three passes were required although up to 5 passes were used where necessary in order to give a uniform sheet which held together as a single dough piece. Then this combined sheet was allowed to rest for 30 min. For this purpose, the sheet was covered with aluminium foil and then sealed in a plastic bag to firstly exclude light and secondly to prevent moisture loss after resting. The thickness of the sheet was reduced stepwise by passing between the rollers of the noodle machine. The roll gap settings used were: 2.2, 1.8 and 1.4 mm.

Cutting: The sheet was cut into strands using the cutting roll attachment of the noodle machine having a cutting width of 2.0 mm. The noodle strands were then cut into 25 cm lengths using a knife before drying.

Drying: The fresh noodles were arranged upon trays lined with aluminium foil. The noodles were placed loosely in order to facilitate effective drying and the trays were stored in a fan forced oven at 40 °C for 24 h. The product was then allowed to cool for

30 min in the ambient conditions of the laboratory prior to being placed in airtight plastic bags or containers for storage.

6.3.4 Preparation of yellow alkaline noodles

Ingredients used for yellow alkaline noodles

The flours used for preparation of yellow alkaline noodles were either bakers strong or P-farina, depending upon the specific purpose of the experiments as described in the results chapters. The ingredient formulation was: 300.0 g flour, 96.0 g water and 3.0 g sodium carbonate.

Method for yellow alkaline noodles

The procedure for making yellow alkaline noodles was basically the same as that described above for white salted noodles with the following modification:

Mixing: alkaline salt (rather than common salt used for white noodles) was dissolved in the required volume of water.

6.3.5 Preparation of instant noodles

Ingredients used for instant noodles

The basic ingredients used to make instant noodles were: 200.0 g flour, 60.0 g water, 0.2 g sodium carbonate. The oil used to deep fry the noodles was palm oil (Auroma Pty Ltd, Australia).

Method for instant noodles

The procedure for making instant noodles was the same as that for yellow alkaline noodles at the mixing and rolling steps. However, following these, the resultant sheet was not rested but was immediately passed a further four times between the rollers to reduce the sheet thickness before cutting into noodle strands for the further preparation steps of steaming, frying and draining.

Steaming: fresh noodles strands were placed in a steamer and steamed over vigorously boiling water for 2 min. Then they were removed from the steamer and placed onto dry paper towel for 30 s.

Frying: The noodles from the steaming step were then immediately placed into a wire basket and deep fried in palm oil for 45 s. The temperature of the oil was carefully checked and noodles were only placed into the oil once it had attained 150 °C.

Draining and cooling: The fried noodles were removed from the oil using the wire basket and allowed to drain for 30 s. Noodles were then transferred to absorbent paper and allowed to cool in the air flow created by a fume cupboard for 20 min prior to placing into a sealed bag or container for storage.

6.3.6 Fortification of noodle samples with B group vitamins

Samples of noodles were also prepared in which additional B group vitamins had been incorporated into the formulation. Each of three different noodle styles was fortified under controlled conditions. This was achieved by first dissolving the vitamin in an appropriate solvent and a suitable volume was then used as part of the liquid portion of the formulation. Other aspects of noodle preparation followed the procedures described above. It is noted that here the fortification levels are expressed on the basis of the fresh weight of flour. For both riboflavin and PN.HCl the levels added were 12 mg/kg of flour and water was used as solvent. In the case of folic acid the level was 2.86 mg/kg flour and the compound was first prepared in an aqueous solution of ethanol (20 percent) which also contained NaOH (0.01 M). Dried folic acid (20 mg) was dissolved in 500 mL and incorporated into the formulation at a rate of 7.15 mL per 100 g fresh weight flour. For studies of noodles containing fortification levels of thiamin it was not necessary to specifically incorporate the vitamin. Instead a commercial flour which had been fortified at the flour mill was selected. Thiamin had been added to this flour in accordance with current Australian legal requirements for breadmaking flours.

A further experiment was carried out for noodles fortified with folate in which varying levels of fortification were compared. For this purpose, flour was fortified using a standard folic acid solution at a concentration of 40 μg/mL. This solution was prepared by dissolving 20 mg of dried folic acid into 500 mL of the solvent described above. Fortified noodles were made in the laboratory by adding different volumes of the folic acid solution (3.57, 7.15 and 10.0 mL per 100 g fresh weight flour) into the typical

formulation of white salted noodles. These corresponded to levels of fortification of 1.43, 2.86 and 4.0 mg/kg respectively.

6.4 General methods for characterisation of flours and noodle samples

In the analysis of all samples, multiple analyses were carried as described for the individual analysis procedure. In all cases the results for at least duplicate measurements of individual samples were assessed statistically and are reported as the mean value ± standard deviation. In reporting data, the latter is abbreviated as sd and the number of replicate determinations is referred to as n.

6.4.1 Moisture determination

The moisture contents of samples (flours, doughs, dried noodles, steamed noodles, fried noodles, and cooked noodles) were measured following the air oven method (AOAC 1990a). For each sample analyses were carried out in duplicate. It is noted that samples were not ground prior to analysis. Empty aluminium moisture dishes with lids were first placed into a pre-heated oven set at 130 ± 3 °C. After 1 h, the empty dishes were taken from the oven and cooled in a desiccator containing active silica gel desiccant for a period of 20 min and then weighed. Sub-samples (approximately 2.0 g) were accurately weighed into the pre-weighed dishes. Then the covered dishes containing the samples were placed into the oven with the lids placed under the respective dishes and dried at 130 ± 3 °C. The process of drying, cooling and weighing was repeated after 1 h until a constant weight was attained. The loss in weight was used to calculate the moisture content of the samples using the following equation:

$$\text{Moisture content (percent)} = \frac{\text{Loss in weight of dish, lid and sample upon drying}}{\text{Initial weight of sample}} \times 100$$

6.4.2 Measurement of the pH of flours and noodle samples

The pH values of flour and noodle samples were determined by the AACC standard procedure (AACC 1994b). For this, a sample (10 g) was thoroughly blended in 100 mL

of water using the Ultra-Turrax homogeniser. The mixture was then allowed to settle for approximately 30 min after which the supernatant liquid was decanted and tested with a calibrated pH meter. All analyses were carried out in triplicate.

6.4.3 Measurement of instant noodle colour

The colour of instant noodles was determined by using Minolta Chroma Meter. The instrument was first calibrated using the white calibration tile supplied by the manufacturer. For analysis, the three different colour parameters, L^*, a^* and b^* were recorded. The L^* value measures the degree of whiteness/darkness and the higher the L^* value, the lighter the colour. The a^* value indicates the balance between redness and greenness of the sample with positive values corresponding to red colours and negative to green. The b^* value indicates the balance between yellowness (+) and blueness (-). For a^* and b^* readings, values closer to zero indicate less intense colour whereas readings further from zero correspond to more intense chroma characteristics (Hutchings 1999). Multiple sets of readings ($n \geq 10$) were taken on all samples by moving the measuring head on a random basis to different locations on the surface of the sample between readings. Mean values are reported.

6.5 General sampling and extraction procedures used in the analysis of vitamins

6.5.1 Preparation of samples for vitamin analyses

For analysis purposes, flours did not require pre-treatment, while noodle products were prepared as follows:

Grinding

All the noodles investigated, including commercial products as well as those prepared in the laboratory and which were in the dried form (white salted and yellow alkaline noodles), and the fried form (instant noodles) were ground to pass through a 35-mesh screen (sieve aperture: 500 μm) prior to extraction of vitamins.

Cooking

The noodle samples were cooked by placing a small amount (approximately 10 g) into a saucepan of gently boiling water (300 mL). After every minute, a strand of noodle was

removed, immediately placed into water which was at ambient temperature. The strand was then squeezed between two microscope slides. Noodles were considered to be fully cooked at the point where the uncooked core had just disappeared, that is a uniform colour and appearance was observed upon squeezing. The optimum cooking time of both commercial and laboratory product varied considerably depending upon the cross-sectional sizes and also the style of noodles.

6.5.2 Extraction of vitamins from flour and noodle samples

Homogenisation

A small quantity of sample (1-7 g) (including, dough, ground noodles, fresh noodles, steamed noodles, fried noodles and cooked noodles) were weighed and blended with a minimum volume of diluted acid (0.1 M, either HCl or H_2SO_4) for a short period (20 s to 2 min). The volume used was that sufficient to allow effective maceration of the particular sample.

Autoclaving

All the prepared samples were autoclaved at either 105-121 °C for 10 or 15 min, depending upon which vitamin was to be analysed and then cooled to room temperature before application of any further specific treatments. In addition, for analyses of riboflavin and thiamin, if the assay could not be finished in one day, after autoclaving the filtrate was stored in refrigerator (Ellefson 1985b). For folate extracts, the samples were stored frozen.

The instant noodles contained elevated levels of fat. For these samples it was observed that the fat formed a discrete layer above the aqueous extract after autoclaving. The samples were centrifuged at refrigeration temperatures. Where a layer of fat was observed, care was taken in withdrawing aliquots of the aqueous extract in order to exclude the fat material (Ellefson 1985b).

6.6 Procedures and calculations applied generally in the analysis of vitamins

Due to the potential sensitivity of the vitamins to light, all procedures were performed in the absence of direct light. In addition, samples and sample extracts were covered with aluminium foil to exclude light and brown glassware was used wherever possible. Except where otherwise indicated, all steps in analytical methods were performed without delay.

6.6.1 Procedures used in the validation of vitamin analysis methods

In all cases, a variety of approaches were used to ensure the validity of the methods and the resulting analytical data. During the development and establishment of methods the initial approach was to measure standard solutions of either individual vitamins or mixtures of these. Secondly, the AACC reference sample was used and the results evaluated in relation to the specifications supplied with the sample (Table 6.2). The third procedure involved recovery studies in which flour samples were spiked with appropriate amounts of the standard vitamin compound prior to extraction.

Recoveries were calculated as follows:

$$\text{Recovery (percent)} = \frac{(\text{vitamin in spiked sample} - \text{vitamin in unspiked sample})}{\text{vitamin added in spiked sample}} \times 100$$

6.6.2 Calculation of vitamin contents to a dry weight basis

The results obtained for contents of each of the vitamins in flour, dough and noodle samples were routinely adjusted by calculation to a dry weight basis. The purpose was to facilitate the direct comparison of the results particularly for different sample types. All samples analysed for vitamins were also tested for moisture content. The following general equation was applied:

$$\text{vitamin content (adjusted to a constant moisture basis)} = \text{vitamin content (as is basis)} \times \frac{100 - \text{constant moisture figure}}{100 - \text{actual moisture of sample}}$$

In all cases the data were recalculated to a dry weight basis (where the constant moisture figure is zero) so the equation was used in the form:

$$\text{vitamin content (adjusted to a dry basis)} = \text{vitamin content (as is basis)} \times \frac{100}{100 - \text{actual moisture of sample}}$$

6.6.3 Duplication and presentation of analytical results for vitamin contents

In the analysis of samples for vitamin content, at least duplicate sub-samples of each sample was extracted on different days. In addition, multiple analyses were performed on each extract obtained. The results of replicate analyses of each sample have been calculated and presented as mean values ± sd. These calculations were carried out using Microsoft® Excel 2000 software. In the evaluation of results obtained when reference materials were repeatedly analysed, the coefficient of variability of a series of values was also calculated using the following formula:

$$\text{Coefficient of variability (percent)} = \frac{\text{standard deviation}}{\text{mean value}} \times 100$$

6.7 Evaluation of procedures for simultaneous analysis of mixtures of B group vitamins

6.7.1 Methods for extraction and HPLC analysis of vitamins

A series of different HPLC methods for simultaneous determination of water-soluble vitamins were evaluated. For this purpose the published methods were followed quite closely although in some instances the specific column available was not identical to

that used in the original study. The impact of composition of mobile phases on peak separation was also investigated in some cases along with various extraction procedures and the use of solid phase extraction. For these purposes mixtures of standards were first run and in most cases samples of the AACC reference and flour (Maximus) were also used for method evaluation. Standard solutions of each vitamin were prepared at concentrations in the range 1-25 μg/mL and calibration curves prepared using the HPLC conditions described as follows:

Method of Chase and Soliman (1990)

The extraction for this method involved homogenising sample (5 g of flour or 1 g of AACC reference) with 25 mL HCl (0.1 M) followed by sonication at full power until clear. Typically periods exceeding one hr were required. The homogenate was then diluted with mobile phase to give vitamin concentrations in the range 1-15 μg/mL and filtered through 0.45 μm nylon filter. Mobile phase was prepared by dissolving acetonitrile (9.5 percent), 1-hexanesulphonic acid (0.005 M) and ammonium hydroxide (0.5 mL) in water and adjusting the final solution to pH 3.6 with orthophosphoric acid (0.1 M) and the volume adjusted to 1 L. HPLC was carried out on a Waters-Resolve C-18, 5μm (3.9 × 150 mm) column with isocratic elution at the flow rate of 1 mL/min and ultraviolet (uv) detection wavelength at 280 nm for the first 5 min then 254 nm for the remainder of the chromatographic run.

For further assessment of a suitable column, another HPLC column Allsphere ODS 2 was also used along with the above mobile phase at the same flow rate for analysis of a mixture of vitamin only.

Method of Albalá-Hurtado and others (1997)

Flour sample (5 g) and AACC reference sample (1 g) were extracted by firstly homogenising with 10 mL of water, then trichloroacetic acid (TCA, 1 g) was added and mixed thoroughly, centrifuged at 1250 *g* for 10 min to separate the precipitate from the supernatant. After that TCA (3 mL, 4 percent) was added to the residue, mixed and re-centrifuged. The two acid extracts were combined and made up to 10 mL with 4 percent TCA prior to filtering through a 0.45 μm filter. A Waters Spherisorb ODS 2 C-18 column was used with uv detection at wavelengths of 261 nm (0-6 min), 290 nm (6.1-13

min), 282 nm (13.1-16 min), 268 nm (16.1-19.5 min) and 246 nm (19.6-60 min) corresponding to the elution of NTA, PN, folic acid, riboflavin and thiamin respectively. For the mobile phase, an aqueous solution was first prepared (0.005 M octanesuphonic acid sodium salt, 0.5 percent triethylamine, 2.4 percent glacial acetic acid) and adjusted to pH 3.6 using HCl (0.1 M). This was then mixed with MeOH (85:15) and used isocratically at room temperature and a flow rate of 1 mL/min.

Method of Agostini and Godoy (1997)

Extraction of flour (5 g) and AACC reference sample (1 g) involved homogenising sample in dilute H_2SO_4 (0.1 M) prior to sonication for 60 min. Then it was diluted with MeOH and stored at –18 °C for another 60 min. After that the extract was mixed and passed through a 0.45 μm filter prior to chromatography on a Waters Spherisorb ODS 2 C-18 column at room temperature with a flow rate of 0.7 mL/min. For the mobile phase an aqueous solution was first prepared with 1-hexanesulphonate (0.005 M), triethylamine (0.15 percent) and adjusted to pH 2.8 using 0.1 M HCl. This solution was then used in a multi-step gradient. During the initial period of 3 min, a linear gradient was used, starting from 2 percent acetonitrile: 98 percent aqueous solution and changing to 3 percent acetonitrile: 97 percent aqueous solution. This was followed by another linear gradient over a period of a further 20 min and having a final composition of 2 percent acetonitrile, 41 percent aqueous phase and 57 percent MeOH. Peaks were identified using a diode array detector at 254 nm (first 9 min), 278 nm (9-15 min) and finally at 254 nm (after 15 min).

Method of Papadoyannis and others (1997)

It is noted that for was evaluation of this particular method only a mixture of standards was used. The uv detector was set at 270 nm. The column used was Phenomenex ODS 3 and elution was effected with a multistep gradient as described in Table 6.12.

Method of Phenomenex (Phenomenex catalogue chromatography 00/01)

In addition, a mixture of B group vitamins was also analysed by using HPLC column Phenomenex ODS 3. For the mobile phase, two solutions were prepared:

A) Water/MeOH/H_3PO_4 (95:5:0.2) containing 0.005 M hexanesulphonate

B) Water/MeOH/H_3PO_4 (50:50:0.2) containing 0.005 M hexanesulphonate

Elution involved a linear gradient having an initial composition of 100 percent A and final of 100 percent B, run over 30 min. The equilibration time between runs was set for 20 min and other conditions used were a flow rate of 1.5 mL/min and uv detection at 275 nm (Phenomenex catalogue chromatography 00/01).

Table 6.12 Description of gradient used in HPLC of vitamins using the method of Papadoyannis and others (1997)

Time (min)	MeOH (percent)	Ammonium acetate (0.05 M) (percent)
0	5	95
6	15	85
13	30	70
20	30	70

6.7.2 Evaluation of solid phase cartridges for preparation of vitamin extracts prior to HPLC analysis

A variety of cartridges were used according to the specific recommendations of the individual manufacturer. The procedures are summarised in Table 6.13. The samples investigated were a mixture of standard B group vitamins (at a concentration in the range 5-25 μg/mL depending on the vitamin concerned) and also the AACC reference sample prepared by homogenising 2 g of AACC sample in 50 mL of diluted acid. In all cases, the solutions from the cartridge was collected and during each step, including loading, washing and elution, separate fractions were reserved for individual analysis. The HPLC procedure used was that of Albalá-Hurtado and others (1997) described above.

Table 6.13 Conditions used with solid phase extraction cartridges for mixtures of vitamins and AACC reference sample

Cartridge type	Conditioning/ equilibration	Load	Wash 1	Wash 2	Elution
OASIS HLB	1 mL MeOH/1 mL water	1 mL sample	1 mL (5% MeOH in water)	—	1 mL MeOH *
OASIS MCX	1 mL MeOH/1 mL water	1 mL sample	1 mL 0.1 M HCl	1 mL MeOH	1 mL 5 % NH_4OH in MeOH *
OASIS MAX (procedure 1)	1 mL MeOH/ 1 mL water	1 mL sample	1 mL 0.05 M sodium acetate/5% MeOH	—	1 mL MeOH followed by 2 mL 0.1 M orthophosphoric acid/ acetonitrile 1:1 (v/v)
OASIS MAX (procedure 2)	1 mL MeOH/ 1 mL water	1 mL sample	1 mL 50 mM sodium acetate/5% MeOH	—	1 mL MeOH followed by 2 mL 2% formic acid/MeOH
Sep-pak plus	1 mL MeOH/ 1 mL water	1 mL sample	1 mL water	—	1 mL MeOH
C-18	2 mL MeOH/ 2 mL water	1 mL sample	1 mL water	—	1 mL MeOH *
SAX	3 mL hexane/ 3 mL MeOH/ 5 mL phosphate buffer	4 mL of sample diluted in 2 mL buffer	2 mL diluted buffer (0.02 M)	—	2 mL diluted buffer (0.1 M)
Silica	2 mL hexane/ 2 mL MeOH/ 2 mL water	5 mL sample	1 mL hexane/ 1 mL water	—	1 mL MeOH

Note — Not applied

* Evaporated and reconstituted in 100 μL of either extracting solution or mobile phase
OASIS MAX cartridges were trialled using two different sets of elution solutions and these are designated as procedures 1 and 2
Higher loading rates were also evaluated as a potential concentration procedure in some cases

6.7.3 Calculation of results for individual vitamins

The individual vitamin contents of the samples were calculated using the external standards. At both the start and end of a series of HPLC measurements, at least replicate analyses of the standard test solutions were performed and the average peak areas were calculated. These were then compared with those of the sample test peak areas. For this comparison, the weighed portion, the amount of aliquot used and the dilutions were taken into account.

Using a spreadsheet prepared in Microsoft® Excel, the average peak areas obtained for the individual standards at different concentration were entered and calculations performed as follows:

Preparation of standard curve

Firstly, the peak areas of individual standard was used directly and plotted using the scatter option in the software with concentration of individual vitamin (μg/mL) on the x axis and the corresponding peak areas on the y axis. A linear regression equation of the form [y = mx + c] typically gave the best statistical fit and the equation as well as the r squared value were recorded. The latter value was then considered and the analyses was repeated if the value was lower than 0.98.

Calculation of individual vitamin contents

The average peak areas for each sample tested were then used in the calculation of vitamin concentrations of the sample solutions using the linear equations. The appropriate dilution factor was applied and allowance made for the original sample weight to express the result per kg of sample. The following equation was used:

$$\text{Vitamin content (mg/kg)} = \frac{T \times D \times 1000}{S_w \times 1000^*}$$

Where

T = The concentration of vitamin calculated using the regression equation for the standard curve (expressed in μg/mL)

D = dilution factor

S_w = amount of sample originally weighed (expressed in g)

1000* = conversion factor so that result is expressed in units of mg of individual vitamin

1000 = conversion factor so that result is expressed per kg of sample

After determination of individual vitamin contents, the data were expressed on a dry weight basis, following the calculation described in section 6.6.1.

6.8 Procedures for analysis of B-6 vitamer contents in Asian noodles by HPLC

In the analysis of B-6 vitamers, the method was performed so that all steps were completed without any delays and no storage of partially treated samples was required. At least duplicate determinations were carried out on the same day.

6.8.1 Preparation of solutions

***Sulphuric acid** (0.1 M)*: 5.45 mL of concentrated H_2SO_4 was diluted with water to make1 L of solution.

Mobile phases: were prepared as mixtures of an aqueous solution (Solution A) containing phosphoric acid 0.033M and octanesulphonate (0.008 M), adjusted to pH 2.2 with KOH (6M) and *iso*-propanol (solution B). These were mixed in ratios of 98:2 and 80:20.

Preparation of standards of B-6 vitamers

Standards of B-6 vitamers were prepared at 3 different concentrations which included: stock solutions (1 mg/mL), standard solutions I (100 μg/mL), standard solutions II (10 μg/mL). Each was prepared in a similar way as follows:

***B-6 vitamer stock solutions** (1 mg/mL)*: 154.1 mg of PM, 121.8 mg of PL and 121.6 mg of PN were weighed separately and each was prepared in individual stock solution by

dissolving in H_2SO_4 (0.1 M) mixed well and made up to 100 mL. These solutions were stable for approximately 4 wk in the refrigerator.

B-6 vitamers standard solutions I *(100 μg/mL)*: 10 mL of stock solutions were pipetted in 100 mL volumetric flasks and made up to the mark with H_2SO_4 (0.1 M). These solutions were freshly prepared every week.

B-6 vitamers standard solutions II *(10 μg/mL)*: 10 mL of standard solutions I were pipetted in 100 mL volumetric flasks and made up to the mark with H_2SO_4 (0.1 M). These solutions were freshly prepared before use.

6.8.2 Procedures used in preliminary comparison of HPLC methods for analysis of B-6 vitamers

For preliminary evaluation of HPLC condition for analysis of B-6 vitamers, individual standards of B-6 vitamers were analysed using two columns (Waters Spherisorb ODS 2 and Waters Resolve C-18) and three mobile phases (Table 6.14).

Table 6.14 Comparison of mobile phases used for analysis of B-6 vitamers by HPLC

Mobile phase	Reference
Hexanesulphonate (0.005 M), acetic acid (2.4%), triethylamine (0.5%), MeOH (15%) (pH 3.6)	Albalá-Hurtado and others 1997
Solution A (Water containing phosphoric acid 0.033M and octanesulphonate 0.008 M) adjusted to pH 2.2 with KOH (6M) and solution B *iso*-propanol mixed in ratios of 98:2 and 80:20	Gregory and Feldstein 1985; Ollilainen 1999
Octanesulphonate (0.005 M); acetic acid (2.4%), triethylamine (0.5%), MeOH (5%) (pH 3.7)	See section 6.7.1 based on Albalá-Hurtado and others (1997)

6.8.3 Procedures in validation of HPLC analysis of B-6 vitamers

Validation of the HPLC method for B-6 vitamers involved analysis of AACC reference samples along with a recovery experiment. These were done by extraction and measurement of AACC reference sample (1 g) and by adding a known amount of PN solution (corresponding to 20 μg) to flour samples (7 g).

6.8.4 Extraction of B-6 vitamers from flour and noodle samples

The procedures used for extraction were based on those described by Brubacher and others 1985b.

Preparation of the sample

Approximately 50 g each sample was homogenised with 100 mL of H_2SO_4 (0.1 M) using the Ultra-Turrax for 1 min. Sub-samples of 10-15 g of the homogenate were weighed for analysis of B-6 vitamers.

Autoclave treatment of sample extract

An amount of 10-15 g of sub-sample material was weighed to an accuracy of ± 0.01 g in a 100 mL volumetric flask, 30 mL of H_2SO_4 (0.1 M) was added and mixed with 10 drops of paraffin oil for antifoaming purposes before autoclaving for 30 min at 121 °C.

After cooling to room temperature, the solution was made up to 100 mL with water (paraffin oil was above the mark), mixed thoroughly and filtered through a dry pleated filter, the first 10 mL of the filtrate was discarded. For further removal of particularly fine particles, aliquot of this was further treated by passing through an ultra filter (Nylon membrane 0.45 μm) and the resultant solution designated as sample test solution.

Preparation of external standard test solution

For the external standards, 1-2 mL (≡ 10-20 μg) of each of the B-6 vitamer standard solutions (II) were pipetted into individual 100 mL volumetric flasks. H_2SO_4 (0.1 M, 30 mL) was added to each prior to autoclaving for 30 min at 121 °C. After cooling to room temperature, these were made up to 100 mL with water (referred to as standard test solutions).

6.8.5 Method of HPLC adopted for routine analysis of B-6 vitamers

The sample and standard test solutions were injected into the HPLC and the fluorescence of the separated B-6 vitamers measured. Standardisation and stability checks were performed using the standard test solutions.

The HPLC system was run with the fluorescence detector at wavelengths of 290 nm for excitation and 396 nm for emission. The separation was performed on an analytical column Waters Resolve C-18 column 150 × 3.9 mm, 5 μm with a matching guard cartridge. Analyses were carried out at 30 °C with a column heater and a flow rate of 1.5 mL/min. The eluting solvent consisted of Solution A (Water containing phosphoric acid 0.033M and octanesulphonate 0.008 M) adjusted to pH 2.2 with KOH (6M) and solution B *iso*-propanol mixed in ratios of 98:2 for 12 min followed by 80:20 for 10 min. Re-equilibration was carried out using the 98:2 solution for 7 min. The injector loop used had a volume of 100 μL.

6.8.6 Calculation of results for B-6 vitamers

The B-6 vitamers content of the analysis sample was calculated using the external standards. At both the start and end of a series of HPLC measurements, at least a replicate analysis of the standard test solutions were performed and the average peak areas were calculated. These were then compared with those of the sample test solutions. For this comparison, the weighed portion, the amount of aliquot used and the dilutions were taken into account.

Equation used for the calculation of B-6 vitamers:

$$\text{B-6 vitamer content (mg/kg)} = \frac{F_p\, C_s\, H \times 1000}{F_s\, q\, E \times 1000^*}$$

Where

F_p	=	peak areas for B-6 vitamers in the sample test solution
F_s	=	peak areas for B-6 vitamers in the standard test solution
C_s	=	concentration in the standard test solution (μg/100mL)
H	=	total quantity of sulphuric acid homogenate
q	=	Total weight of the homogenate
E		weight of homogenate taken for analysis
1000*		conversion to 1000 g (1 kg)
1000		conversion to mg of B-6 vitamers

After determination of B-6 vitamers contents, these data were expressed on a dry weight basis, following the calculation described in section 6.6.1.

6.9 Methods used in the determination of riboflavin contents of Asian noodles by HPLC

The procedures followed for riboflavin analysis were based upon various publications including those of Brubacher and others (1985c), Dong and others (1988) and Albalá-Hurtado and others (1997). These procedures were adopted with modifications. The samples analysed included flours and noodles in the fresh, dried, fried and cooked forms. For studies of stability, samples which had been fortified with riboflavin were analysed.

All steps in the analysis were performed without delay and were completed within a period of 6 h from commencement of extraction until extracts were loaded into the HPLC for analysis. For each sample at least duplicate extractions were prepared and individual extracts were analysed at least in duplicate.

6.9.1 Preparation of solutions used for riboflavin analysis

Sodium acetate solution *(2.5 M):* sodium acetate (102.54 g) was dissolved in water, allowed to cool and made up to a volume of 500 mL.

***Sulphuric acid solution** (2.5 M):* concentrated H_2SO_4 (136.25 mL) was carefully diluted to a final volume of 1000 mL using water.

***Sulphuric acid solution** (0.1 M):* concentrated H_2SO_4 (5.45 mL) was diluted to a final volume of 1000 mL using water.

***Clara-diastase** (10 percent):* Clara-diastase (5.0 g) was dissolved in 50 mL of water with gentle mixing on a magnetic stirrer. This solution was prepared freshly before use.

Mobile phase for HPLC: The mobile phase was based upon MeOH-water (15:85) and consisted of 1-hexansulphonic acid (0.005 M) in water which also contained glacial acetic acid (2.4 percent), triethylamine (0.5 percent) and MeOH (15 percent). This was made by dissolving 1-hexansulphonic acid sodium salt (0.94 g) in water (approx 500 mL) and then glacial acetic acid (24 mL) was added, followed by triethylamine (5 mL) and MeOH (150 mL). The solution was made up to 1.0 L and the pH of the mobile phase was found to be 3.6 ± 0.1. After mixing, the mobile phase was filtered through a 0.45 μm filter before use in the HPLC analysis.

6.9.2 Preparation of riboflavin standard solutions

Preparation of stock solutions

The stock solutions were prepared as follows:

Stock solution a: Riboflavin (25 mg) was dissolved in H_2SO_4 (0.01 M) in a 500 mL volumetric flask and made up to the mark (the concentration of this solution was 50 μg riboflavin/mL).

Stock solution b: Solution a (2 mL) (equivalent to 100 μg) was then diluted to 500 mL with H_2SO_4 (0.01 M) to give a standard solution equivalent to 0.2 μg riboflavin/mL or 2 ng/10 μL.

Preparation of standard test solutions

Solutions I: A volume of 4 mL riboflavin stock solution a (≡ 200 μg) was diluted with H_2SO_4 (0.1 M) and made up to 100 mL. Aliquots of this solution (10 mL) were subjected to the complete analysis procedure including disintegration with H_2SO_4 and enzymatic digestion (see section 6.9.6). This overall procedure gave standard test solution I which was 100 mL with 0.2 μg /mL (≡ 200 ng/mL).

Solution II: A volume of 10 mL riboflavin stock solution b, corresponding to 2 μg riboflavin was diluted in the same way as for solution I but without being subjected to the analysis procedure. The resulting solution was designated as standard test solution II with 2 ng/10 μL.

6.9.3 Procedures used in the preliminary evaluation of riboflavin extraction from samples

Method of Ndaw and others (2000)

There was 3 extraction protocols used to extract riboflavin from the flour and AACC reference samples. These were:

Trial 1: 50 mL of HCl (0.1 M) was added to each flask containing either flour sample (6 g) or AACC reference sample (0.5 g) and mixed thoroughly and autoclaved for 30 min at 121 °C. These samples were allowed to cool, adjusted to pH 4.5 with 2.5 M sodium acetate prior to addition of 500 mg of Clara-diastase and incubation for 18 h at 37 °C. Following digestion each solution was diluted to 100 mL with water and filtered through a cellulose acetate filter (0.2 μm) before injection to HPLC.

Trial 2: 50 mL of 0.05 M sodium acetate (pH 4.5) was added to each flask containing the same amounts of flour and AACC reference sample as in Trial 1, followed by 500 mg of Clara-diastase and incubation for 18 h at 37 °C. Before injection to HPLC, these digested samples were treated as in Trial 1.

Trial 3: Here, all treatments were the same as described in Trial 2 except that Clara-diastase was replaced with a mixture of papain (100 mg), 1 percent glutathione (500 μL), acid phosphatase (20 mg) and α-amylase (10 mg).

Method of Chase and others (1993b)

The extraction was prepared by homogenisation of samples of flour (6-7 g) and AACC reference sample (0.5-1 g) with 60 mL of water prior to adding 2 mL of perchloric acid to each solution and stirring for 30 min. Then the pH was adjusted to 3.2 with potassium hydroxide (6 M), diluted to a final volume of 200 mL with mobile phase and

refrigerated overnight to allow complete precipitation of the protein and perchlorate. The solutions were then filtered before HPLC analysis.

Method of Brubacher and others (1985c)

Samples of flour (6-7 g) and AACC reference sample (0.5-1 g) were homogenised with 30 mL of H_2SO_4 (0.1 M) and autoclaved at 121 °C for 15 min. After cooling to room temperature, the solutions were adjusted to pH 4.5 with sodium acetate solution (2.5 M) prior to adding 5 mL of 10 percent Clara-diastase suspension and incubated 90 min at 45 °C. Then the digested mixtures were acidified with 4 mL of H_2SO_4 (2.5 M) diluted to a final volume of 100 mL and filtered (0.45 μm) for HPLC analysis.

The HPLC conditions for analysis of riboflavin were adopted from the earlier work which introduced clear, symmetrical peaks and these were readily separated from the other vitamins studied. The conditions involved a Waters Spherisorb ODS 2 C-18 column and the mobile phase was MeOH:H_2O (15:85) containing hexanesulphonate (0.005 M), acetic acid (2.4 percent), triethylamine (0.5 percent) (pH 3.6) (Albalá-Hurtado and others 1997). These HPLC conditions were used as a basis during the development of a method for the specific analysis samples prepared using the extractions described above.

6.9.4 Procedures used in comparison of HPLC conditions for riboflavin analysis

For further evaluation of parameters for HPLC analysis, the various mobile phases were used and these were prepared from the following solutions and the details of the trials are presented in Table 6.15.

1) Solution A: An aqueous solution of hexanesulphonate (0.005 M), triethylamine (0.5 percent), glacial acetic acid (2.4 percent) and MeOH (15 percent).
2) Solution B was MeOH (100 percent)

Table 6.15 Description of mobile phases used in the preliminary evaluation of HPLC procedures for analysis of riboflavin

Trial number	Composition of mobile phase	Flow rate (mL/min)
1	Solution A (85%) Solution B (15%)	1.5
2	Solution A (85%) Solution B (15%)	1.0
3	Solution A (90%) Solution B (10%)	1.0: initial to 15 min 0.7: thereafter
4	Solution A only	0.7
5	Solution A only	1.0

6.9.5 Method applied used for extraction of samples and standards in the routine analysis of riboflavin contents

For routine use the procedure of Brubacher and others (1985c) was found to give the best results and was therefore adopted. Flour and noodle samples (approx 6-7 g) as well as AACC reference sample (approx 0.5-1 g) were weighed into 250 mL wide-neck conical flasks. Approx 30 mL of H_2SO_4 (0.01 M) was added and the samples homogenised for between 20 s and 2 min using the Ultra-Turrax set on medium speed. The time used for each particular sample was based upon that required for the achievement of a homogeneous extract which flowed freely. For cooked noodles, short times were sufficient. In the case of dried noodles both ground and unground samples were analysed and the recovery of riboflavin compared.

Within each batch of samples prepared for analysis, aliquots of standard test solution I (10 mL) were also prepared in duplicate and sufficient H_2SO_4 (0.01 M) added to give the same volume as that used for noodle samples. The standard flasks were then subjected to the steps used for samples. Homogenates and standards were autoclaved for 15 min at 121 °C. After cooling to room temperature, the pH of the mixture was adjusted to pH 4.5 ± 0.1 by adding 6 mL of sodium acetate solution.

6.9.6 Routine procedure adopted for enzymatic digestion for extraction of riboflavin

Clara-diastase solution (10 percent, 5 mL) was added to each of the extracts and standards and mixed thoroughly. This was carried out in order to digest the samples. The mixtures were allowed to incubate for 90 min at 45 °C. After cooling to room temperature, the mixtures were acidified H_2SO_4 (2.5 M, 4 mL). The contents of each flask were quantitatively transferred to a 100 mL volumetric flask and made up to the mark with water. The resulting solutions were filtered through a pleated filter paper (number 1) and the initial 10 mL of filtrate was rejected. The extracts were then further filtered through 0.2 μm membranes before injection to HPLC for analysis of riboflavin.

6.9.7 Method of HPLC analysis adopted for routine analysis of riboflavin contents

Sample test solutions were analysed chromatographically using isocratic elution and a reversed-phase HPLC system. The separation was performed on an analytical column Spherisorb ODS 2 C-18 column, length 250 × 4.6 mm, 5 μm pore size, with a matching guard cartridge. Analyses were carried out at 30 °C with a column heater used to control the temperature and the flow rate of the mobile phase was 1.0 mL/min. The injector loop used had a volume of 25 μL and the column eluate was monitored with the spectrophotometric detector set at a wavelength of 268 nm. The total run time required for each analysis was less than 35 min.

The chromatograms were recorded using the Star Chromatography (Star WS 5.31) software package which provided retention times for each peak along with peak areas. For quantitation of riboflavin contents, samples of standard test solutions I and II were also injected with each batch of sample extracts analysed. In addition, the calibration and stability of the detector were monitored at the beginning of each set of samples and during the course of analyses. This was done by injecting a sample of standard test solution I after every set of 5 analyses of noodle or flour samples.

6.9.8 Calculation of riboflavin data

The riboflavin content in the sample test solution was calculated by direct comparison with the peak area values obtained for the external standard. At the beginning of each series of analyses, at least three injections were included for each of standard test solution I and also for standard test solution II. Both of these had been prepared and processed in parallel with the unknown samples in a single batch. The peak areas of standard test solution II corresponded to 2 ng riboflavin per 10 μL. The peak areas of the sample test solutions were compared with those of standard test solution I. Account also was taken for the weight of the sample portion originally taken, the volume used for the analysis and the dilution. The equation used to calculate the riboflavin content in samples is as follows:

$$\text{Riboflavin content (mg/kg)} = \frac{F_p \times C_s \times 1000}{F_s \times S_w \times 1000^*}$$

Where

F_p = peak area of riboflavin in sample test solution

F_s = peak area of riboflavin in standard test solution I

C_s = amount of riboflavin in 10 mL volume of standard test solution I (20 μg)

S_w = amount of sample originally weighed (expressed in g)

1000* = conversion factor so that result is expressed in units of mg of riboflavin

1000 = conversion factor so that result is expressed per kg of sample

After determination of riboflavin contents, these data then were expressed on a dry weight basis, following the calculation described in section 6.6.1.

6.10 Procedures used in the analysis of folate contents in Asian noodles by the microbiological method

The approach used in folate analysis was based upon published procedures, particularly the AOAC method (AOAC 1990c) as more fully described by Angyal (1996). In the

current study modifications were considered and evaluated for the analysis of Asian noodles.

6.10.1 Preparation of solutions

Reagent solutions

Phosphate ascorbate buffer *(0.05 M phosphate, 1.0 percent ascorbic acid)*: The buffer was prepared by mixing 92 mL Na_2HPO_4 (0.1 M), 407 mL NaH_2PO_4 (0.1 M), 501 mL water and 10 g of ascorbic acid. The pH was adjusted using 4 M NaOH to either 6.1 ± 0.1 or 7.8 ± 0.1 required at different stages during folate extraction.

Folic acid casei medium: 9.4 g of *L casei* medium was dissolved in 100 mL of water.

AOAC agar: 4.8 g agar was dissolved in 100 mL of water and autoclaved for 15 min at 121 °C.

Lactobacillus broth AOAC: The broth was prepared by dissolving 39 mg in 100 mL of water. Aliquots (volume of 10 mL) were dispensed into a series of tubes and autoclaved for 15 min at 121 °C.

Standard solutions

Stock solution *(200 μg/mL):* 100 mg of pure folic acid was weighed and dissolved in a solution of NaOH (500 mL, 0.01 M, also containing 20 percent ethanol). This solution was stored in the refrigerator under toluene.

Working standard solution *(200 ng/mL):* 0.5 mL of stock solution was made up to 500 mL with the solution of 0.01 M NaOH in 20 percent ethanol and stored in the refrigerator.

Assay standard solution *(1 ng/mL):* 0.5 mL of working standard solution was diluted to 100 mL with phosphate ascorbate buffer pH 6.1.

Preparation of saline solution *(0.85 percent):* 0.85 g of NaCl was dissolved in 100 mL of water and autoclaved for 15 min at 121 °C.

Glycerol solution *(80 percent):* 80 mL of glycerol was made up to 100 mL with water and autoclaved for 15 min at 121 °C.

Preparation of enzyme solutions

Each of the enzyme solutions was prepared freshly on the day of use.

Protease solution *(2 mg/mL):* 1 mL protease solution (50 mg/mL) was diluted with water to a volume of 25 mL.

α-Amylase solution *(20 mg/mL):* 1.0 g α-amylase was suspended in 50 mL of water and stirred gently with a magnetic stirrer for 5 min and centrifuged at 3,000 rpm (1,200 × *g*) for 10 min. The supernatant was filtered through glass wool into a beaker and covered with Parafilm®.

Chicken pancreas *(5 mg/mL):* 500 mg of chicken pancreas was suspended in 100 mL of 0.1 M phosphate ascorbate buffer pH 7.8 and stirred for an hour. The suspension was centrifuged for 10 min at 3000 rpm, filtered through glass wool into a beaker and covered with Parafilm®.

6.10.2 Procedures used in preparation of bacterial culture

Method for gram staining of bacteria

The morphological type of the culture was observed by applying gram stain. For this, a smear of the *L casei* culture was dried and fixed on a microscope slide prior to sequentially applying solutions of crystal violet (30 s), iodine (30 s), acetone-ethanol (50:50) (3 s) and safranine (1 min). The smear was washed with water after application of each individual solution. Following the final wash, the slide was dried gently between sheets of clean blotting paper and allowed to dry in air prior to examination under the oil immersion objective of a laboratory microscope. Under these conditions gram-positive bacteria stain purple.

Cryoprotection using glycerol

These procedures are based upon those described by Grossowicz and others (1981); Wilson and Horne (1982); Shrestha and others (2000). Culture of *L casei* was transferred to the 10 mL sterilised *L casei* broth, vortex and incubated at 37 °C for 24 h. Folic acid casei medium for cryoprotected culture was prepared by dissolving 9.4 g of folic acid casei medium, 0.3 mL (200 ng/mL) of working standard solution and 50 mg of ascorbic acid with water to have a final volume of 200 mL. The mixture was autoclaved at 121 °C for 10 min and cool immediately in running water. 0.5 mL of the *L casei* culture from the broth which was incubated prior at 37 °C for 24 h was inoculated to the prepared cryoprotected culture medium, vortex and incubated at 37 °C for 20 h. An equal volume of sterilised glycerol solution (80 percent) was thoroughly mixed with

the incubated culture solution and this was designated as stock culture. Aliquots of this were taken and stored in small sealed centrifuge tubes at -20 °C (Grossowicz and others 1981; Wilson and Horne 1982; Shrestha and others 2000). Before inoculation, the culture was diluted with saline solution to give an appropriate absorbance which was established as follows.

Determination of optimum inoculum concentration

In order to determine the optimum conditions for assay, a series of standard curves were set up. For this different amounts of stock culture were diluted with sodium chloride (0.85 percent) to give an initial range of absorbances in the range 0.01-0.09 at a wavelength of 540 nm when read against sodium chloride solution. An inoculum volume of 50 μL of each diluted culture was used to inoculate into each set of a series of folic acid standards at concentrations in the range of 0-1.0 ng/tube. All of the inoculated tubes were then incubated at 37 °C for 20 h. The absorbances were measured and the individual standard curves plotted and compared. Bacterial cultures having initial absorbances in the range of 0.05–0.08 were found to give standard curves which could be used for estimation of folate extracts from noodles. However, inoculation with cultures having an absorbance of 0.08 provided optimal standard curves.

6.10.3 Calibration of spectrophotometer prior to establishment of folate analysis procedure

These procedures are based upon those of Angyal (1996).

Preparation of dried cells

The calibration for the folate assay on the specific spectrophotometer was prepared by adding 1 mL of inoculum to 300 mL sterile *L casei* medium containing 1 mL standard stock solution (100 μg/mL) then incubated at 37 °C for 20 h.

After incubating, the cells were centrifuged and washed three times with 50 mL of saline solution and then resuspended in saline solution to a final volume of 25 mL. A volume of 10 mL of cell suspension (in duplicate) was evaporated on a steam bath and dried to a constant weight in a vacuum oven at 110 °C. Dry weight of cells in mg/mL of suspension was calculated by correcting for weight of NaCl.

Preparation of calibration curve

After calculation, a volume of cell suspension was diluted with saline solution to have an equivalent of 0.5 mg dry cells/mL. The test tubes were set up in triplicate with the addition of different volumes of resuspended cells: 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 mL; then sufficient saline solution was added so that each tube contained a volume of 5 mL. Finally, *L casei* medium (5 mL) was added to have a total volume of 10 mL/tube. The absorbance was measured and used in preparing a calibration curve by plotting against the level of diluted cell suspension expressed in terms of the weight of cells in the individual tubes.

6.10.4 Evaluation of procedures for extraction and preparation of samples

Two procedures were compared for extraction and deconjugation of folate from samples of flour and noodles. These were based upon methods described by Shrestha and others (2000) and Angyal (1996) are presented in Figure 6.1. It is noted that the conjugase used in this comparison was from chicken pancreas.

6.10.5 Preparation of extracts for routine analysis by the microbiological method

The overall approach in preparation of extracts involved homogenisation in phosphate-ascorbic buffer, followed by a heat treatment using the autoclave. The resulting solution was sub-sampled to give separate fractions which were then treated in one of two ways. The first was the tri-enzyme treatment and effectively measures 'total folate'. For the second sub-sample no enzyme treatment was applied and the folate measured in these extracts is commonly referred to as 'free folate' in the scientific literature.

Homogenisation and autoclave treatment of samples

The same procedure was applied to flours, noodles, reference samples and to samples used in the assessment of recoveries from spiked samples. In order to extract folates from the food matrix, 1 to 10 g samples (depending on the concentration of folate content in the sample) were weighed in duplicate in 250 mL beakers and 50 mL of 0.1 M phosphate ascorbate buffer (pH 6.1) added to each sample. The mixtures were then homogenised using the Ultra-Turrax (1-3 min depending on the sample). An additional 30 mL of buffer was used to wash down the sample from the sides of the Ultra-Turrax

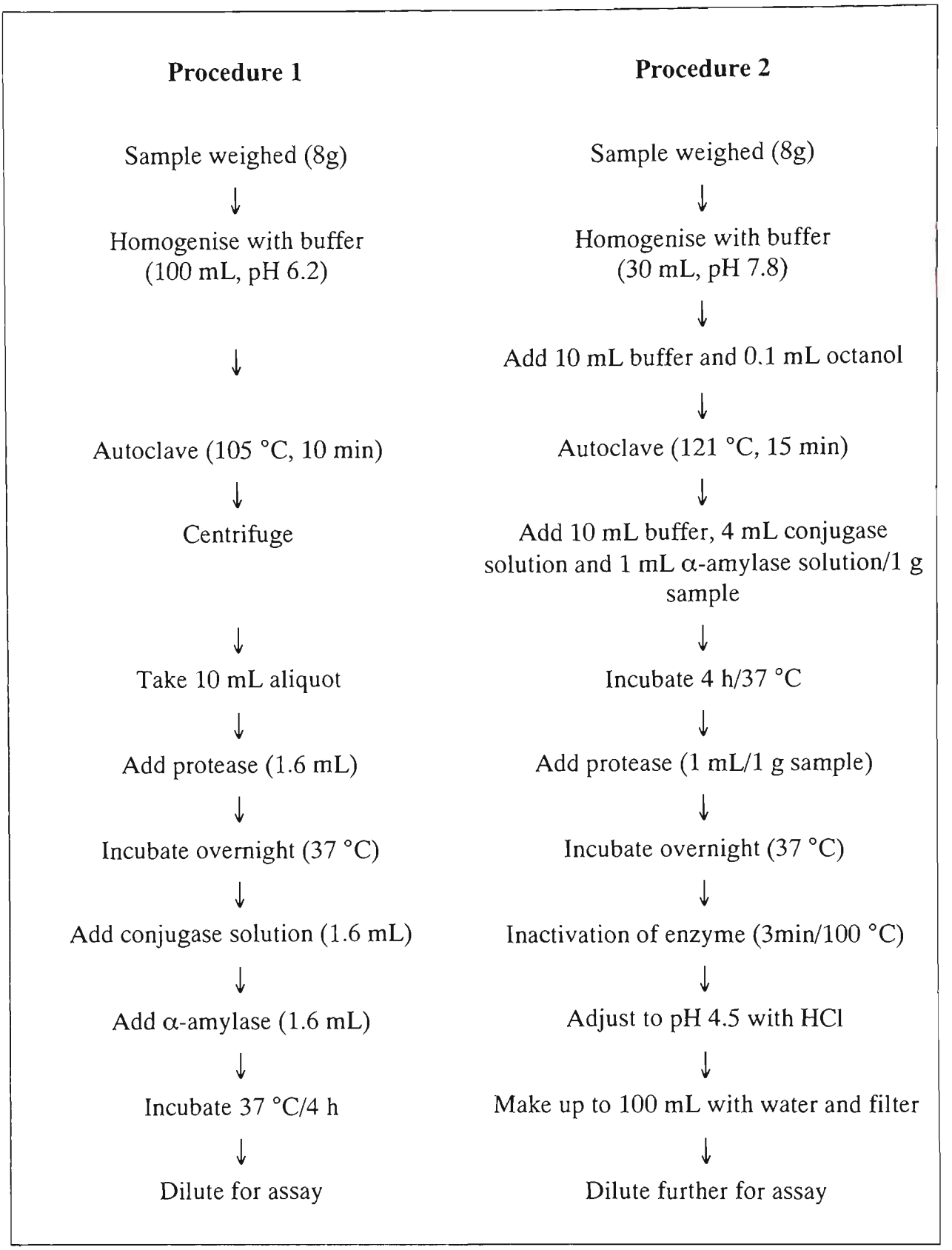


Figure 6.1 A comparison of the procedures evaluated for the extraction of folate from noodle samples

Note Procedure 1 was that of Shrestha and others (2000) and procedure 2 was from Angyal (1996) and in both cases the conjugase was from chicken pancreas

prior to making up to a volume of 100 mL with the phosphate ascorbate buffer. Antifoaming agent, octanol (0.1 mL) was added to the flask before covering with aluminium foil and autoclaving at 105 °C for 10 min.

Following autoclaving, the homogenised samples were allowed to cool to room temperature and centrifuged at 4,000 rpm (2,100 × *g*) for 15 min. Aliquots of the supernatant were then taken and handled in one of two ways. One set of aliquots was stored (–20 °C) and used for subsequent measurement of free folate. The second set was treated by the tri-enzyme method for measurement of total folate.

Further preparation of folate extracts using the tri-enzyme method

After the autoclave treatment, 10 mL of each sample extract (including reference and recovery samples) prepared above was placed into a 50 mL centrifuge tube. For each tube protease was added (1.6 mL, 2 mg/mL), vortexed and incubated overnight at 37 °C. This enzyme was then inactivated by placing the tubes in a boiling water bath for approx 5 min and allowed to cool to room temperature. The extract was adjusted to pH 7.2 with 2 M NaOH prior to adding α-amylase solution (1.6 mL, 20 mg/mL) and chicken pancreas solution (1 mL, 5 mg/mL). After incubation for a further 4 h, the enzymes were inactivated in a boiling water bath (approx 5 min). The resultant extract was cooled to room temperature and centrifuged for 10 min at 4,000 rpm (2,100 × *g*). The supernatant was diluted to a concentration of approx 0.2–1.0 ng folate/mL and analysed directly using the microbiological assay.

Preparation of enzyme-only blanks

For each batch of analyses involving tri-enzyme treatments duplicate blank extractions were prepared and designated as enzyme-only extracts. For these, 10 mL of buffer was used in place of sample extract and run in parallel with product samples using the method described above.

6.10.6 Procedures for routine microbiological assay of sample extracts, standards and blanks

It is noted that in the published AOAC method, for each assay tube, the final volume is made up to 10 mL. This comprised 5 mL of a solution of folate (standard or sample or

blank) and 5 mL of *L casei* medium. In the current study the final volume of the assay was reduced to 3 mL. For this the same ratio was used corresponding to 1.5 mL of solution containing folate and 1.5 mL of medium. This modification was made in order to reduce the amounts of reagents as well as the time of preparation required.

Test tubes (16 × 110 mm) were washed carefully prior to use in the assays. Each concentration/sample was prepared in triplicate. The assay was set up by preparing a series of standard tubes, blanks, sample tubes and recovery tubes as follows:

Standard tubes were set up for the folate standard curve. Assay standard solution (1 ng/mL) was pipetted into the test tubes in triplicate at the volumes of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mL. Then the appropriate volume of buffer was added to each test tube to give a volume of 1.5 mL prior to addition of 1.5 mL of *L casei* medium so that the final volume in each tube was 3 mL.

Enzyme-only blanks: In all cases where tri-enzyme treatments were applied, a series of blanks were prepared by firstly diluting the enzyme only extract described above to give a folate concentration of between 0.2–1.0 ng/mL. Then 0.5 mL of the diluted extract, 1.0 mL of buffer and 1.5 mL medium were added to the test tubes giving a final volume of 3 mL.

Inoculated and uninoculated blanks: For each of these, sets of three tubes were prepared with 1.5 mL buffer and 1.5 mL medium.

Sample tubes and recovery tubes: these were prepared by diluting the extracted samples to a concentration of between 0.2–1.0 ng/mL with dilution buffer. An appropriate volume of each diluted extract was taken together with diluted buffer to make to a volume of 1.5 mL and then made up to a final volume of 3 mL with medium.

After preparation of all of the tubes, a plastic cap was placed on each and aluminium foil was used to cover the test tube racks. These were autoclaved at 121 °C for 5 min and then cooled quickly in cold water to minimise colour formation. The prepared culture (50 μL prepared as described in Section 6.11.2) was inoculated into each of the

tubes with the exception of the uninoculated blank tubes to which no addition was made. Then all tubes were incubated at 37 °C for 18-20 h.

Growth responses were recorded with the spectrophotometer and the wavelength used was 540 nm. The instrument was adjusted to zero using the uninoculated blank. The absorbance of the inoculated blank was first read against the uninoculated blank as a check that the assay was working satisfactorily. In those cases where the absorbance of the inoculated blank was relatively high, this indicated problems with the bacterial culture. Accordingly, if the absorbance exceeded 0.02 then no further readings were taken and the batch was discarded. The analyses were repeated and a fresh vial of cryoprotected culture was used. When satisfactory readings were obtained for the blanks, the absorbance of each enzyme-only blank, standard and sample was then measured against the inoculated blank.

6.10.7 Calculation of results for folate contents

Using a spreadsheet prepared in Microsoft® Excel, the spectrophotometer readings obtained for the enzyme-only blanks, standards and the samples were entered and calculations performed as follows:

Preparation of standard curve

Firstly, the absorbance values for the folate standards were used directly and plotted using the scatter option in the software with concentration of folate (ng/mL) on the x axis and the corresponding absorbance values on the y axis. A logarithmic regression equation of the form [$y = a \ln(x) + c$] typically gave the best statistical fit and the equation and the r squared value of the curve were recorded. The r squared value was then considered and the whole batch of assays was rejected if the value was lower than 0.98.

Calculation of free folate contents

The absorbance values for each sample tube in which the sample extract was not treated by the tri-enzyme procedure were then considered. Any individual result having an absorbance either below or above the effective useful range of the calibration curve was rejected and not used for further calculation. It is noted that the curves were not linear

and the slope was highest at low folate concentrations thereby limiting the reliability of low absorbance values. After consideration, the folate concentrations of the sample solutions were calculated using the regression equation.

These values were then calculated using the appropriate dilution factor and for original sample weight to express the result as μg of folate per kg of sample. The following equation was used:

$$\text{Folate content } (\mu g/kg) = \frac{T \times D \times 1000}{S_w \times 1000^*}$$

Where

T = The concentration of folate calculated from the standard curve (expressed in ng/mL)

D = dilution factor

S_w = amount of sample originally weighed (expressed in g)

1000* = conversion factor so that result is expressed in units of μg of folate

1000 = conversion factor so that result is expressed per kg of sample

Calculation of total folate contents

The absorbance values for tubes representing extracts that had been processed using the tri-enzyme procedure were also discarded if outside the range of the standard curve. Then the mean of the absorbances for the enzyme-only blanks and also those for each of the tri-enzyme treated sample extracts were separately read from the standard curve and each calculated back to the amount of folate present in the 10 mL of extract originally taken for assay. The value for the tri-enzyme blank was then subtracted from those of the samples prior to calculation of the data per kg of sample using the equation described above.

Expression of folate contents on a dry weight basis

Finally data for both free and total folate were expressed on a dry weight basis, following the calculation described in section 6.6.1.

6.11 Procedures for analysis of thiamin contents in Asian noodles by the fluorimetric method

The extraction and assay of thiamin were performed using the Fluorimetric method published by AOAC (1990d), in which:

1. The reactions were carried out in triplicate for each of purified extract.
2. A thiamin free blank was prepared for each sample to which the oxidising reagent was not added.
3. The fluorescence emitted by this blank was subtracted from that emitted by the sample, and the difference was calculated according to the AOAC method.

This procedure is summarised as follows: Samples were prepared (ground, cooked, steamed and fried noodles), acidified (homogenised with 0.1 M HCl) and the slurry autoclaved for 20 min at 109 °C. The mixture was allowed to cool before centrifugation for 20 min at 3,500 rpm (1,600 × *g*), oxidation of thiamin to thiochrome with $K_3Fe(CN)_6$, extraction of thiochrome with 2-methyl-1-propanol and finally determination of thiochrome by using the fluorescence spectrophotometer. In detail, the method involved:

6.11.1 Preparation of reagents

It is noted that in the storage of many of the solutions used in analysis of thiamin and other vitamins, it was necessary to protect the solution from light. This was achieved through the use of brown bottles and these were wrapped carefully in aluminium foil and were stored in closed cupboards.

Bromophenol blue indicator: 0.1 g indicator was dissolved with 3.0 mL 0.05 M NaOH, and diluted to 250 mL with H_2O.

Sodium hydroxide solution *(15 percent)*: 15 g NaOH was dissolved in water, then allowed to cool and diluted to 100 mL.

***Potassium ferricyanide solution** (1 percent)*: 1 g $K_3Fe(CN)_6$ was dissolved in water to make 100 mL. This solution was prepared freshly on the day when the analysis was to be performed.

Oxidising reagent: 4.0 mL of the 1 percent $K_3Fe(CN)_6$ solution was mixed with 15 percent NaOH solution and made up to a volume of 100 mL. This solution was used within 4 h.

2-methyl-1-propanol (*iso*-butyl alcohol): this was used as supplied.

Quinine sulphate stock solution: 10 mg quinine sulphate was dissolved in 0.1 M H_2SO_4, made up to 1.0 L and stored in light-resistant container. This solution was used to monitor the performance and reproducibility of the fluorimeter.

Quinine sulphate standard solution: 1 volume of quinine sulphate stock solution was mixed with 39 volumes of 0.1 M H_2SO_4. This solution was stored in a light-resistant container.

Preparation of standard solution

Thiamin hydrochloride was used to prepare the standard solutions as follows:

***Stock solution** (100 μg/mL):* Thiamin hydrochloride (50 mg) was dried to constant weight over fresh active silica gel in a desiccator and then dissolved in 20 percent alcohol which had been adjusted to pH 3.5-4.3 with 0.1 M HCl and diluted to 500 mL with the acidified alcohol. This solution was stored in a light resistant bottle.

***Intermediate solution** (10 μg/mL):* 100 mL stock solution was diluted to 1 L using 20 percent alcohol adjusted to pH 3.5-4.3 with 0.1 M HCl and it was stored in a light resistant bottle.

Working standard solution: 5 mL thiamin.HCl intermediate solution was diluted to 250 mL with 0.1 M HCl to give a solution for which 1 mL was equivalent to 0.2 μg of thiamin.HCl.

6.11.2 Procedures for routine analysis of thiamin contents of Asian noodles

Autoclave digestion

A sufficient quantity of each sample was weighed in order to ultimately give a final assay solution having a thiamin concentration of approximately 0.2 μg/mL (approx 3.125 g of flour sample for 100 mL final volume).

The measured amounts of samples were placed into 250 mL beakers and approximately 50 mL of 0.1 M HCl was added to each sample. The samples were thoroughly mixed and then autoclaved for 20 min at 121 °C. After autoclaving, they were allowed to cool and diluted with 0.1 M HCl to a final measured volume of 100 mL. The extracts were then centrifuged at 3,500 rpm (1,600 × *g*) for 20 min. The supernatants were used as the sample extracts of determination of thiamin.

Oxidation of thiamin to thiochrome

Firstly, a series of four 50 mL centrifuge tubes were set up to act as standards for each set of samples to be analysed. KCl (2.5 g) and working standard solution (5 mL) were added to each. These tubes were covered with aluminium foil to protect solutions from light, and swirled gently to dissolve most of the salt. Oxidising reagent (3 mL) was added to tubes 1 and 2, then 2-methyl-1-propanol (13 mL). The mixtures were shaken for adequate mixing. Tubes 3 and 4 were designated as standard blanks and these were treated in a similar way to tubes 1 and 2 except that 15 percent NaOH was added rather than oxidising reagent.

For each sample to be analysed a further four 50 mL centrifuge tubes were set up. These tubes were treated in the same way as for those tubes containing working standard solution except that sample solution was included instead of standard. The tubes were then also centrifuged at low speed until a clear supernatant was obtained. The upper of the two layers was taken and the fluorescence measured.

Measurement of thiochrome by fluorimetry

Cuvettes for use with the fluorimeter were of 1 cm dimensions with four transparent sides for the entry and emergence of the light beam. Polystyrene cuvettes were chosen as these are suitable for wavelengths above 350 nm (Harris 1996). In preliminary studies the three alternative slit widths (slits 3, 5 and 10) were compared to determine which was suitable. Slit 5 was found to be satisfactory and so was adopted for routine use along with wavelengths set at 365 nm for excitation and 435 nm for emission. The intensity of fluorescence of the sample solution was compared with that from the oxidised working standard solution. The results were calculated as follows:

$$\text{Thiamin content (mg/kg)} = \frac{(I - b) \times 20 \times 1000}{(S - d) \times S_w \times 1000^*}$$

Where

I	=	measured fluorescence of 2-methyl-1-propanol extract from oxidised assay sample solution
b	=	measured fluorescence of extract from assay sample solution which has been treated with 3 mL 15 percent NaOH solution
S	=	measured fluorescence of extract from oxidised working standard solution
d	=	measured fluorescence of extract from oxidised working standard solution which has been treated with 3 mL 15 percent NaOH solution (standard blank)
S_w	=	amount of sample originally weighed (expressed in g)
1000*	=	conversion factor so that result is expressed in units of mg of thiamin
1000	=	conversion factor so that result is expressed per kg of sample
20	=	conversion factor so that result is expressed in μg/100 mL

After determination of thiamin contents, the data were expressed on a dry weight basis, following the calculation described in section 6.6.1.

Chapter 7

Preliminary assessment of procedures for simultaneous extraction and quantitation of B group vitamins from flour and cereal-based foods

The purpose of this chapter is to describe and discuss the results obtained during the evaluation of procedures for simultaneous determination of B group vitamins in Asian noodles.

7.1 Introduction

In reviewing procedures for analysis of B group vitamins (Chapter 4) the potential of newer methods has been outlined. Some of these are claimed to allow for the simultaneous separation and quantification of a number of vitamins. Generally HPLC or, in some cases capillary electrophoresis, have been used offering significant advantages particularly speed and simplicity of analysis (Woolard 1984; Rees 1989; Chase and Soliman 1990; Albalá-Hurtado and others 1997; Agostini and Godoy 1997; Papadoyannis and others 1997).

In the initial phase of the current study, selected published methods have been investigated and evaluated for their potential application to grain-based foods. Various extraction procedures, analytical columns and mobile phases have been compared and in addition solid phase extraction options have been studied. The overall objective has been to simultaneously extract and quantitate up to six different B vitamins in flour and flour-based foods.

7.2 Evaluation of chromatographic methods for B group vitamins

Various HPLC procedures were set up and evaluated firstly using standard vitamins. Those considered at this stage of the study were thiamin (in the hydrochloride form), riboflavin, NA, NTA, PN and folic acid. These were chosen as representing the forms of

the vitamins most likely to be present in the samples of interest in this study or as being the form of the vitamin that is most commonly used in fortification of food products.

In the assessment and subsequent application of vitamin methods in this study, it was decided that the highest grade of purified water readily available in the laboratory would be used for all procedures (see also Section 6.1). The reason for this was to eliminate the potential influence of varying water quality, particularly in the context of the relatively low levels of vitamin compounds being analysed in this investigation. In addition, to assist in the validation of the analytical procedures, a reference sample containing the vitamins of interest was obtained from AACC (see Section 6.1 and Tables 6.1 and 6.2).

In the preliminary evaluation of HPLC methods for simultaneous analysis of the vitamins, four published approaches were found to provide some potential and these are summarised in Table 7.1. Additional details are provided in Section 6.7. In setting up each of the column systems, the recommended procedures provided by the column manufacturers were followed in order to assess the column performance. Thus a mixture of known compounds was chromatographed under specified conditions and the results obtained were compared with those of the column supplier. In the case of the Waters Spherisorb ODS 2 C-18 column, a reversed-phase test mix containing uracil, phenol, N,N-diethyl-m-toluamide and toluene, was used to assess the consistency of retention time, symmetry of the peaks as well as column efficiency. HPLC condition using mobile phase with a mixture of 58:42 acetonitrile:water, flow rate 1 mL/min and uv detection at 254 nm at ambient temperature. The test chromatogram showed symmetrical peaks and the efficiency of separation was the same as that reported in the quality assurance data originally supplied by the column manufacturer.

For the analysis of mixtures of vitamin standards, detection utilised either a diode array system or a programmable spectrophotometric detector. It was possible to program the system so that a different wavelength was used for each individual vitamin and those required were in the uv region in the range between 246 and 290 nm.

An example of the chromatographic separation achieved with standards is presented in Figure 7.1. In this the compounds eluted over a period of 35 min and the separation was effective. Following the approach of Chase and Soliman (1990) an internal standard of

Table 7.1 Description of methods and conditions assessed for simultaneous determination of B group vitamins

No	Column	Sample extraction	Mobile phase	Reference
1a	Waters-Resolve C-18	Homogenise with HCl (0.1 M), sonicate, filter through 0.45μm nylon filter	Acetonitrile, 1-hexanesulphonic acid (0.005 M), ammonium hydroxide and phosphoric acid, pH 3.6. Isocratic elution was used	Chase and Soliman 1990
1b	Allsphere ODS 2	Mixture of B vitamins	As above	As above
2	Waters Spherisorb ODS 2 C-18	Homogenise with water, TCA, centrifuge, make up the final volume with 4% TCA, filter with 0.45 μm filter	MeOH-water (15:85), octanesuphonic acid sodium salt (0.005 M), TEA, acetic acid, pH 3.6. Isocratic elution was used	Albalá-Hurtado and others 1997
3	Waters Spherisorb ODS 2 C-18	Homogenise with H_2SO_4 (0.05M), sonicate, adding methanol, store at –18 °C/1h and filter with 0.50 μm membrane	Multistep gradient from 2% acetonitrile and an aqueous phase with hexanesulphonic acid and TEA at pH 2.8 with increasing MeOH levels up to 57%	Agostini and Godoy 1997
4	Phenomenex ODS 3 C-18	Mixtures of standards used for evaluation of method	MeOH, CH_3COONH_4 (0.05 M) in multistep gradients from 5-30% MeOH	Papadoyannis and others 1997

Note Further methodological details are provided in Section 6.7

3-hydroxybenzoic acid was incorporated in the sample prior to injection and this compound was clearly separated from each of the vitamins. In addition, when varying levels of each vitamin were injected and standard curves plotted, linear relationships were obtained over a wide range of concentrations. Typical calibration curves are presented in Figure 7.2.

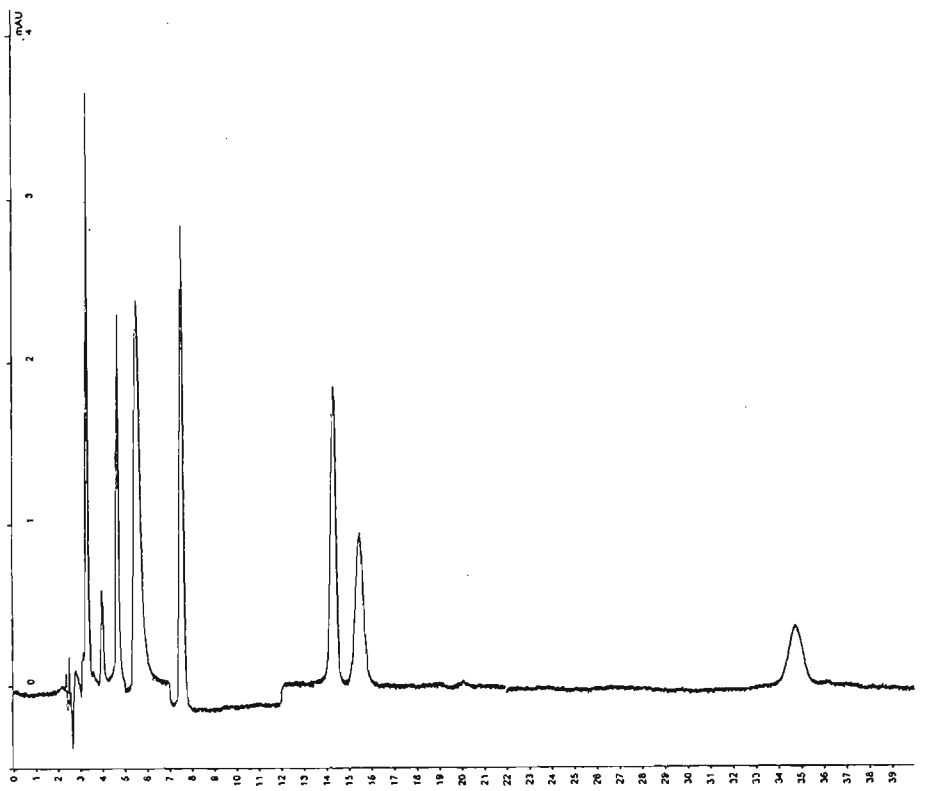
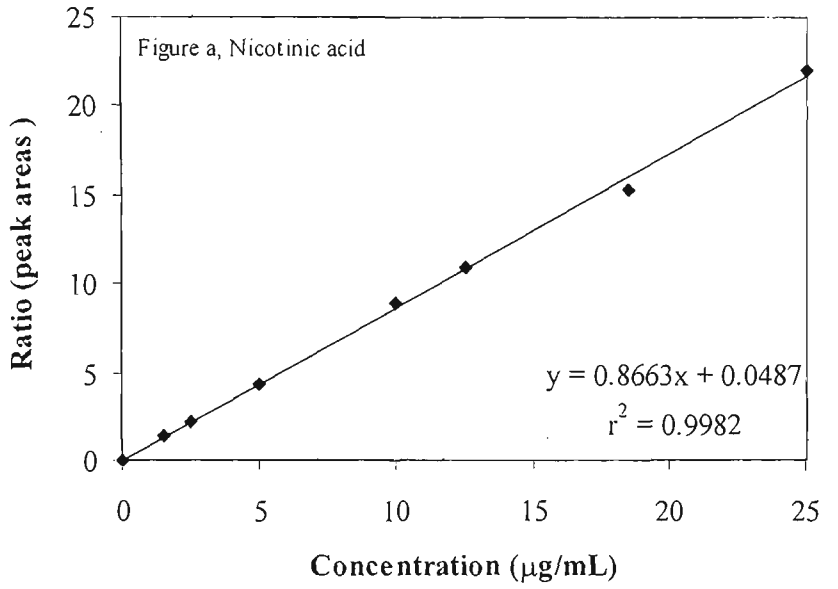


Figure 7.1 Chromatogram obtained for a mixture of vitamin standards and an internal standard of 3-hydroxybenzoic acid using method 2

Notes 1 For a description of method 2 refer Table 7.1

2 The vitamins eluted at retention times of 3.3 min for NA, 4.7 min for NTA, 5.6 min for PN, 7.6 min for thiamin, 15.5 min for folic acid, 34.8 min for riboflavin and 14.4 min for the internal standard

In order to further evaluate the procedures, samples of the AACC reference and of flour were used following a variety of approaches to extraction and to analysis. The results obtained in these studies indicated that the vitamins extracted from the samples were generally quite difficult to quantitate. For the AACC sample, the peaks for NA and NTA overlapped with solvent peak and eluted with other unidentified peaks during the first 6 min of the chromatographic run. For this sample the other vitamins did give peaks which were clear, symmetrical and sharp. In addition these were separated from other peaks including that for the internal standard. However when samples of wheat flour were analysed, it was found that the vitamin peaks overlapped with other peaks in the chromatograms or, in the case of riboflavin and PN, no peak was obtained. The application of various extraction procedures as well as a range of chromatographic columns and eluants do not readily overcome these problems encountered with flour



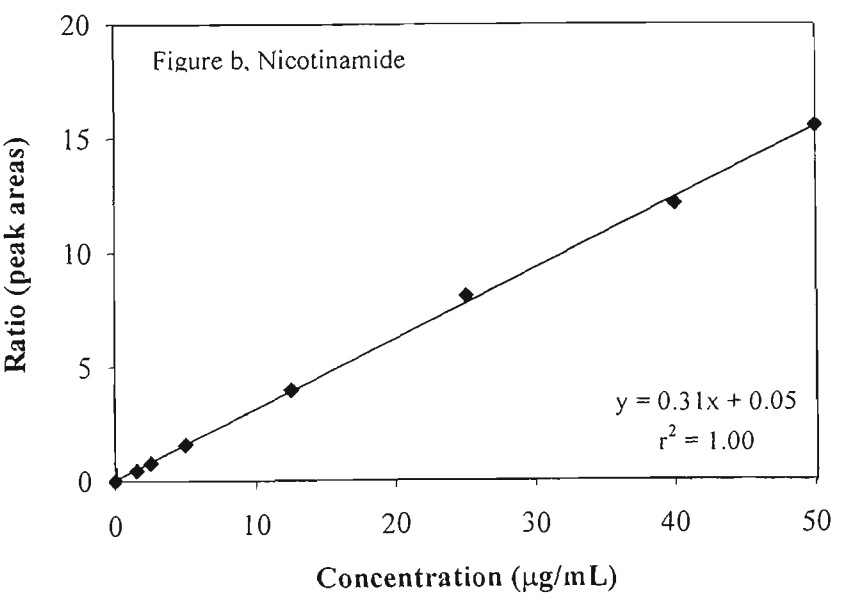
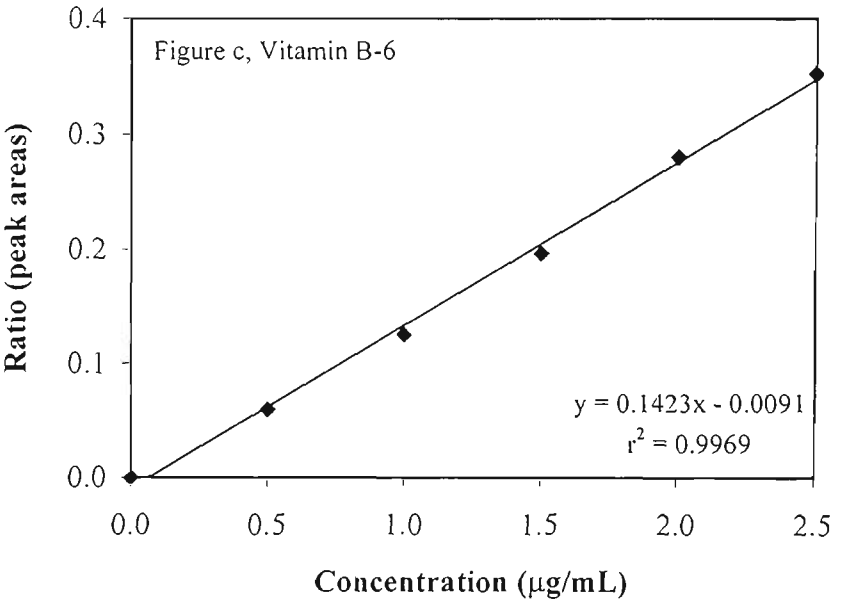


Figure 7.2 Parts a and b



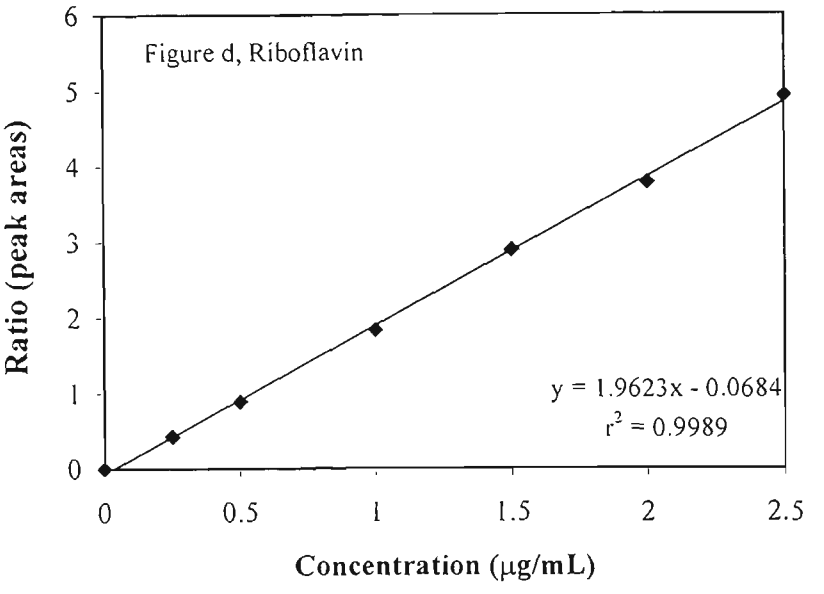
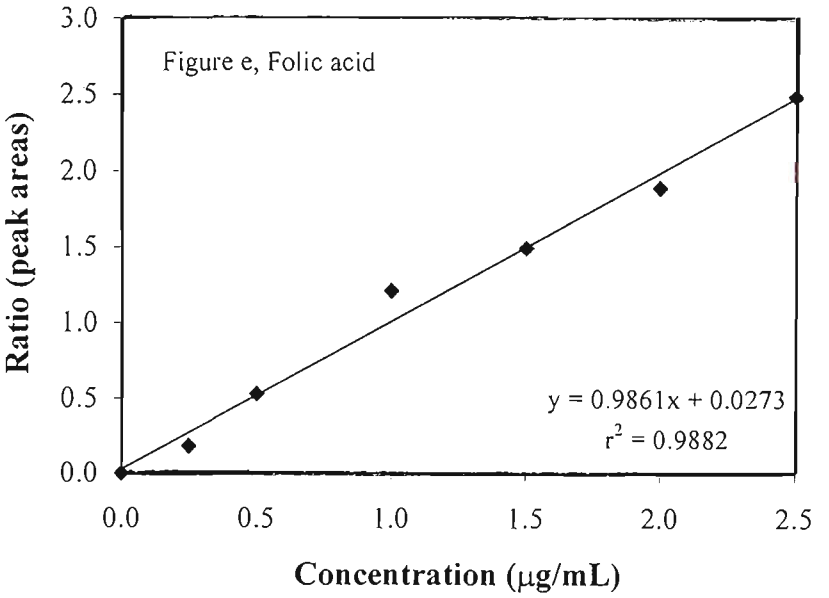


Figure 7.2 Parts c and d



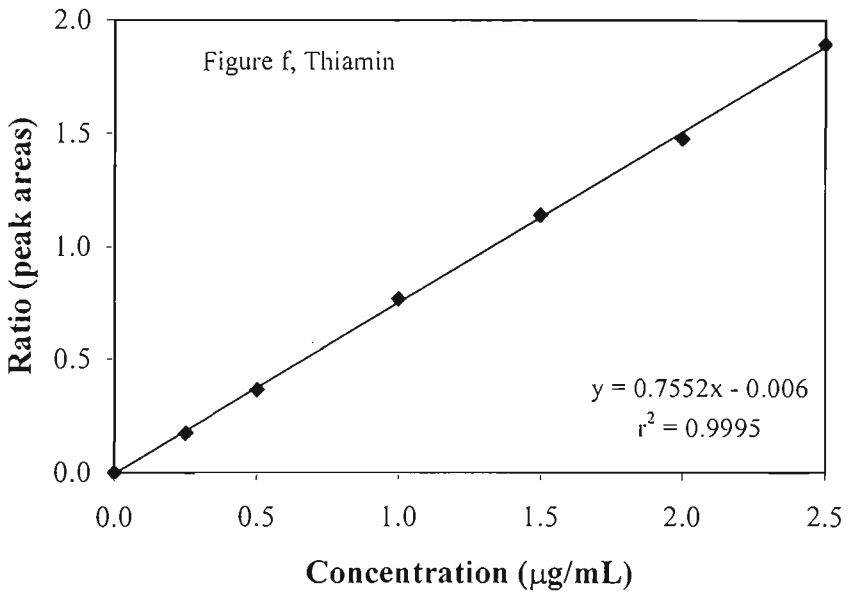


Figure 7.2 Calibration curves for each vitamin obtained on HPLC using method 2 and the internal standard of 3-hydroxybenzoic acid

Notes 1 For a description of method 2 refer Table 7.1

2 Ratio values were calculated by dividing the peak areas of individual vitamins by the peak areas of internal standard

samples. Accordingly, a variety of sample concentration and clean up procedures were investigated.

7.3 Evaluation of solid phase extraction/clean up procedures

In an attempt to remove unidentified peaks which overlapped with those identified as B vitamins and to facilitate quantitation of the vitamins, a variety of solid phase extraction procedures were trialled. The studies involved mixtures of standards, the AACC sample of a fortified breakfast cereal as well as a flour sample. The AACC and flour samples were extracted using HCl solution prior to application to a range of different commercial cartridges. In each case these were prepared and used according to the instructions provided by the manufacturer The specific details are presented in Table 6.13. Separate eluates were collected after washing and the proportions recovered were measured using the HPLC method based on that of Albalá-Hurtado and others (1997). It was found that hexanesulphonic acid gave clearer chromatograms than the octanesulphonic originally suggested and a MeOH gradient of 5-15 percent was used. The results obtained for the AACC reference sample are summarised in Table 7.2.

Table 7.2 Comparison of different solid phase extraction cartridges for clean up of AACC reference sample

	NA	NTA	PN	Thiamin	Folate	Riboflavin
Sep-pak plus	W	E	W	W	S	S
Plus HLB	S	E	S	U	N	S
Oasis MCX	U	U	U	U	U	U
Oasis MAX	E	E	U	U	N	W
C-18*	S	W	W	U	S	S
SAX*	N	W	W	U	S	W
Silica**	N	S	S	N	N	S

Notes
- E Vitamin eluted upon initial application to column and partial elution occurred with washing and further recovery when eluted with solvent
- N Not eluted from solid phase
- S Eluted from solid phase only by with solvent
- U Vitamin only partially absorbed and eluted during washes
- W No vitamin eluted upon initial application to column but partial elution occurred with washing and further recovery when eluted with solvent
- * Analysis performed only on the wash and elution fractions
- ** Analysis performed only on the elution fraction

In the case of each cartridge type some of the vitamins were not effectively bound onto the column as at least some of the material was eluted with the original solution. In addition, particular vitamins were detected in more than one of the fractions which were obtained. For example thiamin was not fully retarded on most cartridges but was recovered in various washes as well as in the initial eluate. Furthermore, following collection of a number of fractions precipitation was observed for Sep-pak plus, C-18 and Silica cartridges. In no case was each of the vitamins concentrated into one fraction.

As a further example of the patterns typically obtained upon elution from the cartridges, the proportion of each vitamin recovered in eluate fractions from Sep-pak plus cartridges are presented in Table 7.3. These data show that three of the vitamins were distributed between washes and eluate so that effectively no concentration was achieved. When the first eluate was run on HPLC there was considerable overlap of NA, NTA and PN with unidentified peaks (Figure 7.3).

Overall, the results show that the vitamins were retained to varying extents for each of the cartridges tested. In no case were all vitamins retained prior to elution with solvent. Therefore it was concluded that none of the cartridges showed potential for concentration and/or removal of interfering peaks in the chromatography of the B vitamins. Accordingly further studies focussed on optimisation of the HPLC procedures.

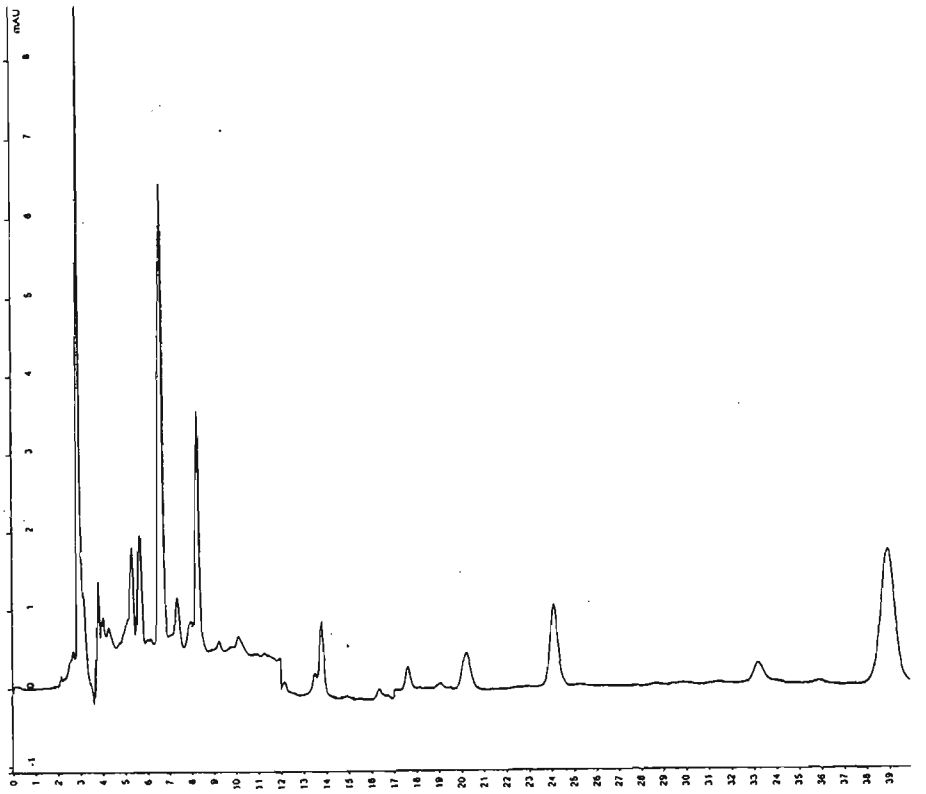


Figure 7.3 Vitamins in the AACC reference sample eluted from Sep-pak plus cartridges and chromatographed using method 2

Notes 1 For a description of method 2 refer to Table 7.1

2 The vitamins eluted at retention times of 4.0 min for NA, 6.6 min for NTA, 8.3 min for PN, 20.2 min for folic acid, 39.0 min for riboflavin and thiamin was not detected

Table 7.3 The distribution of vitamins from the AACC reference sample in various fractions obtained from Sep-pak plus cartridges

	NA	NTA	PN	Thiamin	Folate	Riboflavin
Load	0	44.1	0	0	0	0
Wash	35.9	15.0	0	35.3	0	0
Elute - 1st	38.1	29.4	93.5	21.9	100	100
Elute- 2nd	26.0	11.5	6.5	42.8	0	0

Note Results are the proportion recovered in the fraction, expressed as a percentage of the total which was calculated by summing the individual fractions

7.4 Further comparison of HPLC methods for B vitamins

The four published HPLC methods described in Table 7.1 were studied and in particular, their usefulness in quantifying the vitamins in flour samples was evaluated. Varying conditions of elution, detection and extraction were compared in an effort to optimise the separation and quantitation of the vitamins. The results are summarised in Table 7.4.

For each method, attempts were made to vary the composition of the mobile phase to optimise separations. For method 3 (Agostini and Godoy 1997) it had been claimed that a pH of 2.8 gave good results. In the current study this could not be confirmed (Figure 7.4). The most promising results were obtained with method 2. Controlling the temperature at 30 °C, replacing octanesulphonic acid with hexanesulphonic acid and using gradient elution improved reproducibility and shortened the analysis time for mixtures of standards of the vitamins (Figure 7.1). However with this method the results for samples of cereals included many unidentified peaks (Figure 7.5) and for most of the vitamins of interest quantitation was not possible.

Table 7.4 Summary of results for simultaneous determination of B vitamins using four different HPLC methods

No.	Description of results
1	Unable to clearly separate peaks for thiamin, riboflavin, NA and PN for cereal products.
2	Good separation of standards but unable to use for flour-based product due to overlapping peaks. Retention of PN very variable. Total analysis time is 50 min.
3	Poor resolution with NA and NTA co-eluting with unidentified peaks.
4	The mobile phase (ammonium acetate:MeOH) did not elute all the vitamins from the column used (Phenomenex ODS 3 C-18).

Note Numbers refer to methods described in Table 7.1

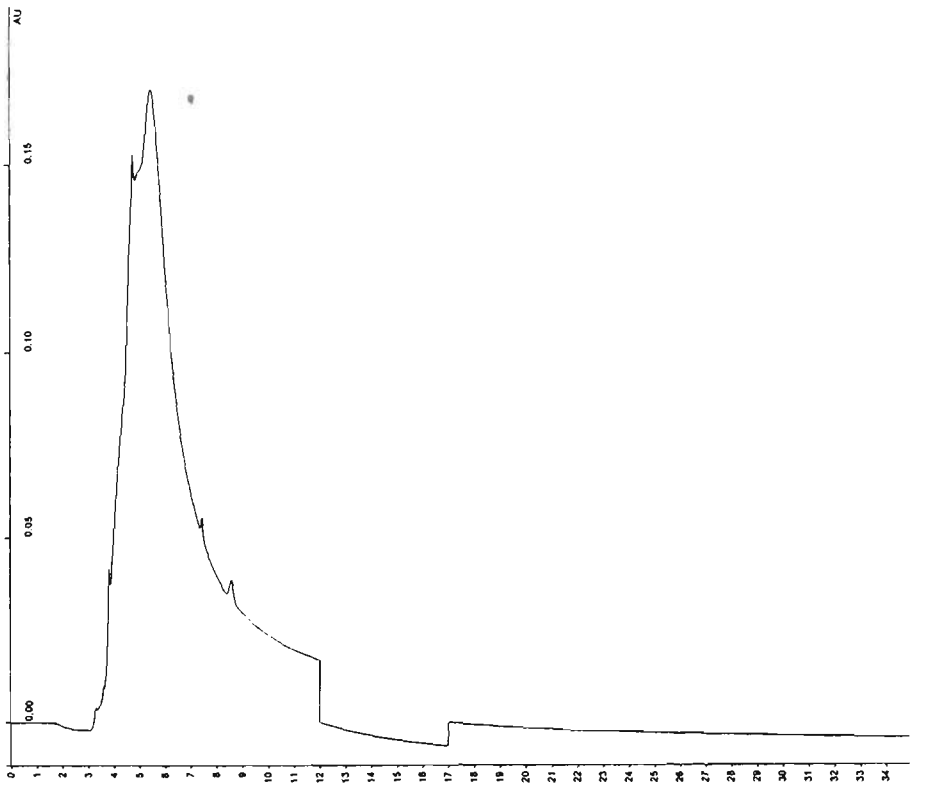


Figure 7.4 Chromatogram of standard vitamins analysed at pH 2.8 using HPLC method 3

Notes 1 For a description of method 3 refer to Table 7.1
2 The retention times of the vitamins could not be readily determined

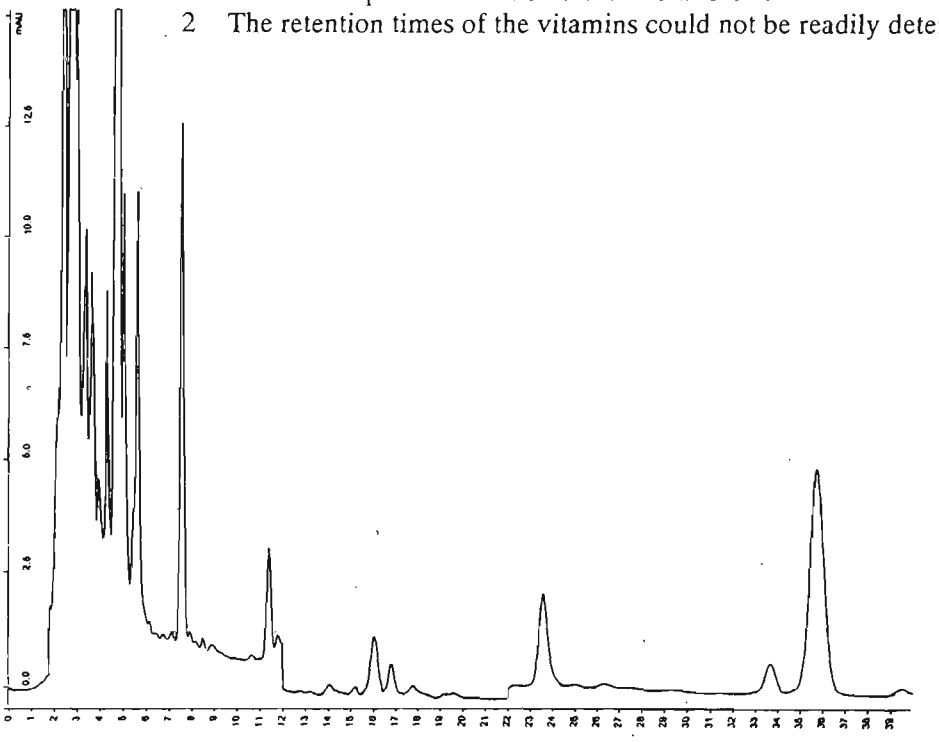


Figure 7.5 Chromatogram of AACC reference sample analysed using method 2 following modification

Notes 1 For a description of method 2 refer to Table 7.1 – the modifications involved the ion pairing reagent, temperature control and gradient elution
2 The vitamins eluted at retention times of 3.4 min for NA, 4.7 min for NTA, 5.6 min for PN, 7.6 min for thiamin, 16.1 min for folic acid, and 35.8 min for riboflavin

7.5 Conclusion on the investigation of procedures for the simultaneous analysis

Based upon extensive trials (including data not described above) it was found that some of the published methods could be modified to give good separations of all the vitamins studied when standards were analysed. In addition, good recoveries were obtained and total analysis times were less than one hour. However, despite extensive efforts to optimise the procedures, these did not give useful results for samples of flour and cereal foods. Solid phase procedures did not provide assistance for the simultaneous analysis of each of the B vitamins.

This conclusion may reflect the fact that some of the published procedures were developed specifically for analysis of vitamin supplements. In these there are relatively high concentrations of vitamins, fewer contaminants present and sample preparation is relatively simple. For the samples investigated here and the levels of the vitamins present in the current study, it was concluded that simultaneous measurement was not practical. For further investigation of vitamin stabilities in flours and Asian noodles the approach of analysing vitamins individually has therefore been adopted. It is noted also that the results obtained in these preliminary evaluations of methods indicated that the NA and NTA were present at relatively low levels. In addition, the measurement of these in flour samples by each of the HPLC procedures resulted in difficulties in quantitation due to the small peak areas, the similar elution times of the two different forms of the vitamin and the elution of the peaks in the early stages of the chromatogram where a large number of overlapping, unidentified peaks occurred. Accordingly the results reported in subsequent chapters are focused on B-6, riboflavin, folates and thiamin.

Chapter 8

The measurement and stability of vitamin B-6 in three different styles of Asian noodles prepared in the laboratory

The purpose of this chapter is to describe and discuss the results obtained during the analysis of Asian noodles for vitamin B-6. Following validation, the analytical method has been applied to a study of noodles prepared under controlled conditions in the laboratory.

8.1 Introduction

Vitamin B-6 exists as a series of vitamer forms (PN, PL and PM) together with various derivatives of these. Plant-derived foods contain primarily PN, a significant proportion of which may be present as PN-glucoside or other conjugated forms which appear to be largely unavailable to humans (Vanderslice and others 1979; Ball 1998).

In relation to stability of vitamin B-6, very few studies have examined this issue in food products (Navankasattusas and Lund 1982; Sampson and others 1996). Generally the vitamin appears to be stable under most conditions other than those involving heat and light (Gregory 1996). It has previously been reported that significant vitamin B-6 losses occur upon heating low moisture food products (Yen and others 1976). However, there have been no specific reports on stability of B-6 during the processing of Asian noodles. Therefore the objective of this phase of the project was to investigate the stability of vitamin B-6 in three different styles of Asian noodles prepared in the laboratory and particularly to evaluate the influence of heat and pH on the loss of vitamin B-6 during processing.

8.2 Selection of a suitable method for vitamin B-6 analysis

In reviewing procedures for analysis of vitamin B-6 (Chapter 4) it was evident that a wide variety of procedures have been reported. The first objective of this work was to select a method suitable for the determination of vitamin B-6 in Asian noodles.

The use of HPLC coupled with fluorimetric determination has enabled the development of specific and sensitive methods for the determination of B-6 as the preferred method for routine food analysis. One such approach has been proposed as an appropriate analytical method for adoption by the European Community (Brubacher and others 1985b). Accordingly, this HPLC method was selected as the starting point for the evaluations reported here.

8.3 Preliminary evaluation of an HPLC method for vitamin B-6

For HPLC a reversed-phase system was used with a stationary phase (Brubacher and others 1985b; Ollilainen 1999) based on ODS. The procedures used here are described more fully in Section 6.8. The solvent originally suggested included sulphuric acid and this was too acidic (pH of approx 1.4) for use with the Waters Spherisorb ODS 2 column used in the current study. Two different reversed-phase C-18 columns were considered (Waters Spherisorb ODS 2 and Waters-Resolve C-18) and a series of solvent systems were evaluated for the separation and quantitation of the three forms of vitamin B-6 using isocratic elution. The solvents studied were 1) hexanesulphonate, acetic acid, triethylamine, methanol (pH 3.6) (Albalá-Hurtado and others 1997); 2) phosphoric acid 0.033M containing octanesulphonate and *iso*-propanol (pH 2.2) (Gregory and Feldstein 1985; Ollilainen 1999); and 3) octanesulphonate; acetic acid, triethylamine, methanol (pH 3.7) (refer to Section 6.8.2). The results obtained with solvent 1 showed that the three standards were not clearly separated with retention times of between 3.7 and 4.4 min. Trials indicated that enhancement of the separation of the peaks could not be readily achieved. With solvent 2 it was found that reasonable separations were achieved but that the best results were achieved using the Waters-Resolve C-18 column. The resolution of the standards with solvent 3 was less satisfactory. The use of the ion-pair reagent has been reported to enhance the retention of the compounds of interest on the stationary phase and also to alter the selectivity of the method for particular compounds (Dong and others 1988; Ollilainen 1999).

On the basis of these results it was decided to adopt the procedure based on solvent number 2 above and the column Waters-Resolve C-18. For this method it was possible to readily separate standards of the three forms of the vitamin. The order of elution was

PL, PN and PM and all were readily separated within 11 min and a typical chromatogram of the standards is presented in Figure 8.1.

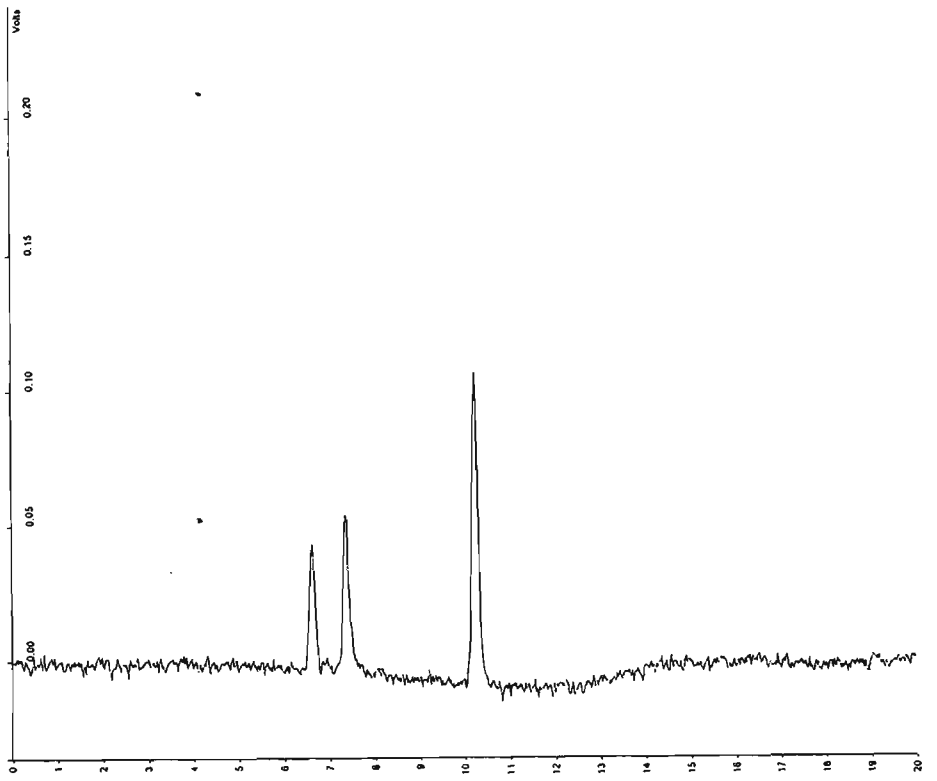


Figure 8.1 Chromatogram obtained when standards of three forms of vitamin B-6 were analysed using HPLC

Note The B-6 vitamers eluted at retention times (min) of 6.6 for PL, 7.4 for PN and 10.2 for PM

In the current study, the use of the conditions described resulted in HPLC traces in which the peaks standards typically eluted at retention times of between 7 and 11 min. However, from day to day retention times varied considerably. In an attempt to overcome this problem, a column heater was used to control the temperature. A further approach used in the current study was to place the solvent reservoir in a water bath controlled at a temperature of 30 °C. It was found that these two strategies successfully reduced the variation in retention times due to the change of the ambient room temperature particularly from daytime to night. Using the conditions described, good separations of the three forms of vitamin B-6 were consistently achieved. The significance of temperature control has previously been highlighted by Welsch and others (1996), who evaluated a model HPLC system. More recently, in the analysis of

B-6 vitamers using HPLC, Ollilainen (1999) also reported that temperature control is of significance.

During the course of these evaluations, samples of standards were run repeatedly over an extended period. As a further step in validation of the HPLC method used here, the reproducibility of the data obtained for PN was assessed and, for this purpose, the same concentration was used (0.066 μg/mL) and the peak areas compared. The results are presented in Table 8.1. These demonstrate some variability which is attributed to the fact that during this time various parameters were varied during the establishment of the optimum chromatographic conditions. However, the overall level of reproducibility in peak area results for PN was regarded as satisfactory. Similar results were obtained for the other two forms of the vitamin studied here. A typical standard curve obtained using a range of concentrations of PN is shown in Figure 8.2. These results further confirm the suitability of the method evaluated here and so it was next applied to the analysis of a reference food sample.

Table 8.1 Reproducibility of data obtained when PN standard was analysed on HPLC over a 12 week period

Parameter	Results
Time span of analyses	12 weeks
Number of replicates	32
Mean peak area (counts)	520,764
Range of results	449,320-585,002
Standard deviation	45,188
Coefficient of variability (%)	8.7

Note The PN solution injected had a concentration of 0.066 μg/mL

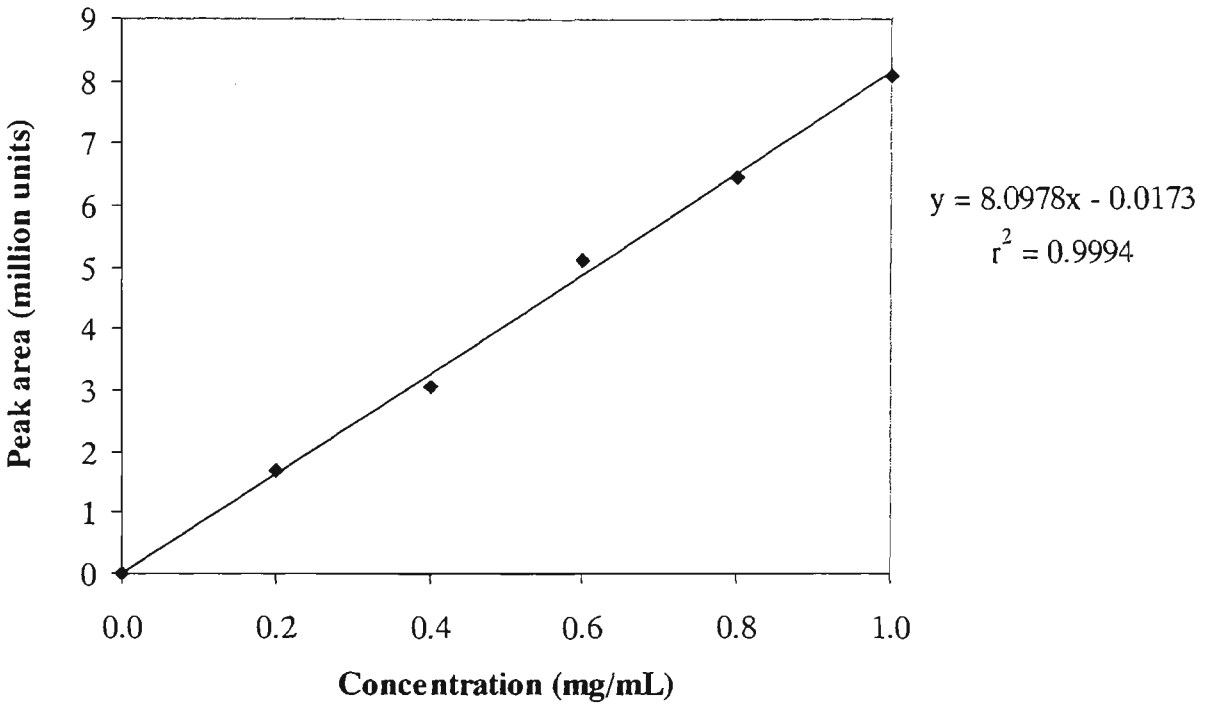


Figure 8.2 Standard curve of PN analysed using HPLC

8.4 Evaluation of sample extraction and validation of HPLC analysis of vitamin B-6

The method for vitamin B-6 analysis of Brubacher and others (1985b) involves sample preparation by homogenisation in a medium of 0.1 M H_2SO_4 prior to autoclaving for 30 min at 121 °C. In addition, the original procedure recommended that quantitation be carried out by an approach referred to as external standardisation. For this, a solution of standard PN was subjected to the same extraction conditions as those used for food samples. The procedures are described more fully in Section 6.8.4. A typical chromatogram obtained for the AACC reference sample is shown in Figure 8.3 and the analytical results obtained over a period of a number of weeks are presented in Table 8.2.

For analyses of the reference sample the mean value (92.1 ± 5.3 mg/kg) is similar to that obtained originally by the laboratories which participated in the AACC collaborative survey (mean 85.0 mg/kg) (compare with Table 6.2). Each of the values found for the reference sample were within the range of the values (73.0–100.5 mg/kg) indicated for the AACC sample. It is noted that the amount of PN in the sample represented approx

88 percent of total which is consistent with the sample having been fortified with the vitamin specifically in the form of PN.

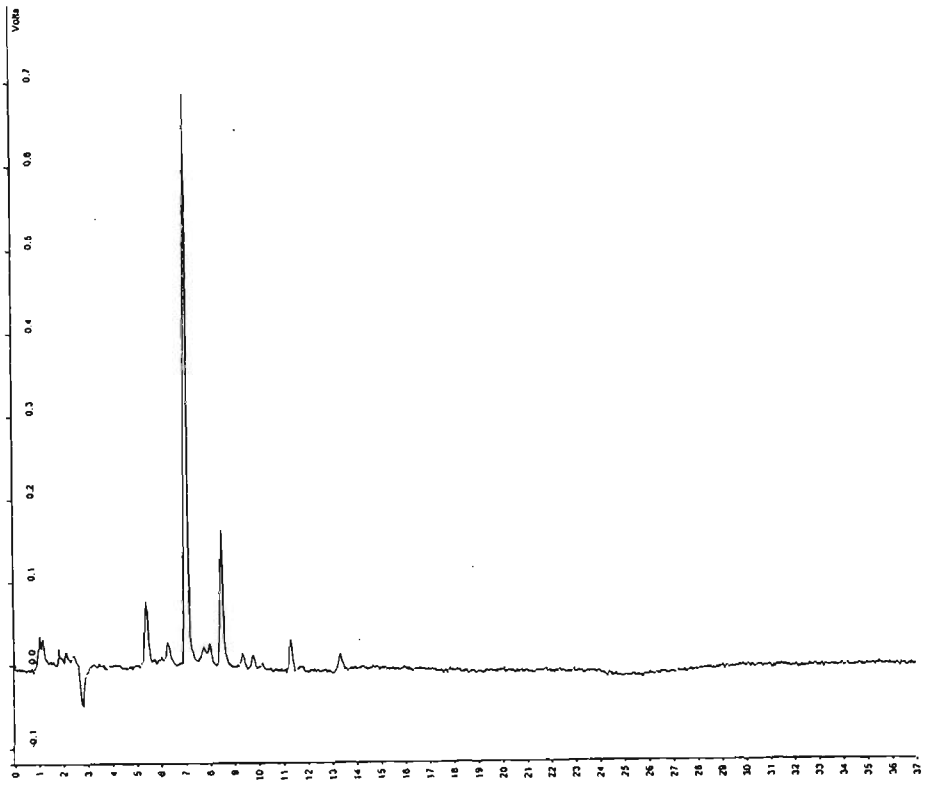


Figure 8.3 Chromatogram obtained when the AACC reference sample was analysed using HPLC
Note PN eluted at a retention time of 7.1 min

The validity of the procedure for routine food analysis was further evaluated by measuring the recovery of PN with a spiking study. For this, the recovery was determined by spiking a flour sample with PN. The flour sample chosen was that used in preparation of noodles in later parts of this study. The results of replicate experiments are presented in Table 8.3 and show a mean value very close to 100 percent recovery. These are comparable with the recovery rates stated in the original publication on the method at between 94–102 percent (Brubacher and others 1985b).

Table 8.2 Results of repeated analysis of B-6 vitamers in the AACC reference sample

Description	PL	PN	PM	Total vitamin B-6
Time span of analyses	3 weeks	3 weeks	3 weeks	3 weeks
Number of replicates	12	12	12	12
Mean value	8.92	81.2	1.92	92.1
Range of results	4.77-12.0	78.2-85.9	0.76-2.66	86.2-97.3
Standard deviation	2.3	2.3	0.72	5.3
Coefficient of variability (%)	25.3	2.8	37.5	5.7

Notes 1 Results for B-6 contents are expressed in units of mg/kg on an as is moisture basis
2 Total vitamin B-6 was calculated by summing the values found for PN, PL and PM

Table 8.3 Recovery of standard PN from a flour sample spiked at a rate of 20 μg in 7 g flour

Injection	Recovery (%)
1	99.2
2	99.8
3	106.0
4	103.3
Mean	102.1
Standard deviation	3.2
Coefficient of variability (%)	3.1

8.5 Vitamin B-6 contents of wheat flour

In analysing the spiked flour samples it was found that the levels of PL and PM were relatively low and therefore the flour sample was tested repeatedly for all three vitamers of B-6. A typical chromatogram is shown in Figure 8.4 and the calculated results for flour are presented in Table 8.4. The results show that all three forms are present at relatively low levels decreasing in order from PL to PN and PM. Some difficulty was experienced in obtaining repeatable peak areas for the vitamers and manual re-integration using the Star Chromatography works station (Varian, version 5.31) software was utilised in an attempt to enhance identification of the peak from the noise associated with the base line. The amounts present in the flour extracts were approaching the limit of detection reported previously for the method (Brubacher and others 1985b). In assessing options to optimise the determination of vitamin B-6 in flours various approaches were considered. It was not possible to increase the ratio of flour to extractant as the resulting solutions became very viscous and hence could not be analysed satisfactorily.

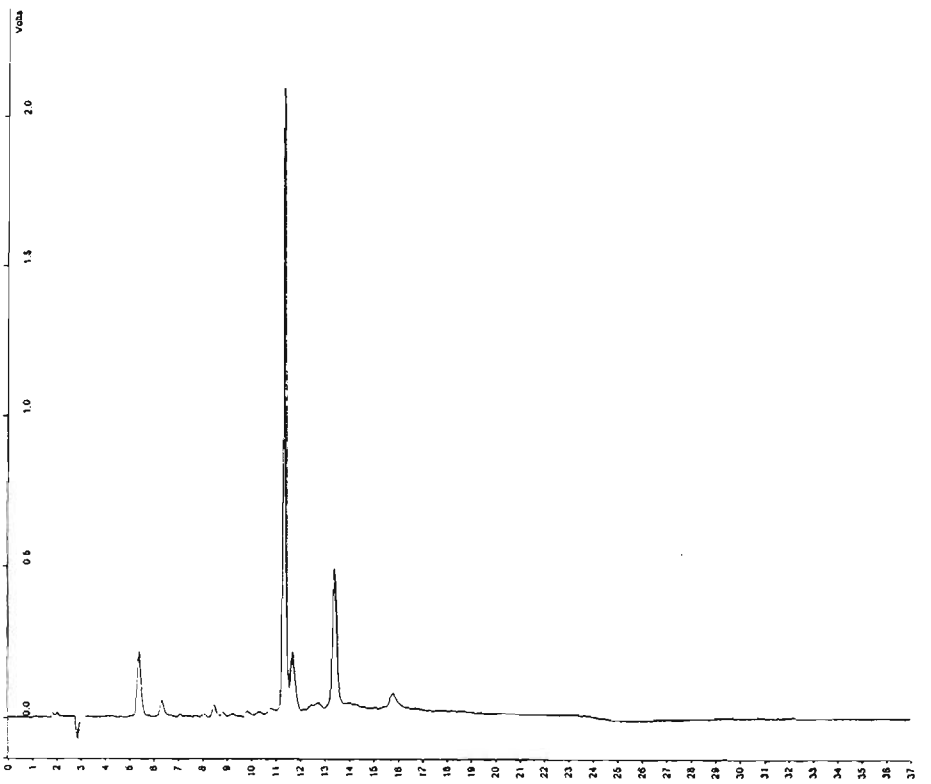


Figure 8.4 Chromatogram obtained when flour was analysed using HPLC
Note PN eluted at a retention time of 7.1 min

Table 8.4 Results of repeated analysis of total B-6 in a flour sample

Analysis number	PL	PN	PM	Total Vitamin B-6
Time span of analyses	3 weeks	3 weeks	3 weeks	3 weeks
Number of replicates	15	15	15	15
Mean value	1.96	0.63	0.34	2.93
Range of results	1.70-2.23	0.47-0.90	0.20-0.50	2.57-3.35
Standard deviation	0.20	0.14	0.10	0.44
Coefficient of variability (%)	10.2	22.2	29.4	15.0

Note Results for B-6 contents are expressed in units of mg/kg on a dry weight basis

The results obtained for the unfortified flour analysed here can be compared with the relatively few values available in food composition tables and the literature. These are summarised in Table 8.5 and show a wide range of values. The analytical results found in the current study are within the range of values reported elsewhere. In considering the differences in results found by various researchers it is noted that in some cases considerable variability was found when one analytical procedure was applied to samples within one country (Sampson and others 1996; Scherz and Senser 2000). This range may reflect inherent variability in flours due to environment and genotypic factors. Alternatively it may reflect the potential impact of milling, particularly varying extraction rates. In some cases data is available for the vitamin B-6 contents of whole wheat and also for bran and germ samples (Leklem 1991; Sampson and others 1996; Ollilainen 1999; Scherz and Senser 2000; USDA ARS 2002). In all cases these indicate that bran and germ have higher levels than the corresponding wholemeal and this in turn is much higher than values for the white flours.

Table 8.5 A comparison of results found in the current study with published values for total vitamin B-6 contents of milled (white) wheat flours

Description and origin of samples	Total vitamin B-6	Reference
Current study – one flour sample	2.6 ± 0.4	Table 8.4 (see note 1)
Single flour sample (Japan)	0.29	Toukairan-Oda and others 1989
Based on data published in 1969 (US)	0.60	Leklem 1991
Glycosylated forms included (Finland)	1.3	Ollilainen 1999
Single flour sample (France)	1.06 –1.42	Ndaw and others 2000
Only PN and glycosylated PN found (North America)	0.08 – 0.37	Sampson and others 1996
Six different flours (US)	0.33 – 0.50	USDA ARS 2002
Data from four European countries	3.5 – 4.1	Ollilainen 1999
Four different flours (Germany)	1.00 – 2.80	Scherz and Senser 2000

Notes
1 Results are expressed in units of mg/kg on an as is moisture basis
2 Only values for white flours are summarised here as reported wholewheat values are typically much higher
3 Where necessary values have been recalculated from those in original references to facilitate direct comparison
4 Data from Table 8.4 was recalculated and expressed an as is basis
5 Data of Sampson and others recalculated in terms of PN
6 A range of values is given where either data for different flour samples were reported or a range of values was found using different analytical procedures

In considering the current analytical results there is little published information on the relative amounts of different vitamers of B-6 in white flours. The available data are summarised in Table 8.6. The results in the current study are similar to those of one earlier report in which PL represents approximately two thirds of the vitamin in flour. However, two other sets of data indicate that more than half of the vitamin is in the form of PN. The findings of Sampson and others (1996) serve to further complicate the

picture. Using a derivatisation procedure to enhance detection, they showed that the only forms found in North American flours were PN and glucosylated PN.

Table 8.6 A comparison of results found here with published values for the proportion of PL, PN and PM in milled (white) wheat flours

PL	PN	PM	Reference
66.9	21.5	11.6	This study: Table 8.4
17.6	58.8	23.5	Ollilainen 1999
0	100	0	Sampson and others (1996)
24.0	55.0	21.0	Leklem 1991
72.6	14.5	12.8	Toukairan-Oda and others 1989

Notes
1 All values are expressed as percentage of total vitamin B-6 found
2 Only values for white flours are summarised here
3 Where necessary values have been recalculated from those in original references to facilitate direct comparison
4 Different analytical procedures were applied in these studies

In the studies reported by Sampson and others it was found that glucosylated PN was subject to hydrolysis during extraction prior to analysis. It is also known that the bound forms have nutritional activity. Based on the evaluations undertaken here it appears that the Australian wheat flour analysed here contains a number of B-6 vitamers. The total amount of the vitamin in flour (Table 8.4 and 8.5) is relatively low. In nutritional terms it can be estimated that consumption of one serving of a flour-based product including Asian noodles might provide up to 4.1 percent of RDI. This figure is based upon the current Australian RDI values for adults which are up to 2.2 mg of vitamin B-6 per day and the serving size assumed here is equivalent to 35 g of a food. It is noted that this discussion also assumes no loss of the vitamin during processing or storage, although it has been reported that losses of up to 40 percent of B-6 might be expected to occur during cooking of typical foods (Gregory 1996).

The first conclusion from the analysis of the flour sample is that this does not represent a particularly good source of vitamin B-6. It was also found that the levels of two of the vitamers were sufficiently low that reliable quantitation was difficult. The method did, however, provide repeatable and reliable results over a period of some months when applied to the reference sample which was a fortified cereal grain food. In addition the method provided results close to the mean value provided with the AACC reference sample.

At this point in the investigation preliminary analyses of some commercial noodles indicated that the levels of B-6 were sufficiently low that quantitation by the method used here was not practical. Further studies were commenced on noodles prepared in the laboratory. Accordingly the analysis method validated here has been utilised in further studies of the use of flour fortified with vitamin B-6 for the preparation of the three styles of Asian noodles under controlled conditions.

8.6 The preparation of three styles of Asian noodles in the laboratory

Laboratory methods for noodle preparation were selected and set up to reflect, as far as possible, typical commercial formulations and processing practices for each of the three styles of noodles. The procedures were based upon published approaches (Moss and others 1987) and are described in detail in Sections 6.3.2-6.3.5. It is emphasised that for all steps here the water used was of MilliQ grade. The basis of this choice was to minimise the potential impact of impurities in the water upon the measurement of the vitamin compounds. For all three styles, samples of the noodles prepared in the laboratory were taken at each stage of processing and also following cooking to the optimum point which was assessed for each batch of noodles prepared. The samples were then analysed for moisture as well as vitamin contents. The results obtained for moisture are presented in Table 8.7.

These data show relatively little variation in moisture contents of the three styles of noodles at corresponding stages of processing. The primary value of these results has been in the calculation of vitamin content results to a dry matter basis. These calculations were used in all subsequent phases of this research in order to facilitate the comparison of results at different stages of processing.

The pH values of the noodles were also measured and typical results were 5.9 for the white salted, 10.6 for yellow alkaline and 7.4 for the instant noodle products.

Table 8.7 Moisture contents for noodle samples prepared in the laboratory and analysed at different stages of processing

Processing step	White salted noodles	Yellow alkaline noodles	Instant noodles
Dough	33.4 ± 0.6	35.5 ± 0.08	34.3 ± 0.1
Steamed	na	na	31.8 ± 1.2
Dried noodle	9.8 ± 0.2	9.80 ± 0.07	10.4 ± 0.6
Noodles cooked to the optimum point	68.4 ± 0.7	66.4 ± 0.2	64.6 ± 0.4

Notes 1 Results are the mean of duplicate analyses and are expressed as mean ± sd in units of g per 100g
2 na indicates not applicable
3 The optimum cooking times varied for each style of noodle – this issue is discussed further in Chapter 11

8.7 The stability of vitamin B-6 in three styles of Asian noodles

The form of vitamin B-6 generally used in fortification is PN. This is reported to reflect the enhanced stability of this vitamer (Gregory 1996). In selecting a suitable level of fortification for the current study, consideration was given to the regulatory framework applying in Australia. PN, as the hydrochloride, is the only form of the vitamin which may be used for fortification and the upper limit of addition is expressed in terms of 25 percent RDI per serving. This level corresponds to approximately 12 mg of PN /kg flour sample. Therefore this was chosen and the vitamin added to the formulations after dissolution in a suitable volume of water. In all cases the vitamin solution was prepared and added separately to either the salt or kansui solution, as appropriate in the particular formulation. Solution concentrations were adjusted so that the final doughs incorporated the appropriate levels of the required salt(s) and the vitamin. The procedures are described in Section 6.3.6.

A series of fortified products was prepared in the laboratory and samples taken at various stages during processing for analysis of PN contents by HPLC. Similar chromatographic patterns were obtained for the various dough, dried noodle and cooked products. The patterns obtained for dried and cooked yellow alkaline noodles are shown in Figures 8.5 and 8.6. These are similar to those obtained for samples of the other styles of noodles. The moisture contents of the samples (Table 8.7) were used in the calculation and expression of vitamin content values on a dry weight basis. The results for white salted and yellow alkaline noodles are presented in Table 8.8 and those obtained for instant noodles in Table 8.9.

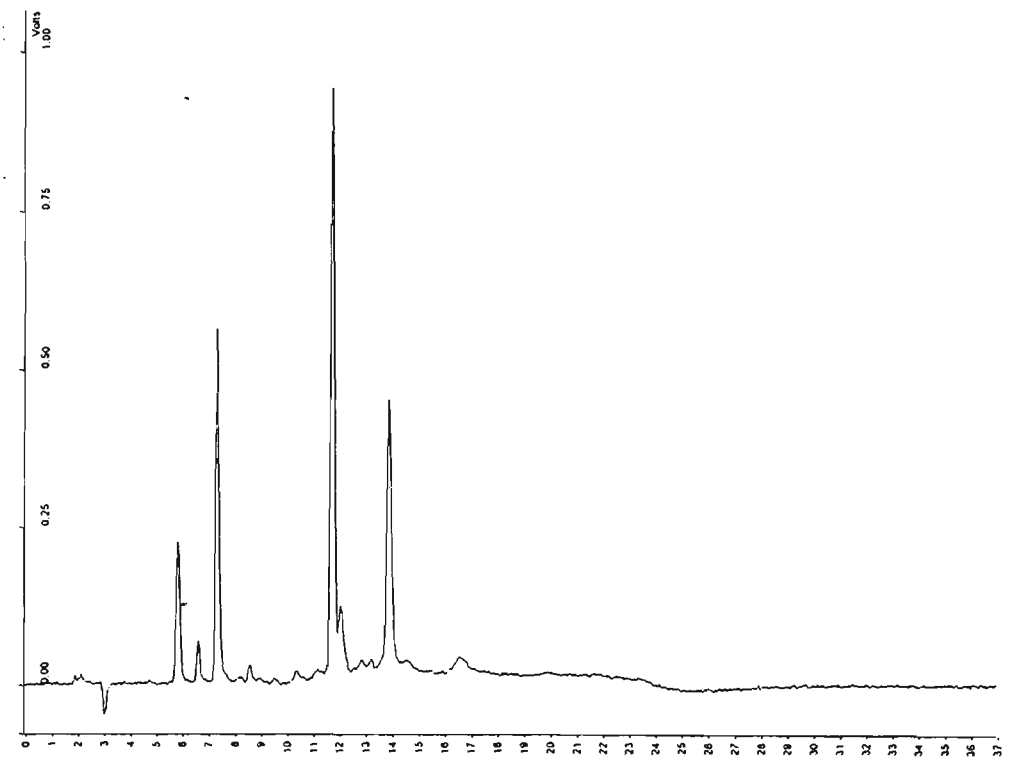


Figure 8.5 Chromatogram obtained for PN when dried yellow alkaline noodles were analysed using HPLC
Note PN eluted at a retention time of 7.3 min

For white salted and yellow alkaline noodles a similar pattern of results was found. It is noted that the amount of PN calculated as 12 mg for addition resulted in values of 14.3 mg in the tables of results due to the recalculation of experimental values to a dry weight basis (the flour moisture content averaged 12.6 g per 100g). The results obtained here have also been summarised in Table 8.10 to show cumulative and relative losses of the vitamin.

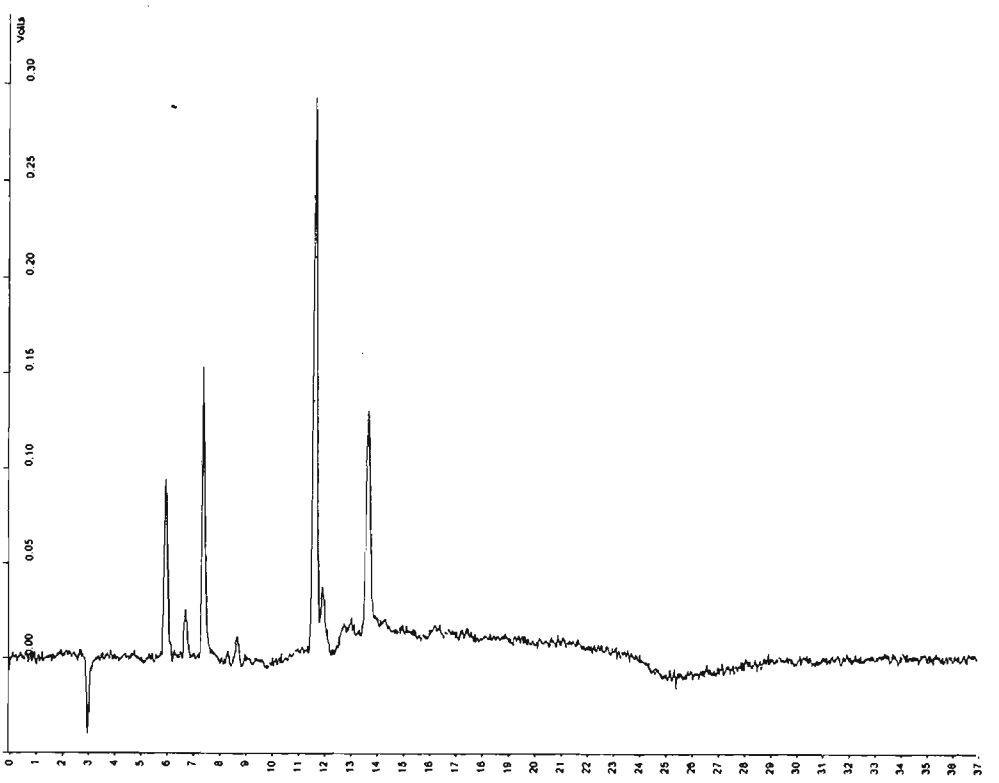


Figure 8.6 Chromatogram obtained for PN when cooked yellow alkaline noodles were analysed using HPLC

Note PN eluted at the retention time of 7.3 min

Table 8.8 PN contents at different stages during processing of white salted and yellow alkaline noodles

	White salted noodles	Yellow alkaline noodles
Flour	0.6 ± 0.1	0.6 ± 0.1
Vitamin added to formulation	14.3	14.3
Dough	11.2 ± 0.4	11.4 ± 0.3
Dried noodles	11.3 ± 0.4	11.4 ± 0.3
Cooked noodles	6.2 ± 0.2	5.4 ± 0.4

Notes 1 Results are presented as mean ± sd and are expressed as mg/kg on a dry weight basis
2 The values presented for the amounts added to flour have been calculated based upon the known concentration and volume of vitamin B-6 solution used

The results demonstrate that, overall, considerable losses in PN occur in all three styles of Asian noodles. The pattern of loss in white salted and yellow alkaline noodles are very similar with significant losses during dough mixing and no losses upon subsequent drying at 40 °C. However, substantial losses then occurred in the final procedure of cooking the noodles. The losses observed for boiling of the white products are apparently lower than those for the alkaline style. This probably reflects the longer period of boiling required for the latter type (3.50 min compared to 6.10 min respectively). The similarities in the results for these two styles of noodles indicate that for PN the pH of the noodles is not a primary determinant of vitamin stability. Whilst lower temperatures (40 °C) caused no loss, it does appear that heating at boiling temperatures is the major factor influencing the high overall rate of losses found.

Table 8.9 PN contents at different stages during processing of instant noodles

	PN content
Flour	0.6 ± 0.1
Vitamin added to formulation	14.3
Dough	11.2 ± 0.2
Steamed noodles	11.0 ± 0.2
Fried noodles	8.2 ± 0.1
Cooked noodles	4.9 ± 0.7

Notes 1 Results are presented as mean ± sd and are expressed as mg/kg on a dry weight basis
2 The values presented for the amounts added to flour have been calculated based upon the known concentration and volume of vitamin B-6 solution used

Table 8.10 Relative losses of PN during processing of laboratory noodles

Noodle style	Processing stage			PN loss	Cumulative PN loss
White salted	Flour	→	dough	21.7	21.7
	Dough	→	dried noodles	0	21.7
	Dried noodles	→	cooked noodles	35.7	57.3
Yellow alkaline	Flour	→	dough	20.3	20.3
	Dough	→	dried noodles	0	20.3
	Dried noodle	→	cooked noodles	41.9	62.2
Instant	Flour	→	dough	21.7	21.7
	Dough	→	steamed noodles	1.4	23.1
	Steamed	→	fried noodles	19.6	42.7
	Fried noodles	→	cooked noodles	23.1	65.7

Note Losses are expressed as a percentage relative to the total amount of PN in the ingredient formulation (from flour plus that added during fortification)

In the preparation of instant noodles some losses were observed during dough mixing. However no decrease in PN content was seen upon steaming of the product. Substantial losses occurred at the steps involving deep-frying and boiling even though these required only 45 s and 2 min respectively for this style of noodle. In relation to heat stability the results indicate differences when varying conditions are applied. The current laboratory results indicated that drying noodles at a temperature of 40 °C had no affect on PN. This is consistent with the study of Saidi and Warthesen (1983) who reported no significant degradation of PN.HCl when aqueous solutions protected from light were held at 40 and 60 °C for up to 140 days at pH levels ranging from 4-7.

One of the unexpected findings here was that approximately 20 percent of the total PN in the formulations appeared to be lost during the dough mixing stage for each of the three styles of noodles. The only likely explanations which might account for this are the known susceptibility to light and the chemical reactivity of the vitamin (Gregory

1996). Light was probably not a significant factor in the current study as all steps were carried out in conditions of subdued light. Furthermore, noodles were covered whenever possible. It is more likely that the observations are the result of other factors. The vitamers are known to react with a range of other food components including lysyl groups in proteins. In addition, examples of interconversions of the different forms have been reported in some food systems particularly from PL to PM (Leklem 1991). More recently in a review of the chemistry of the B-6 vitamer forms their reactivity has been highlighted (Ollilainen 1999). In relation to the apparent losses found in the current study, further investigations would be required to clarify the mechanism (or mechanisms) of the losses in the dough mixing stages of Asian noodles.

In order to further consider how losses in PN occurred during cooking, samples of the water were taken at the point of optimum cooking. These were then analysed for PN content using HPLC. A typical chromatogram for cooking water is presented in Figure 8.7 and the results are summarised in Table 8.11.

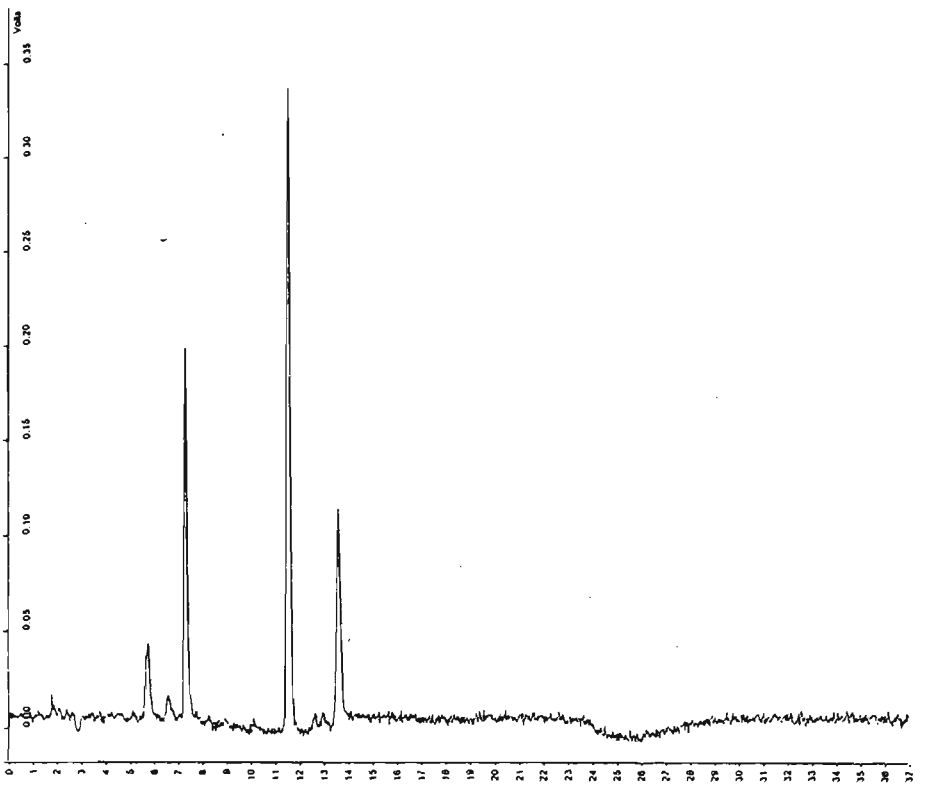


Figure 8.7 Chromatogram obtained for PN when cooking water for yellow alkaline noodles was analysed using HPLC
Note PN eluted at a retention time of 7.4 min

The data indicate leaching of PN into the cooking water for each of the styles of noodles. The amount of PN not recovered in either the noodles or in the water was calculated and can be considered as an indication of the extent of chemical decomposition occurring during boiling. In each case approximately one third of the PN originally present was lost in this manner. In addition, these results confirm the influence of heat on PN during cooking. The losses were directly related to the cooking time with longer times resulting in the greater vitamin losses probably due to thermal decomposition.

Table 8.11 Amounts of PN in cooked noodles and cooking water when three styles of noodles were boiled for their individual optimum cooking times

	Cooked noodles	Cooking water	Amount not recovered
White salted	6.2 ± 0.2 (55%)	1.51 ± 0.08 (13%)	3.6 (32%)
Yellow alkaline	5.4 ± 0.4 (47%)	1.56 ± 0.03 (14%)	4.4 (39%)
Instant	4.9 ± 0.7 (60%)	0.73 ± 0.07 (9%)	2.6 (31%)

Notes
1 Results for PN contents are presented as mean ± sd and are presented as mg/kg with respect to one kg of noodles expressed on a dry weight basis
2 The values for amounts not recovered have been calculated from the contents of the dry noodles prior to cooking from Tables 8.8 and 8.9 by subtracting the amounts in the cooked noodles and the water
3 Values in parentheses are the proportions of the PN in comparison with the levels originally measured in the dried noodles prior to cooking and expressed as a percentage

The results for cooking of noodles samples have been recalculated and compared with the available published data (Table 8.12). The results of cooking loss of these flour-based products show a wide range of from 13–67 percent. In the context of this wide range, the values for the Asian noodles reflect relatively high losses of PN but remain within the overall range of values. It is noted that the pasta and noodles were prepared from different wheat flours and may also include other ingredients. Furthermore the term egg noodles refers to a durum-based product (compare with Section 3.3, page 26). The original levels of total vitamin or of individual vitamers may have varied. In addition, the data from the US was for enriched products (Ranhotra and others 1985) or may have been fortified (USDA ARS 2002). Other factors which may have impacted on

the losses of B-6 include the cooking time as well as the thickness of pasta or noodle strands. In one study (Ranhotra and others 1985) a wide range of different levels of loss were found for the same style of pasta product which had been sampled from different cities in the US.

Table 8.12 A comparison of vitamin B-6 contents and cooking losses for pasta and noodle products

Sample	Vitamin B-6 content	Relative loss (%)	Reference
Asian noodles	8.2 - 11.4	23.1 – 41.9	This study (Tables 8.8 – 8.10)
Egg noodles	1.40 - 1.55	35 – 61	Ranhotra and others 1985
Spaghetti	0.82 - 0.86	16 – 50	
Macaroni	1.05 - 1.32	17 – 67	
Egg noodles	1.0	42.1	Holland and others 1991
Spaghetti	1.7	59.5	
Macaroni	1.0	58.8	
Noodles, eggs enriched	1.35 ± 0.09	14.8	USDA ARS 2002
Noodles, eggs, spinach enriched	4.51 ± 0.74	19.8	
Noodles, Japanese, soba	2.58 ± 0.78	42.5	
Noodles, Japanese, somen	0.55 ± 0.06	26.4	
Spaghetti, enriched	1.18 ± 0.07	12.9	
Spaghetti, protein-fortified	1.96 ± 0.30	19.0	

Notes
1 Vitamin contents are for dried noodles as purchased and are expressed in units of mg/kg on an as is basis
2 Loss values are the proportion of vitamin B-6 measured in the dried noodle sample which was lost during cooking and are expressed as a percentage
3 Where necessary loss values have been calculated from data in the original references by first expressing B-6 contents on a dry matter basis
4 The data from this study relates only to PN whereas the literature data were reported as vitamin B-6
5 Different analytical procedures were applied in these studies

8.8 General discussion and summary of results for vitamin B-6 in three styles of Asian noodles

The overall conclusions from the analyses for B-6 in the current study are that the levels of the vitamers in unfortified flour are relatively low in relation to the RDI values. The incorporation of PN into Asian noodle formulations resulted in enhanced levels in the final cooked products. The retention in the three styles of noodles was similar. Losses occurred at most stages during processing including the initial mixing of the dough. The greatest losses occurred during steps involving heating, particularly deep-frying of instant noodles and boiling of the each of the three styles. The length of time required during boiling appeared to be more important than the pH of the noodles.

Despite the losses in PN in the processing of the three styles of noodles, it was possible to effectively increase the amount of the vitamin for the consumer by fortifying the noodles. Allowance for the losses of approximately 57-66 percent would require the incorporation of substantial overages during formulation for each of the styles of Asian noodles.

Chapter 9

The measurement and stability of riboflavin in Asian noodles prepared in the laboratory

The purpose of this chapter is to describe and discuss the results obtained for riboflavin analysis of Asian noodles. This encompasses selection and validation of a suitable method and its application to a study of vitamin stability in these products.

9.1 Introduction

Cereal grain foods are a significant source of riboflavin and for analysis, a wide variety of analytical methods have been applied. In earlier phases of this project it was found that published HPLC procedures for simultaneous determination of all of the B group vitamins in flour-based foods were ineffective. However the methods evaluated showed potential for riboflavin in noodle samples. Accordingly, the initial purpose of this study has been to establish a suitable method for riboflavin assay in a range of noodle products. Secondly, this method has been applied to flour as well as commercial and laboratory noodles in order to document the riboflavin status of this popular group of foods. In addition, the levels of riboflavin at different stages of processing have been measured and related to pH values of the products and ingredient formulation.

9.2 Selection of a method for riboflavin assay

Many different approaches have been applied for analysis of riboflavin (Section 4.5). HPLC procedures typically offer the advantage of reliability. In addition, they can be carried out rapidly, economically and consequently are applicable to routine determination of riboflavin (Ellefson 1985a). Precision is known to be generally very good for these analytical procedures. HPLC methods have been widely applied in determining the vitamin contents in many foods including fruits and vegetables as well as dairy and cereal-based products (Finglas and Faulks 1987) and therefore were chosen for evaluation in this study.

Another issue of relevance is that riboflavin naturally occurs in biological tissues as protein-bound flavin adenine dinucleotide and flavin mononucleotide (Cooperman and Lopez 1991; Combs 1992). Therefore in procedures for total riboflavin analysis thorough sample preparation is necessary and this typically involves hydrolysis as well as enzymatic digestion to release the riboflavin from the matrix (Lambert and DeLeenheer 1992).

9.3 Laboratory assessment of HPLC procedures for riboflavin

From the preliminary studies (Chapter 7) clear chromatograms were obtained for the series of six vitamins when standards were injected into the HPLC (Figure 7.1). The best results were obtained with a Waters Spherisorb ODS 2 C-18 column and a mobile phase based upon MeOH:H_2O (15:85) containing hexanesulphonate (0.005 M), acetic acid (2.4 percent) and triethylamine (0.5 percent) (pH 3.6) (Albalá-Hurtado and others 1997). In the earlier work clear, symmetrical peaks were obtained for riboflavin and these were readily separated from the other vitamins studied. Hence the conditions described there were used as a basis for developing a method for the specific analysis of riboflavin.

Peaks of riboflavin had been detected at a wavelength of 268 nm based upon the work of Albalá-Hurtado and others (1997) (Figure 7.1). A variety of different wavelengths have been suggested in the literature and in addition, fluorescence is also used because it is regarded as a relatively sensitive technique for detection of riboflavin.

Firstly, in order to select a suitable wavelength giving the maximum response, a solution of riboflavin was prepared in 0.01 M H_2SO_4 at a concentration of approximately 5 μg/mL. This was placed into a quartz cuvette in the double beam spectrophotometer and the absorption spectrum recorded against a reference cell containing the blank of 0.01 M sulphuric acid. The results are presented in Figure 9.1 and these show four absorption maxima for riboflavin with a relatively sharp peak occurring at wavelengths of 266 to 268 nm. In addition, a multichannel Varian Polychrom detector was used with the HPLC and a series of wavelengths compared for their response when the riboflavin standard was injected. Wavelengths of 244, 263, 268,

282 and 292 nm have been commonly applied for vitamins in the scientific literature and therefore these were assessed. Peaks were observed for riboflavin for wavelengths of 244, 263 and 268 nm, however, the highest peak area counts were observed for 268 nm and so this was routinely used in the current study.

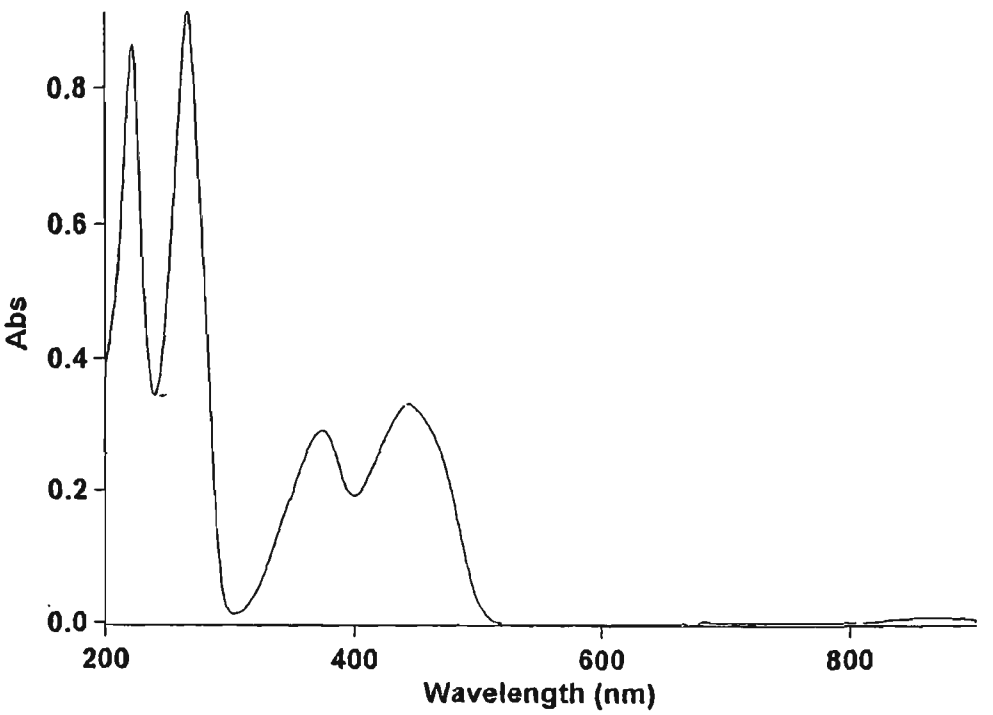


Figure 9.1 The absorption spectrum of riboflavin
Note Riboflavin was dissolved in sulphuric acid (0.01 M) and the spectrum determined with this solvent as reference

In order to further assess the suitability of the HPLC method described earlier, extracts of the AACC reference sample and the strong bakers flour were prepared using the TCA procedure of Albalá-Hurtado and others (1997) which was originally applied to liquid and powdered milk products. Typical chromatograms obtained here are shown in Figures 9.2 and 9.3 respectively. The AACC sample (Figure 9.2) is a fortified breakfast cereal product and for this a clear symmetrical peak was found corresponding to the retention time of standard riboflavin (38.3 min). Furthermore, the peak was well separated from all others in the chromatogram. No corresponding riboflavin peak was found for the bakers flour (Figure 9.3). Based upon these results this HPLC procedure appeared to show potential for further studies of extraction methods specifically suitable for samples of flour and flour-based foods.

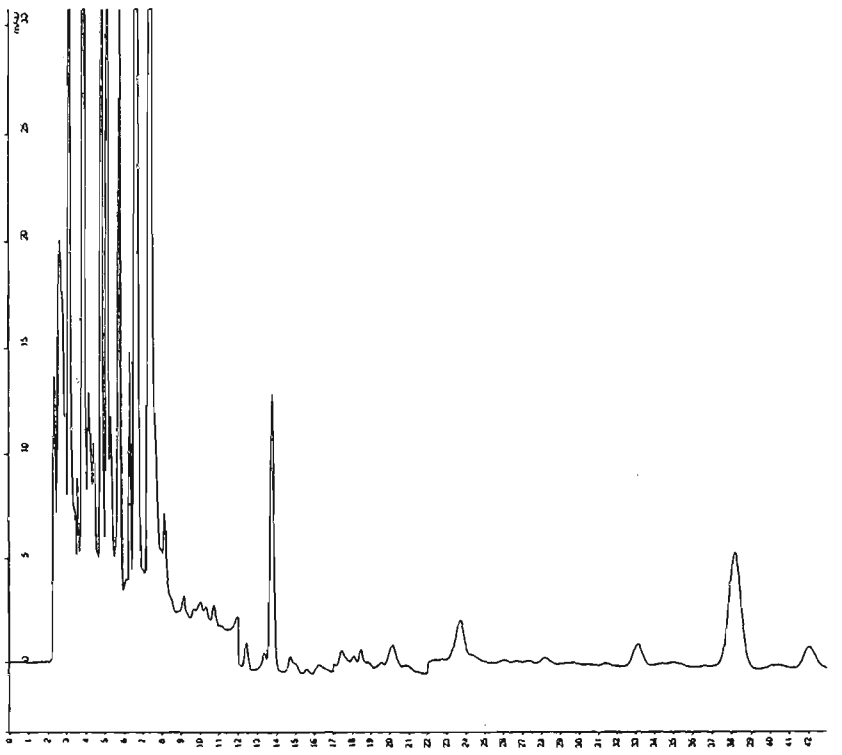


Figure 9.2 **Chromatogram obtained for riboflavin when AACC reference sample was analysed using the method of Albalá-Hurtado and others (1997)**
Note Riboflavin eluted at a retention time of 38.3 min

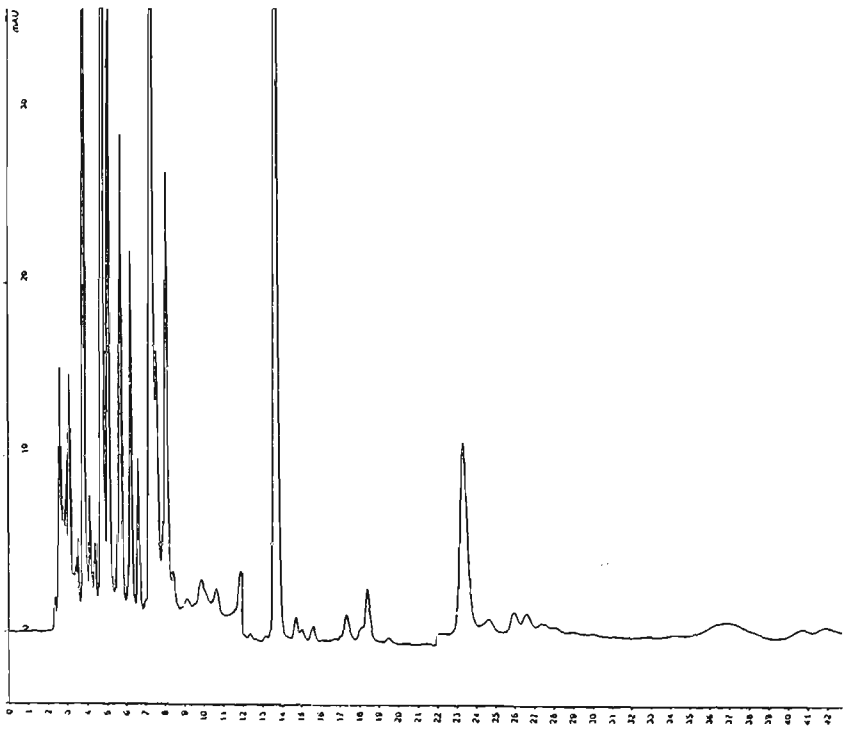


Figure 9.3 **Chromatogram obtained for riboflavin when bakers flour sample was analysed using the method of Albalá-Hurtado and others (1997)**
Note No peak corresponding to riboflavin was found for the bakers flour

9.4 Extraction of riboflavin using the method of Ndaw and co-workers

A significant issue in the optimisation of the procedure for HPLC analysis of riboflavin is extraction. Published approaches for extraction include steps of autoclaving in mild acidic conditions typically in combination with subsequent enzymatic hydrolysis. As virtually all available methods include the acid and autoclaving step this was adopted. The conditions used were 121 °C, 0.1 M HCl for 20 min. This was used in combination with three different digestion methods designed to release riboflavin from the food matrix. These procedures are more fully described in Section 6.9.3.

The enzymatic treatment proposed by Ndaw and others (2000) was applied to samples of strong bakers flour and the AACC reference sample. The three enzymes required for this were amylase, protease (in the form of papain) and phosphatase. These were used in conjunction with different sets of reagents. The resulting extracts were then subjected to HPLC using the parameters and conditions that had already been evaluated in this study. The details of the reagents as well as the results obtained are presented in Table 9.1.

In the case of both procedures, no peaks could be detected for riboflavin in the flour sample. This indicates either that the naturally occurring levels of riboflavin were too low to be detected or alternatively that release of the riboflavin from the food matrix was not achieved by either extraction treatment. The results for peak areas obtained for the AACC reference sample were then compared with calibration curves obtained using solutions of standard vitamins of varying concentrations.

Table 9.1 A comparison of riboflavin results obtained for flour and reference sample extracted using the procedures of Ndaw and others (2000)

Procedure used	Sample	Peak area	Riboflavin content
3 enzymes added plus glutathione	Bakers flour	No peak obtained	None found
	AACC reference	71581	55.6
		72295	56.1
		72152	56.0
		71432	55.5
	Mean	**71865 ± 422**	**55.8 ± 0.3**
As above with glyoxylic acid and ferrous sulphate treatment added	Bakers flour	No peak obtained	None found
	AACC reference	70660	54.9
		63864	49.6
		63471	49.3
		64585	50.1
	Mean	**65645 ± 3375**	**51.0 ± 2.6**

Notes
1 Two separate samples were weighed and extracted for each extraction method
2 The extraction conditions are described as Trial 3 in Section 6.9.3 and follow the specific details originally published by Ndaw and others (2000)
3 Data are expressed on an as is moisture basis
4 Riboflavin content are expressed in units of mg/kg and mean values are expressed as mean ± sd

Over a period of some months a series of standard curves were produced for quantitation of riboflavin in noodle samples. For these, standard solutions were generally prepared to cover the range of 0.25 to 5.0 μg/mL. An example of a standard curve is presented in Figure 9.4 and this represents the curve used in the calculation of data in Table 9.1. Some of the standard curves obtained during the development of the method are summarised in Table 9.2. This shows that excellent correlations were obtained. However, considerable variation in the equations was observed, possibly reflecting the varying conditions of temperature and mobile phases applied during these preliminary studies. Hence, a fresh set of standard solutions was prepared and chromatographed along with each batch of samples analysed during this study.

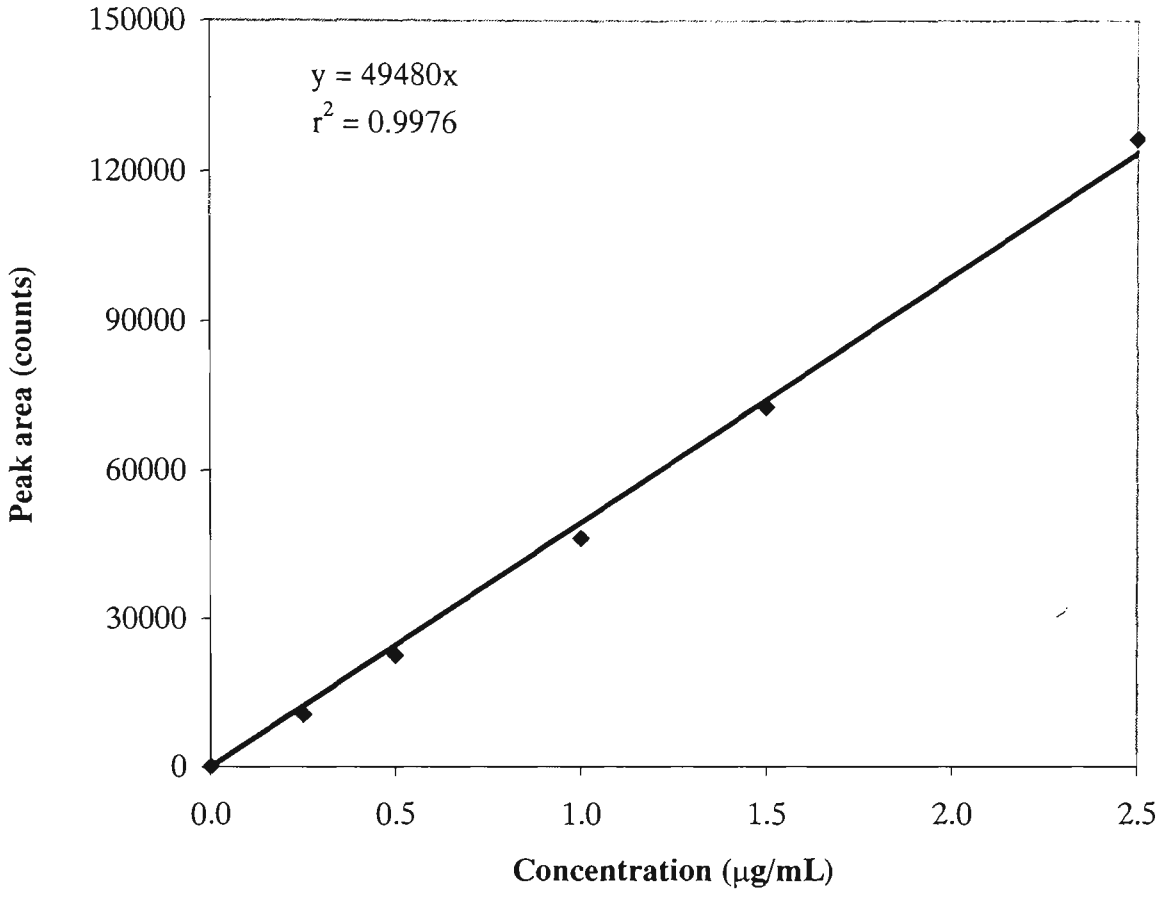


Figure 9.4 A typical standard curve obtained for riboflavin using HPLC analysis

Table 9.2 Description of standard curves obtained during HPLC analysis for riboflavin

Equation	r^2
y = 51707x – 245.15	1.0000
y = 7545.9 x	0.9967
y = 79853x - 2599	0.9998
y = 49480 x	0.9976
y = 52664x + 312.99	1.0000
Mean	**0.9986 ± 0.0015**

Notes 1 In the equations x represents the concentration of the riboflavin and y is the peak area (compare with Figure 9.4)
2 The mean value is expressed as mean ± sd

The results calculated for riboflavin contents of the AACC sample are similar to those obtained by other laboratories and provided with the sample (Table 6.2). The average values for both are close to the average specified and also fall well within the specified range. The results also show good repeatability for both duplicate injections and for duplicate extractions, for the two extraction procedures. It is noted that better results were obtained for the procedure without glyoxylic acid/ferrous sulphate treatment, both in terms of lower standard deviation and closeness to the specified mean values.

Although good quantitative results were found here for the reference sample, the procedures did not allow direct measurement of the levels of riboflavin in unfortified flour samples. Accordingly further evaluations were carried out using alternative extraction methods.

9.5 Extraction of riboflavin using the method of Chase and co-workers

A sample preparation method involving perchloric acid digestion was proposed by Chase and others (1993b) and applied to medical foods and infant formulae. Following digestion for 30 min, extracts were adjusted to pH 3.2 and allowed to stand prior to filtration (see Section 6.9.3). Typical chromatograms for bakers flour and the AACC sample are presented in Figures 9.5 and 9.6 respectively and the results are summarised in Table 9.3.

The results indicate that no riboflavin could be detected in the extracts of flour. In addition, the calculated values for the AACC reference are below the range of results specified for this material. Therefore the procedure did not appear suitable for extraction of flour samples and also gave results below those expected for the reference sample known to have been fortified with riboflavin.

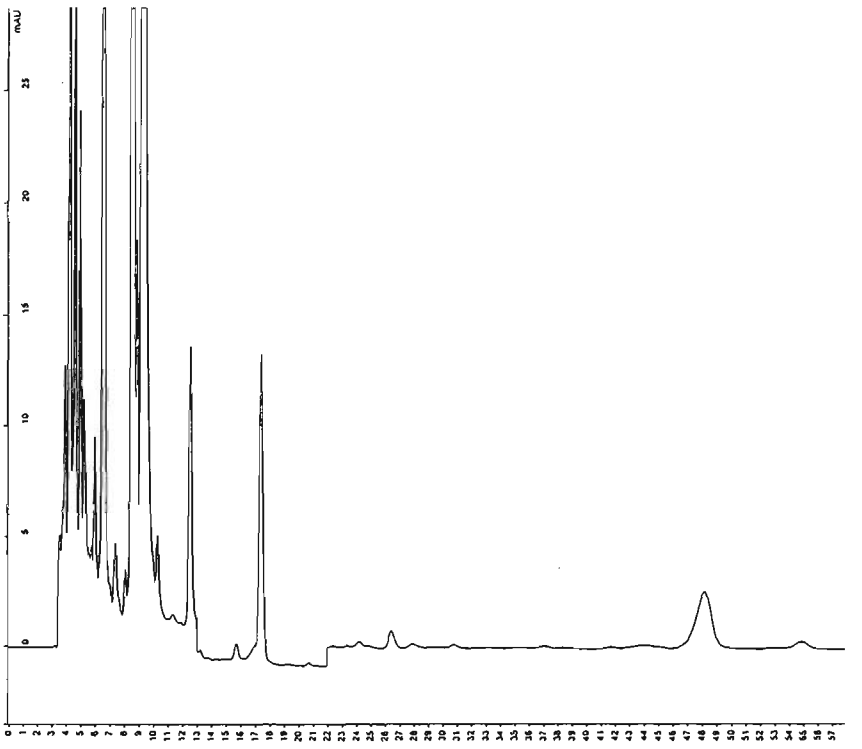


Figure 9.5 **Chromatogram obtained for riboflavin when bakers flour sample was analysed using the method of Chase and others (1993b)**
Note No corresponding riboflavin peak was found for the bakers flour at a retention time of 43.2 min

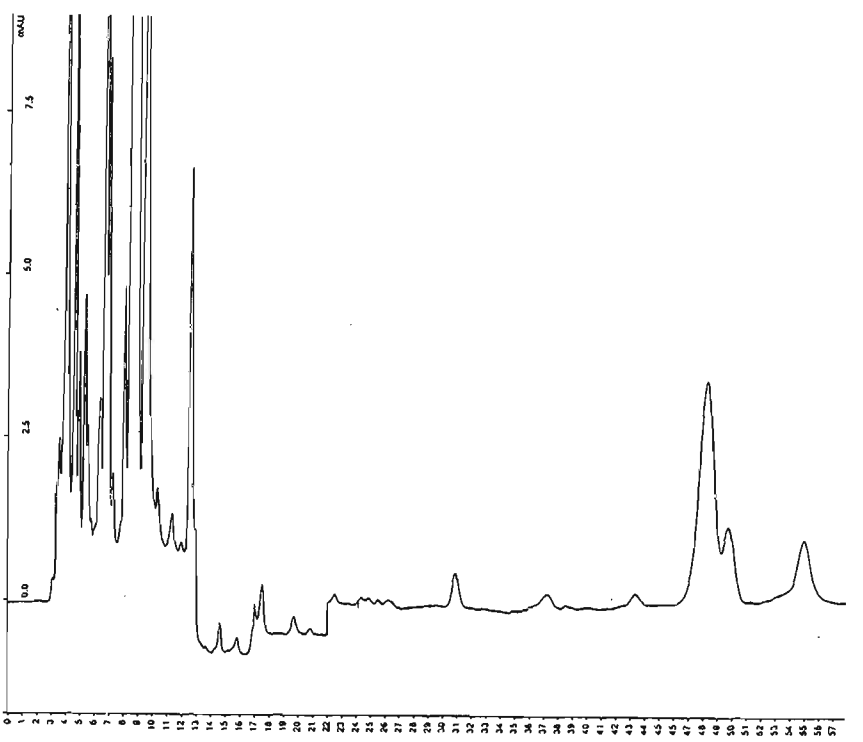


Figure 9.6 **Chromatogram obtained for riboflavin when AACC reference sample was analysed using method of Chase and others (1993b)**
Note Riboflavin eluted at a retention time of 43.2min

Table 9.3 A comparison of riboflavin results obtained for different extracts of flour and reference sample based on the procedure of Chase and others (1993b)

Procedure used	Sample	Peak area	Riboflavin content
Samples digested with perchloric acid, extracts adjusted to pH 3.2 and filtered	Maximus flour	No peak obtained	None found
	AACC reference	31065	42.2
		31804	43.1
	Mean	**31435 ± 522**	**42.6 ± 0.7**

Notes 1 Results are for two different extractions
2 Riboflavin contents are expressed in units of mg/kg on an as is moisture basis
3 Mean values are presented as mean ± sd

9.6 Extraction and analysis of samples by the method of Brubacher and co-workers

The procedure of Brubacher and others (1985c) was originally proposed for use throughout the European Union for analysis of riboflavin and applicable to foods generally. It is based upon autoclaving an acidified sample followed by digestion using the commercial enzyme preparation Clara-diastase (see Section 6.9.3). It is noted that this commercial enzyme preparation probably contains a range of activities which effectively breakdown food matrices and release riboflavin for analysis (Brubacher and others 1985c; Valls and others 1999). The use of a single standard solution which is subjected to the same treatment as sample extracts, has been recommended for quantitation purposes (Brubacher and others 1985c). This approach was applied to samples of bakers flour and AACC reference for evaluation purposes. The results obtained when multiple extracts were prepared and analysed over a two week period are presented in Table 9.4.

These data indicate that relatively consistent results were obtained for a series of different extracts of each sample. Whereas no detectable riboflavin peaks were obtained for the bakers flour using the procedures of Ndaw and co-workers and Chase and co-workers, with the Clara-diastase extracts, clear peaks were found and when quantitated

according to the approach described by Brubacher and others (1985c), the average result was 2.1 mg/kg. This can be compared with literature values (Table 9.5) where a range of results have been published. The variation seen in the data may result from differences in analytical methodology used or from variations in levels due to environmental or genotypic factors. It is also known that milling extraction rate directly influences nutrient composition in wheat flour (Simmonds 1989). The mean value of 2.1 obtained here (Table 9.4) is similar to the figure of 1.5 mg/kg reported for flour in Australian food composition tables (English and others 1990).

Table 9.4 Riboflavin results obtained for different extracts of flour and reference sample prepared by the procedure of Brubacher and others (1985c)

Description	Bakers flour	AACC reference
Time span of analyses	5 weeks	5 weeks
Number of replicates	22	22
Mean value	2.1	62.1
Range of results	1.6-3.0	51.0-73.4
Standard deviation	0.46	8.2
Coefficient of variability (%)	22	13

Notes 1 Riboflavin data presented as mean ± sd and are expressed in unit of mg/kg on an as is moisture basis
2 Each result represents an individual extraction

The data for the AACC reference (Table 9.4) shows that for each extraction, the results are within the specifications for the sample (Table 6.2). This, along with the fact that riboflavin was detected in the flour extracts and gave results similar to those that might be expected indicates that the approach used here warranted further consideration and validation.

Table 9.5 Riboflavin contents of wheat flours reported in various food composition tables

Flour sample	Riboflavin content	Reference
Flour, wheat, white, plain (not enriched with added B vitamins or iron)	1.5	English and others 1990
Wheat flours (two types)	0.7 - 1.7	Scherz and Senser 2000
White (plain and bread making)	0.3	Holland and others 1991
Wheat flour (five types, all enriched)	4.14 - 5.12	USDA ARS 2002

Note Values are expressed as mg/kg on an as is basis and have been recalculated to allow direct comparison with data in Table 9.4.

9.7 Optimisation of riboflavin extraction and analysis

Having established the general suitability of the extraction procedure of Brubacher and others (1985c) (Section 6.9.5) when used in conjunction with the HPLC method adapted earlier in this project, further work was undertaken to optimise the analysis of riboflavin. Firstly, in order to enhance the relative size of riboflavin peaks obtained in chromatograms, the recommended procedure of Brubacher and others was modified so that the extracts were not diluted 1:1. This resulted in peaks having double the area thereby facilitating quantitation. A second step taken to validate the method was then to prepare, treat and chromatograph extracts of standards over a number of weeks. Procedures are described in Section 6.9.5-6.9.7 and the results presented in Table 9.6.

Upon repeated extraction and analysis of the standard riboflavin solution, the overall stability of the method was found to be good. The peak area values obtained showed relatively little variation over a period in excess of two months. Furthermore, the retention time was quite consistent over the same time period. This provided confirmation of the general robustness of the approach used.

Table 9.6 A comparison of peak areas obtained for riboflavin standards prepared using the method of Brubacher and co-workers and chromatographed over a period of two months

Description	Peak area (counts)	Retention time (min)
Time span of analyses	2 months	2 months
Number of replicates	28	28
Mean value	9047	33.1
Range of results	8202-10835	32.0-34.0
Standard deviation	715	0.68
Coefficient of variability (%)	7.9	2.1

A further series of trials were also undertaken in which the flow rate and the composition of the mobile phase were varied. The details of these trials are summarised in Table 9.7 along with the retention times found for riboflavin. Chromatograms are also presented for these trials (Figures 9.7 to 9.15, compare with Table 9.7).

Mobile phases varying in composition have been used in the HPLC analysis of riboflavin. Many of these are based upon MeOH and water and the inclusion of ion pairing reagents has often been recommended. It is noted that the mobile phase originally detailed by Brubacher and others (1985c) included dioxane and this was not considered for use in the current study for safety reasons.

Whilst good separations of riboflavin were obtained with MeOH-water, increased flow rates and higher MeOH concentrations were evaluated. In Trial 1 the flow rates and MeOH concentrations were increased. A sharp symmetrical peak was found to elute at approximately 7.5 min. It is noted that when riboflavin standard was treated with enzyme, a variety of peaks were found in the chromatogram and this was a result of the enzyme treatment because the standard alone gave a single peak. Bakers flour also showed many peaks and riboflavin co-eluted with other components under these conditions (Figure 9.8).

Table 9.7 The mobile phases and flow rates used in the evaluation and optimisation of parameters for HPLC analysis of riboflavin

Trial number	Composition of mobile phase	Flow rate (mL/min)	Retention time of riboflavin (min)
1	Solution A (85%) Solution B (15%) Overall MeOH concentration 30%	1.5	7.55 (see Figures 9.7, 9.8)
2	Solution A (85%) Solution B (15%) Overall MeOH concentration 30%	1.0	11.3 (see Figure 9.9)
3	Solution A (90%) Solution B (10%) Overall MeOH concentration 25%	1.0 for first 15 min, and 0.7 thereafter	17.6 (see Figures 9.10, 9.11)
4	Solution A only	0.7	50.2 (see Figures 9.12, 9.13)
5	Solution A only	1.0	33.3 (see Figures 9.14, 9.15)

Notes 1 Solution A was prepared from an aqueous solution of hexanesulphonate (0.005 M), triethylamine (0.5%), glacial acetic acid (2.4%). This was mixed with MeOH (final MeOH-water ratio of 15:85 v/v)
2 Solution B was MeOH

Mobile phases varying in composition have been used in the HPLC analysis of riboflavin. Many of these are based upon MeOH and water and the inclusion of ion pairing reagents has often been recommended. It is noted that the mobile phase originally detailed by Brubacher and others (1985c) included dioxane and this was not considered for use in the current study for safety reasons.

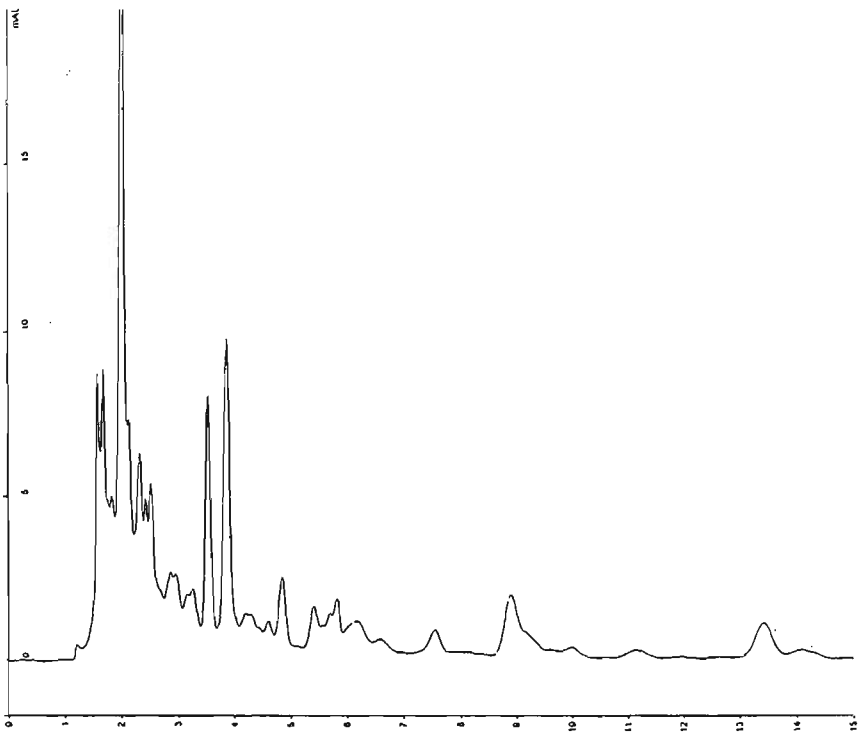


Figure 9.7 **Chromatogram obtained for standard riboflavin using Trial number 1 (refer Table 9.7) following the method of Brubacher and others (1993c)**
Note Riboflavin eluted at a retention time of 7.6 min and other peaks are attributed to the enzyme preparations used for extraction

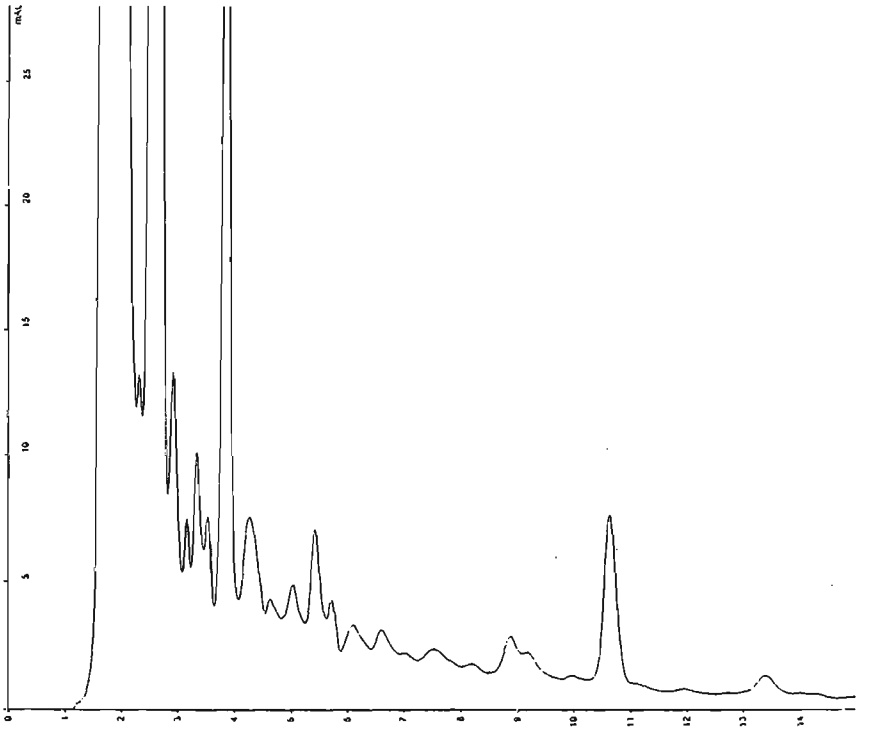


Figure 9.8 **Chromatogram obtained for bakers flour sample using Trial number 1 (refer Table 9.7) following the method of Brubacher and others (1993c)**
Note Riboflavin eluted at a retention time of 7.5 min

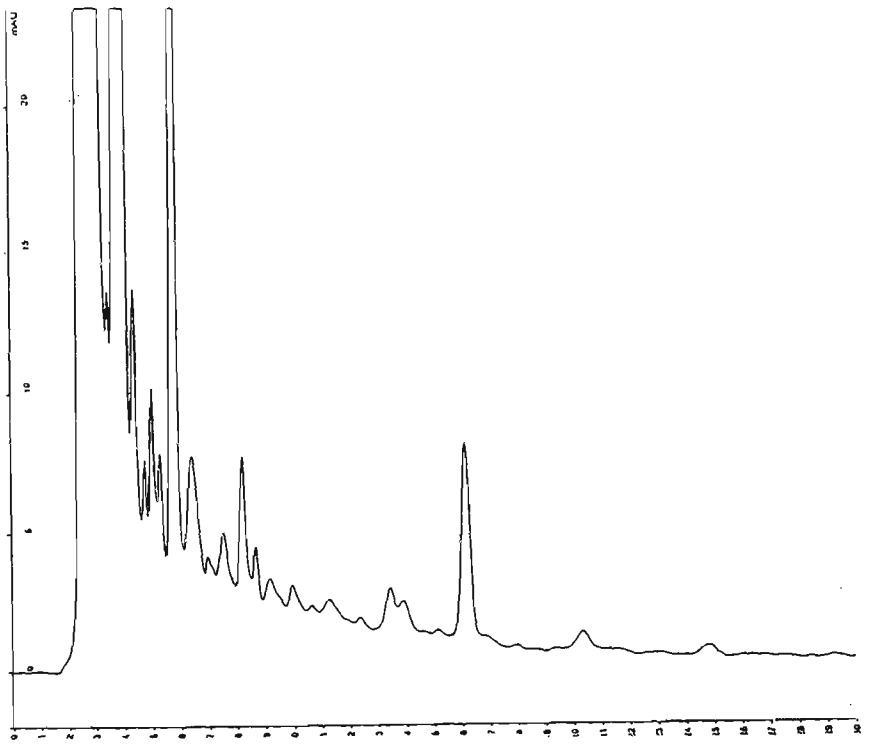


Figure 9.9 **Chromatogram obtained for bakers flour sample using Trial number 2 (refer Table 9.7) following the method of Brubacher and others (1993c)**
Note Riboflavin eluted at a retention time of 11.3 min

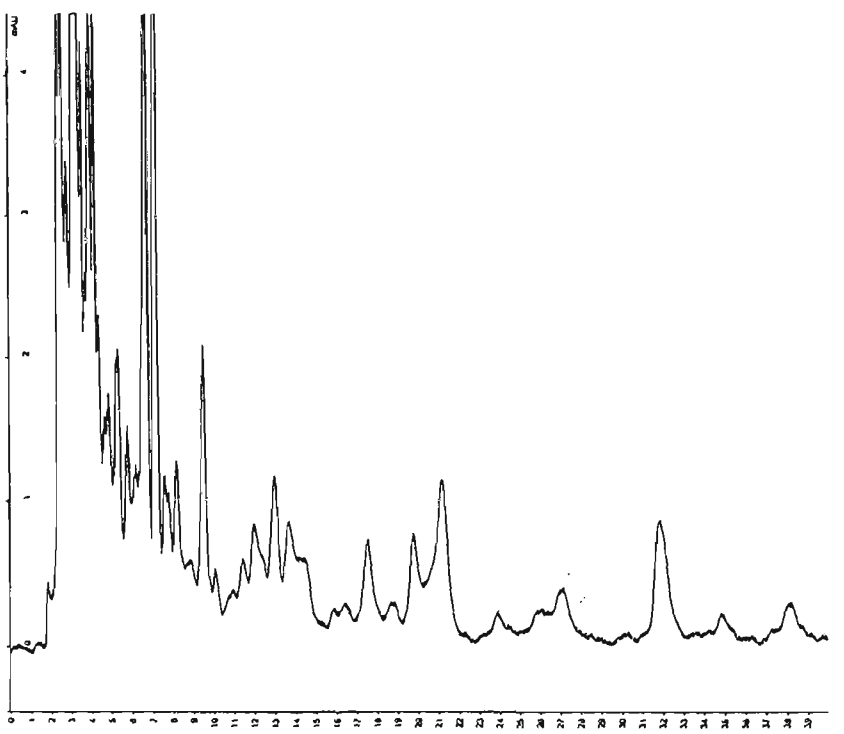


Figure 9.10 **Chromatogram obtained for standard riboflavin using Trial number 3 (refer Table 9.7) following the method of Brubacher and others (1993c)**
Note Riboflavin eluted at a retention time of 17.6 min

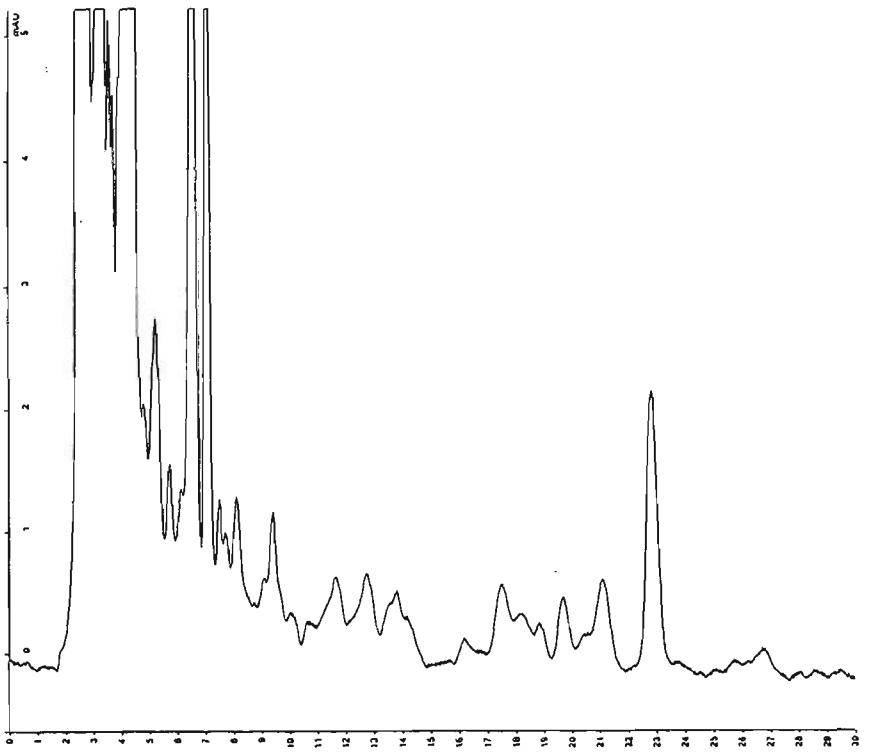


Figure 9.11 **Chromatogram obtained for AACC reference sample using Trial number 3 (refer Table 9.7) following the method of Brubacher and others (1993c)**
Note Riboflavin eluted at a retention time of 17.5 min

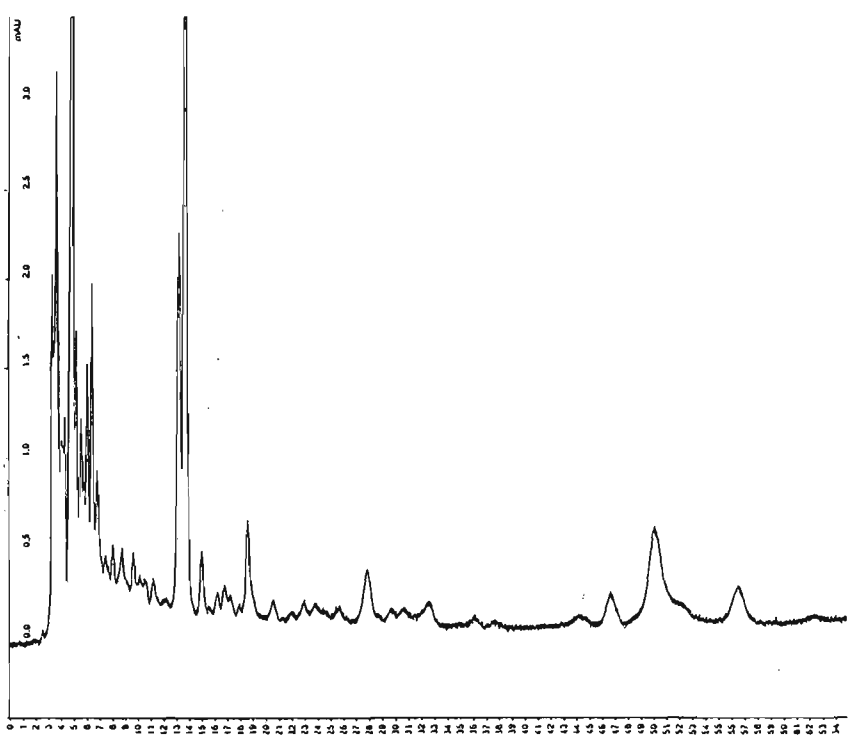


Figure 9.12 **Chromatogram obtained for standard riboflavin using Trial number 4 (refer Table 9.7) following the method of Brubacher and others (1993c)**
Note Riboflavin eluted at a retention time of 50.2 min

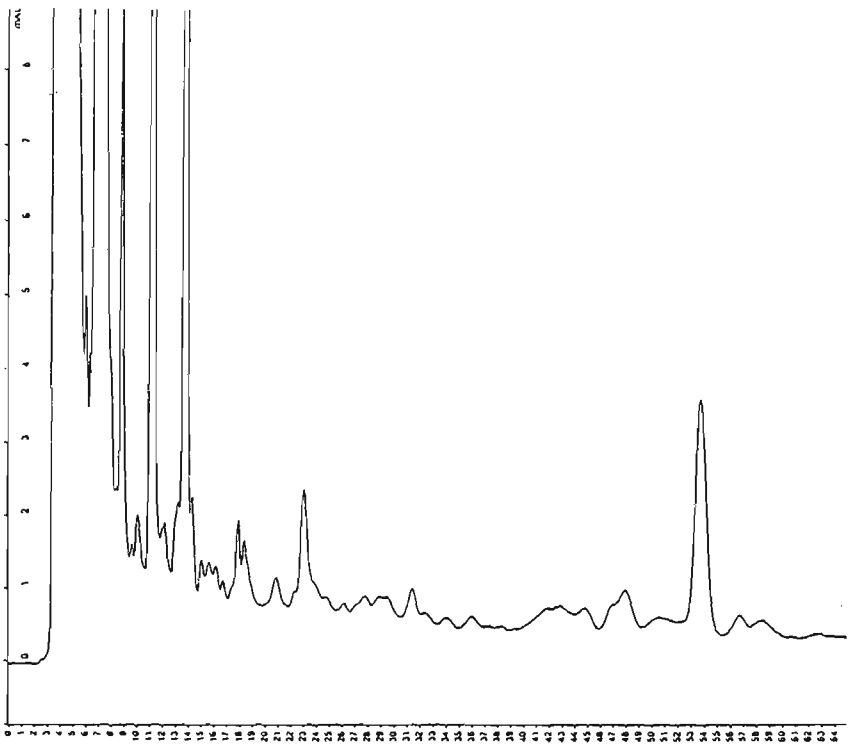


Figure 9.13 **Chromatogram obtained for bakers flour sample using Trial number 4 (refer Table 9.7) following the method of Brubacher and others (1993c)**
Note Riboflavin eluted at a retention time of 50.6 min

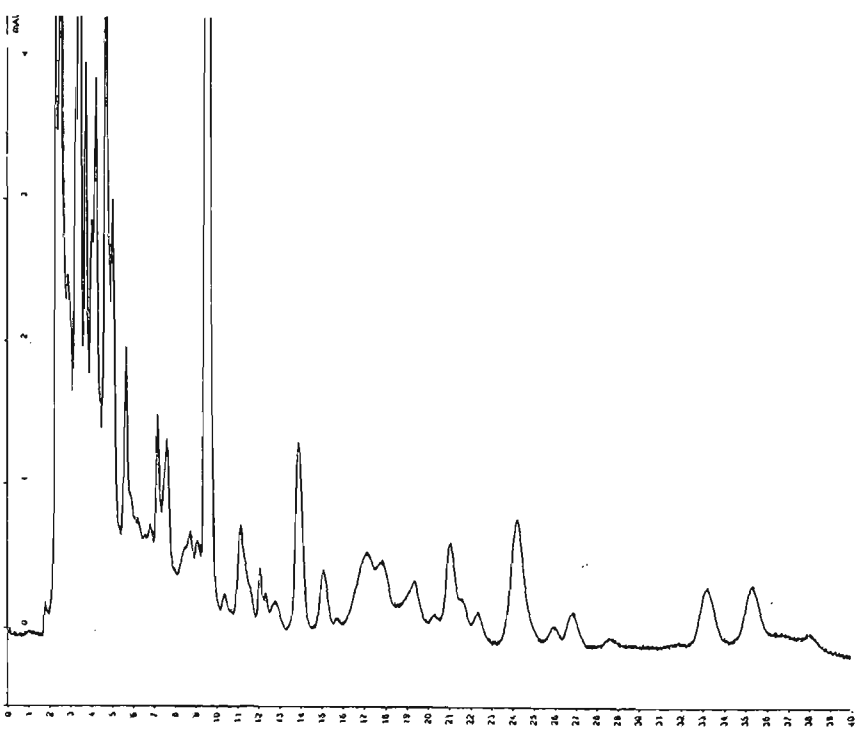


Figure 9.14 **Chromatogram obtained for standard riboflavin using Trial number 5 (refer Table 9.7) following the method of Brubacher and others (1993c)**
Note Riboflavin eluted at a retention time of 33.3 min

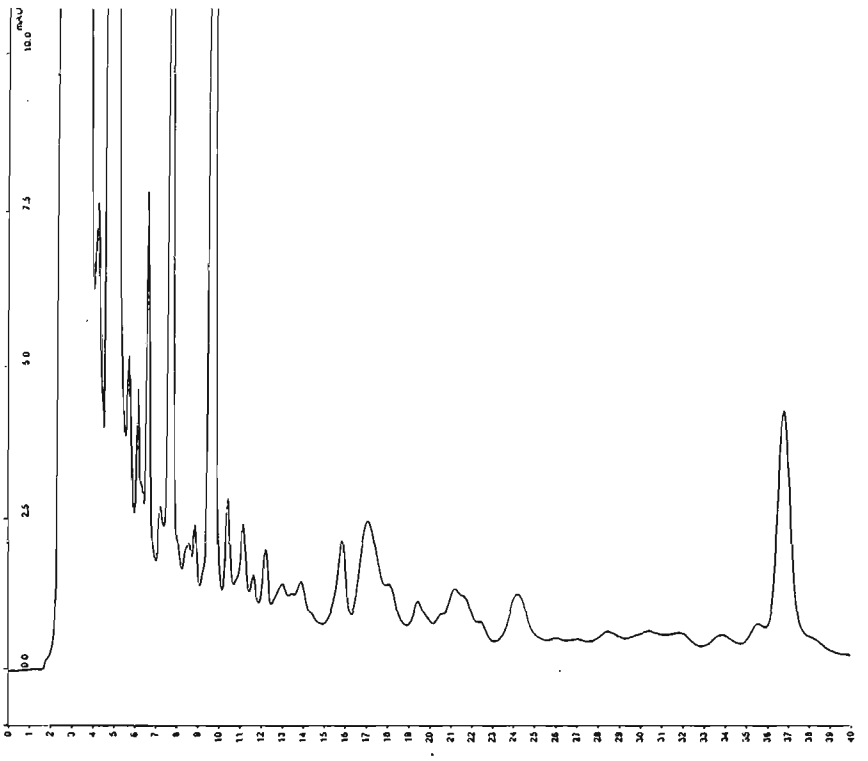


Figure 9.15 **Chromatogram obtained for bakers flour sample using Trial number 5 (refer Table 9.7) following the method of Brubacher and others (1993c)**
Note Riboflavin eluted at a retention time of 33.8 min

Whilst good separations of riboflavin were obtained with MeOH-water, increased flow rates and higher MeOH concentrations were evaluated. In Trial 1 the flow rates and MeOH concentrations were increased. A sharp symmetrical peak was found to elute at approximately 7.5 min. It is noted that when riboflavin standard was treated with enzyme, a variety of peaks were found in the chromatogram and this was a result of the enzyme treatment because the standard alone gave a single peak. Bakers flour also showed many peaks and riboflavin co-eluted with other components under these conditions (Figure 9.8).

When the same mobile phase was used in conjunction with a low flow rate, the retention time was longer (Trial 2, Figure 9.9) but the separation of riboflavin was insufficient to allow quantitation.

In Trial 3, a lower methanol concentration was combined with lower flow rates but the conditions did not result in satisfactory resolution of riboflavin. Therefore it was

necessary to limit the methanol concentration to 15 percent. In comparing flow rates of 0.7 and 1.0 mL/min the separations were good in both cases and no advantage was obtained by using the lower flow rate (Figures 9.12 - 9.15). Accordingly it was concluded that the HPLC conditions and parameters used in Trial 5 (Table 9.7) were suitable for this study.

9.8 Analysis of riboflavin contents of flours and Asian noodle samples

In further preliminary trials, selected commercial dried Asian noodle samples were procured and analysed, along with a commercial flour chosen as one having processing characteristics suitable for the preparation of both white salted and instant styles of product. For the analyses of the dried noodles, the products were first ground to a fine powder prior to weighing and enzyme treatment. In the case of each noodle sample and the flour, detection and quantitation were relatively difficult. For various extracts of the noodles the resulting chromatograms were difficult to interpret. In many cases an additional peak (retention time of 32.9 min) overlapped partially with that of riboflavin (retention time of 32.3 min). In order to facilitate quantitation of the riboflavin it was necessary to re-integrate peaks in the chromatograms. For this the integration parameters were varied for the riboflavin peaks in both sample extracts and also for standards. In some cases it was simply not possible to quantitate the riboflavin. Where the values could be used these were calculated and are presented in Table 9.8.

Table 9.8 Riboflavin contents of P farina flour and two dried commercial noodle samples

Sample	Moisture content	Riboflavin content		Number of analyses
		as is	dry weight	
P farina	12.6	1.24 ± 0.12	1.41 ± 0.14	7
Noodle sample 1	14.4	0.98 ± 0.20	1.14 ± 0.24	4
Noodle sample 2	21.5	0.87 ± 0.17	1.10 ± 0.21	6

Notes 1 Riboflavin data are expressed as mg/kg on both an as is basis and a dry weight basis
2 Mean values are expressed as mean ± sd

The results for the P farina flour are lower than for the bakers flour analysed earlier (Table 9.4), but are within the range of values reported for flours in food composition tables (Table 9.5). It is noted that the chromatograms obtained for some extracts of the P farina flour could not be readily quantified. Based upon these observations, it appears that riboflavin contents of around 1.0 mg/kg represented the lower limit for quantification in flour samples using the procedures employed here.

For the three commercial noodle samples, only two provided chromatograms which allowed quantitation. These two noodles showed similar riboflavin contents (Table 9.8) although in both cases some chromatograms could not be used for calculation even when re-integration was attempted. Again these observations indicated that riboflavin contents below 1.0 mg/kg could not be readily quantitated for noodle samples using these methods.

Based upon these results consideration was given to establishing a model system in which noodles were fortified with riboflavin. For this, a known amount of the vitamin was added into the product formulation following dissolution in the water to be used in preparing the noodle dough. It is noted that the riboflavin solution was prepared separately from the kansui solution such that the riboflavin remained at neutral pH until all of the ingredients were combined, immediately prior to commencement of mixing. This procedure was applied to the preparation of both yellow alkaline and instant noodles in the laboratory.

In establishing a model system for the study of riboflavin stability in noodles consideration was also given to the levels that might be added. In fortification, it is common practice to add an amount which corresponds to one quarter of the RDI for the specific nutrient per serving of a food. Current Australian regulations (Food Standards ANZ 2002) regarding claims of addition refer to 0.4 mg of riboflavin per serving and a serving corresponding to 35 g of dried noodles as purchased. Therefore this amount was used as a basis for the level of addition into the noodle formulations.

Samples of the three styles were prepared using the formulations described in Section 6.3 along with the addition of riboflavin. The resultant noodles were analysed for

riboflavin contents to again establish the suitability and reliability of the extraction and analysis procedures described above.

A further preliminary evaluation was undertaken using dried fortified noodles. Extraction could be achieved using either ground noodles or by high-speed maceration of intact noodles with the Ultra-Turrax. One possible concern with mechanical grinding of noodles could be overheating which might impact upon vitamin levels. The problem would not arise where a pre-cooled solution of acid was used for maceration. A direct comparison of the results obtained by these two alternate procedures is provided in Table 9.9. This data shows that no significant difference was obtained between the two approaches. As the Ultra-Turrax offered a more convenient method, this was used for all dried samples in this study.

Table 9.9 Comparison of using grinder and Ultra-Turrax homogeniser for extraction of riboflavin from dried fortified noodle samples

Sample	Ground	Ultra-Turrax homogeniser
White salted noodles	10.38 ± 0.03	10.33 ± 0.47
Yellow alkaline noodles	9.69 ± 0.26	9.93 ± 0.17

Notes 1 For each noodle style six sub-samples were analysed
2 Riboflavin data are presented as mean ± sd and are expressed in units of mg/kg on a dry weight basis

The riboflavin values then obtained for a series of separate batches of dried noodles are presented in Table 9.10. The results show that there was considerable difference in the riboflavin contents for different style of noodles. There was also some variation from day to day for each analysis. The variability was greater as reflected in the coefficient of variability values for the yellow alkaline noodles which had the higher levels of alkaline salt in the ingredients. The coefficients for these noodles can be compared with published repeatability values. In the ICC standard (1990), it was noted that coefficients of up to 13 percent might be found for vitamin determinations. Therefore, based on the

data in Table 9.10, it was concluded that the level of repeatability is within reasonable and normal levels. Hence the procedure was used for all further studies in this project.

Table 9.10 The repeatability of analyses for riboflavin in dried white salted, yellow alkaline and fried instant noodles

	White salted noodles	Yellow alkaline noodles	Instant noodles
Time span of analyses	6 weeks	6 weeks	2 weeks
Number of batches of noodles analysed	5	5	2
Number of analyses	17	11	8
Mean riboflavin content	11.3	9.1	9.4
Standard deviation	0.62	0.76	0.32
Coefficient of variability (%)	5.49	8.39	3.37

Notes 1 Riboflavin data are expressed as mg/kg on a dry weight basis
2 Each analysis was carried out on an individual extract

In using the method it is noted that particular care was taken to apply uniform technique particularly for the extraction step. In addition, for all analyses, the riboflavin reference standard was analysed along with samples. The purpose of this was to assess the reproducibility of the peak area given by the instrument. These results again indicate good repeatability and show that the performance of the HPLC in conjunction with the extraction method of Brubacher and others (1985c) was satisfactory for this study.

9.9 The influence of noodle processing on riboflavin losses

The next aim was to study the influence of processing and the impact of processing parameters on riboflavin losses after fortification. The riboflavin contents of white salted, yellow alkaline and instant noodles at each stage of processing were analysed and are shown in Tables 9.11 and 9.12.

Table 9.11 Riboflavin contents at different stages during processing of white salted and yellow alkaline noodles

	White salted noodles	Yellow alkaline noodles
Flour	1.41 ± 0.1	1.41 ± 0.1
Vitamin added to formulation	13.7	13.7
Total vitamin level in ingredients	15.1 ± 0.1	15.1 ± 0.1
Dough	13.8 ± 0.4	13.3 ± 0.4
Dried noodles	11.0 ± 0.3	9.6 ± 0.4
Cooked noodles	7.1 ± 0.2	3.9 ± 0.2

Notes 1 Riboflavin data are expressed as mg/kg on a dry weight basis
2 The amounts added to flour are calculated values based upon the known concentration and volume of riboflavin used

The results show that relatively little loss occurred for white salted noodles at each step from flour to dough to the dried noodles. However, almost half of the amount of total riboflavin was lost during the cooking of the dried prepared noodles. In the case of yellow alkaline noodles, there was clearly loss of riboflavin at each stage of noodle processing. Large losses occurred during the preparation before cooking and there was also significant loss during cooking with retention of less than one fourth of the amount of riboflavin originally present in the ingredients. The results of riboflavin analysis of samples taken during instant noodle preparation are shown in Table 9.12.

The results indicate that relatively little riboflavin was lost at the dough preparation stage, with some loss upon steaming and greater loss during the frying step. Besides thermal decomposition, losses of riboflavin may have taken place due to the water solubility of the vitamin. More than half of the total level of riboflavin was lost during cooking of the instant noodles.

Table 9.12 Riboflavin contents at different stages during processing of instant noodles

	Riboflavin content
Flour	1.41 ± 0.1
Vitamin added to formulation	13.7
Total vitamin level in ingredients	15.1 ± 0.1
Dough	13.4 ± 0.3
Steamed noodles	13.0 ± 0.3
Fried noodles	9.3 ± 0.3
Cooked noodles	7.3 ± 0.4

Notes 1 Riboflavin data are expressed as mg/kg on a dry weight basis
2 The amounts added to flour are calculated values based upon the known concentration and volume of riboflavin used

The cumulative losses of riboflavin at each stage during the processing of three different styles of Asian noodles are presented in Table 9.13. Again, the data showed the presence of alkaline salt at high level in the ingredients of yellow alkaline noodles affected the stability of riboflavin. Losses of up to 71 percent of riboflavin were found in yellow alkaline noodles after cooking compared to approximately 53 percent loss found in white salted and instant noodles. The result of white salted, yellow alkaline and instant noodles, as a final product before cooking showed a 27 percent loss of riboflavin found in white salted and 36 percent in yellow alkaline noodles compared to a total loss of 38 percent in instant noodles. The greater loss may be due to the low level of alkaline salt in the ingredients of instant noodles resulting in a neutral pH, in addition of steaming and frying step during processing.

The lowest retention of riboflavin was found during cooking of yellow alkaline noodles compared to white salted and instant noodles. This might be due to the presence of alkaline salt limiting the liberation of riboflavin into cooking water. The extent of

vitamin loss appears to be dependent on the temperature, the time of the treatment and the contribution of the ingredients in the formulation.

Table 9.13 Relative and cumulative losses of riboflavin at each stage during processing of laboratory noodles

Noodle style	Processing stage			Riboflavin loss	Cumulative riboflavin loss
White salted	Flour	→	dough	8.6	8.6
	Dough	→	dried noodles	18.5	27.1
	Dried noodles	→	cooked noodles	25.8	52.9
Yellow alkaline	Flour	→	dough	11.9	11.9
	Dough	→	dried noodles	24.5	36.4
	Dried noodle	→	cooked noodles	37.7	71.1
Instant	Flour	→	dough	11.3	11.3
	Dough	→	steamed noodles	2.6	13.9
	Steamed	→	fried noodles	24.5	38.4
	Fried noodles	→	cooked noodles	13.2	51.6

Note Losses are expressed as % values in relation to the total amount of riboflavin in the ingredient formulation (from flour plus that added during fortification)

Whilst there is relatively little published data with which to compare the results for Asian noodles, a summary is presented in Table 9.14. Most of the products analysed, including the egg noodles, have durum-based ingredients (compare with Table 3.2 and Section 3.3). The only data relating to processing losses reported (Watanabe and Ciacco 1990) are lower than the results found here for Asian noodles.

The influence of cooking of pasta on riboflavin is very variable although the relative losses are typically higher than those found for the three styles of Asian noodles (Table 9.13). The loss calculated for the Japanese soba product from US food composition data is similar to that found in the current study. However the potential losses which might occur during storage of products is not clear. Accordingly the impact of varying storage

conditions was considered in further experiments on Asian noodles prepared in the laboratory.

Table 9.14 A summary of relevant literature values for riboflavin losses during processing and cooking of pasta and noodle products

	Riboflavin content	Riboflavin loss (%)	Reference
Spaghetti			Watanabe and Ciacco 1990
Loss during processing	-	4	
Loss during cooking	-	51	
Spaghetti	3.6-5.5	36.4-52.8	Ranhotra and others 1983
Noodles	3.4-5.9	35.3-56.4	
Macaroni	2.7-3.3	7.4-45.5	
Spaghetti	3.7-5.0	44 (40-56)	Ranhotra and others 1985
Noodles	3.7-6.5	46 (41-49)	
Macaroni	3.2-4.5	37 (37-41)	
Noodles, eggs enriched	5.3 ± 0.22	50.0	USDA ARS 2002
Noodles, eggs, spinach enriched	5.23 ± 0.09	25.2	
Noodles, Japanese, soba	1.40 ± 0.00	31.0	
Spaghetti, enriched	4.95 ± 0.11	41.8	
Spaghetti, protein-fortified	5.23 ± 0.22	23.6	

Notes
1 Riboflavin contents are for dried noodles (as purchased at retail) and are expressed in units of mg/kg on an as is basis
2 Loss values are the proportion of riboflavin measured in the dried noodle sample which was lost during cooking and are expressed as a percentage
3 Loss values have been calculated from data in the original references after first adjusting values to a dry matter basis
4 Loss values are expressed as means or ranges or both in some cases
5 Where precision data was presented results are reported here as mean ± sd
6 Different analytical procedures were applied in these studies

9.10 The changes in riboflavin contents of noodles during short term storage

A series of batches of noodles were prepared in the fresh form and the impact of various storage conditions was studied. Again riboflavin was added at the level of fortification described earlier. It was noted during the analysis of many different batches of noodles that the measured levels of riboflavin initially did appear to be slightly higher than the amounts of riboflavin that had been added. This apparent inconsistency in the data results from the inherent variability in the analysis of riboflavin estimated earlier and described in Tables 9.4 and 9.9.

For the study of storage conditions, white salted and yellow alkaline noodles were prepared in the laboratory. Fresh prepared noodles were placed in sealed plastic bags and covered with aluminum foil to exclude light, prior to storage at room and refrigeration temperatures. For drying, freshly prepared noodles were placed loosely in a tray and dried at 40 °C. The results are shown in Table 9.15.

Table 9.15 Riboflavin contents of white salted and yellow alkaline noodles in different storage conditions

Storage conditions	White salted noodles	Yellow alkaline noodles
Flour	1.41 ± 0.1	1.41 ± 0.1
Vitamin added to formulation	13.70	13.70
Total vitamin level in ingredients	15.1 ± 0.1	15.1 ± 0.1
Dough	14.3 ± 0.1	13.1 ± 0.2
Noodles stored fresh at room temperature for 24 h	13.7 ± 0.4	12.6 ± 0.3
Noodles stored fresh at refrigeration temperature for 24 h	14.10 ± 0.09	12.8 ± 0.2
Noodles dried at 40 °C for 24 h	11.3 ± 0.2	10.1 ± 0.1

Notes 1 Riboflavin data are expressed as mg/kg on a dry weight basis
2 The amounts added to flour are calculated values based upon the known concentration and volume of riboflavin used

These results indicated that minor losses occurred at each stage during processing of white salted noodles. Storage of noodles at room and refrigeration temperature did not affect the retention of riboflavin significantly. However, for this type of noodle, some losses were found after drying the fresh noodles at 40 °C for 24 h.

For yellow alkaline noodles, the amount of riboflavin lost was higher when noodles were dried at 40 °C for 24 h compared to noodles stored fresh at room temperature. There was no significant difference in riboflavin level when fresh noodles were stored at room or refrigerated temperature. However, there was a greater decrease in riboflavin levels for yellow alkaline noodles than that observed for white salted noodles. This might be due to storage of fresh noodles at a condition of high moisture content (33 g per 100g) and high pH level (pH 10) as riboflavin is unstable under alkaline condition (Gregory 1996).

9.11 The riboflavin contents of noodles during storage for up to four days

The preceding experiments showed that more than one third of total riboflavin in the ingredients was lost during the processing of dried yellow alkaline noodles. Therefore, a further study on the loss of riboflavin in yellow alkaline noodles was designed. For this experiment, the noodles were prepared in the laboratory and the fresh noodles were divided into two; one was stored in a sealed container to prevent drying and at room temperature. The other was dried at 40 °C for up to 96 h. During storage and drying, samples of noodles were collected at selected intervals of time for analysis and the results are shown in Figures 9.16 and 9.17.

There was a clear trend of reduction in riboflavin levels when noodles were stored at room temperature for up to 96 h (Figure 9.16). The rate of reduction appeared to be high on the first day of storage at room temperature then the rate became lower. Drying noodles at 40 °C also showed a trend of reduction of riboflavin content (Figure 9.17). The greatest reduction occurred during the first 7 h of drying at 40 °C, then slowed down and almost no change took place after 28 h. There was some batch to batch variation in the riboflavin measurements, however these results confirmed the level of riboflavin found in yellow alkaline noodles (Tables 9.11 and 9.14).

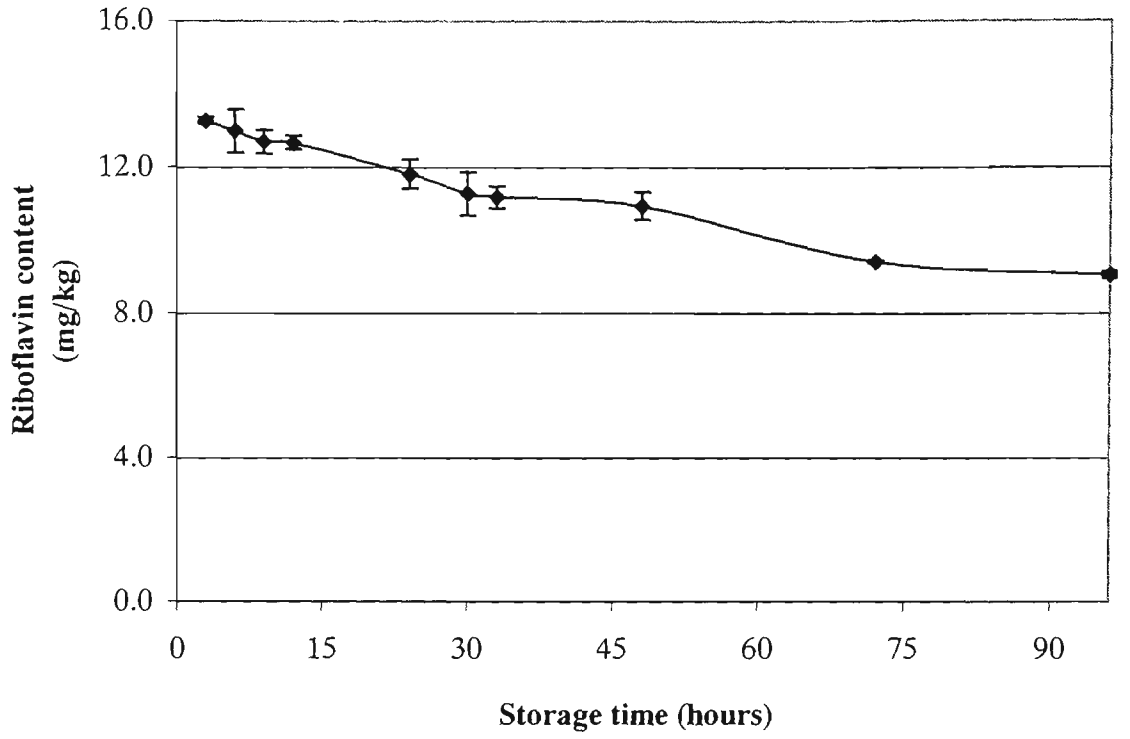


Figure 9.16 Riboflavin content in fresh yellow alkaline noodles stored at room temperature for up to 96 h

Note Riboflavin data are presented as mean ± sd and are expressed in units of mg/kg on a dry weight basis

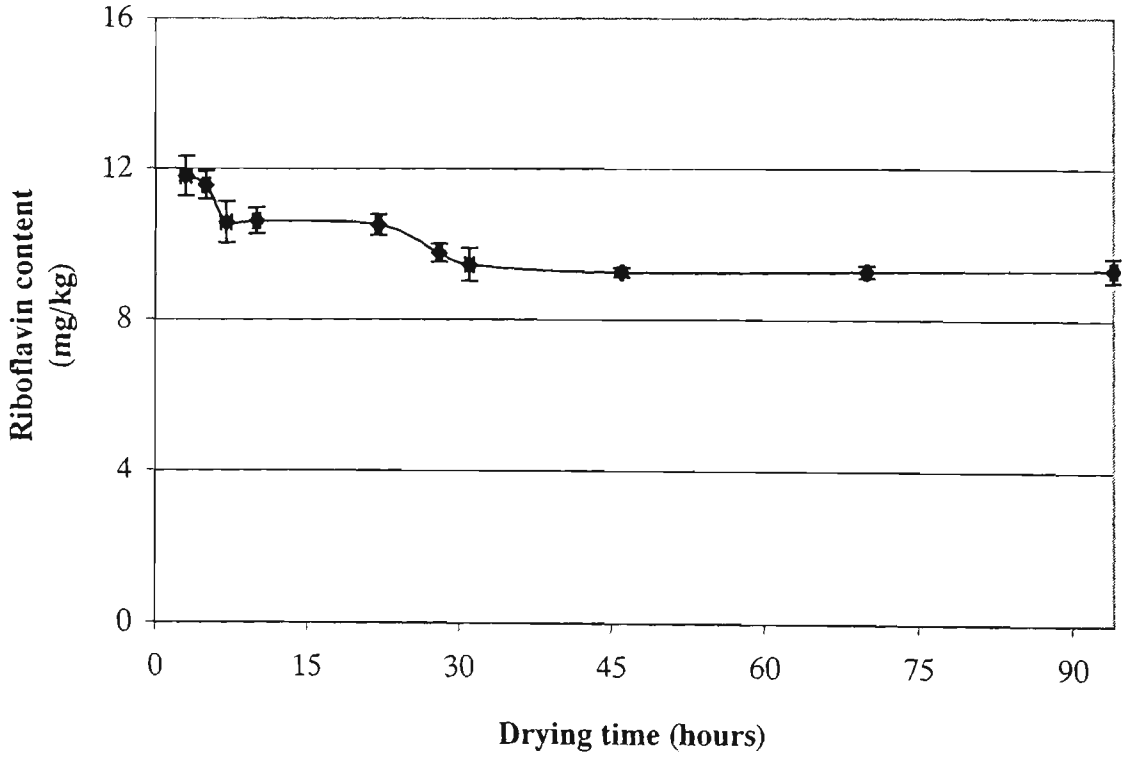


Figure 9.17 Riboflavin content in fresh yellow alkaline noodles dried at 40 °C for up to 94 h

Note Riboflavin data are presented as mean ± sd and are expressed in units of mg/kg on a dry weight basis

9.12 General discussion and summary of results for riboflavin in three styles of Asian noodles

In preliminary studies methods for extraction and analysis of riboflavin were evaluated. Different extraction methods were investigated using the AACC reference sample and bakers flour. The results showed the extraction method of Brubacher and co-workers introduced consistent results. Therefore, this extraction was chosen for further study. The results presented demonstrate that in applying well-established method for vitamin analysis, it is essential to consider extraction procedures effective for the specific food products being analysed. In addition, the modification of the composition of mobile phase was considered in order to optimise the HPLC procedure for quantitation of riboflavin.

It was found that:

- Riboflavin level in flour sample was very low
- In amount in the AACC reference sample was at the high end of the range originally obtained in the AACC collaborative survey.

In order to study the stability of riboflavin in Asian noodles samples were fortified with the addition of riboflavin at a level of 25 percent to flour sample prior to preparation of the products. Fortification was effective in enhancing the total vitamin level in the products after cooking. The riboflavin contents of selected commercial noodle samples were measured and it was found that low levels of riboflavin occurred in commercial noodles. In some cases the peaks of riboflavin was below the limit detection which was estimated to be 0.3 mg/kg.

When samples of the three styles were prepared in the laboratory, the results showed:

- Losses occurred in each step during the processing for all three styles. The highest losses were observed at stages where heating (in the form of steaming and deep-frying) was involved.
- Product pH also appeared to be a factor as greater losses were seen yellow alkaline and instant noodles.
- Cooking resulted in substantial losses with longer cooking times causing greater effect.

For short time storage of white salted and yellow alkaline noodles, the results show higher loss occurred in yellow alkaline noodles. Storage at refrigeration temperature did enhance the stability of riboflavin. There was a clear trend of reduction during storage of yellow alkaline noodles at room temperature for up to 96 h. For drying at 40 °C, a reduction of riboflavin occurred until 28 h

A total loss of 50 percent of riboflavin found after the white salted and instant styles were boiled to the optimum cooking time showed that fortified white salted and instant noodles with riboflavin at levels of 25 percent could be considered as a good source of food to be fortified.

Chapter 10

The measurement, contents and stability of folate in three different styles of Asian noodles

The purpose of this chapter is to describe and discuss the results obtained during the analysis of Asian noodles for folates. These encompass the validation of the analytical method, along with results for commercial noodles and those prepared in the laboratory.

10.1 Introduction

The naturally occurring forms of folates are available from a large variety of different foods. This group of compounds has a structure based on pteroic acid and cannot be synthesised by mammalian cells. In recent years, the roles of folates in human nutrition have received increased interest and attention. There are many different methods for folate analysis including bioassays, microbiological, HPLC, ligand-binding and radio-immunoassay have also been developed (Eitenmiller and Landen 1995).

As for most naturally occurring vitamins, folate derivatives in foods are reported to be highly sensitive to various parameters including temperature, oxygen and light. Their stability is also affected by processing conditions. Therefore, the purposes of this study were firstly to evaluate and validate a method of analysis suitable for folate in flour and Asian noodles. Secondly, to quantitate the level of folate in flour, and noodles as well as to compare the effect of ingredients and processing conditions to the loss of folate during the processing.

10.2 Selection of a suitable method for folate assay

The first step here has been to evaluate suitable methods for analysis of folate contents and to select one for this study. The low concentration of folates present in most foods in addition to their relative instability to heat, low pH, oxygen, and enzymatic breakdown. which limit the applicability of any technique and emphasise the need for sensitive detection techniques (Hawkes and Villota 1989). Although the minimum

analysis time to obtain a result for microbiological assay is about five days compared to one day working of HPLC analysis, most laboratories internationally prefer the use of microbiological assay for determination of folates naturally present in foods, as well as pure folic acid added for fortification purposes (NHMRC 1995). The reason is that naturally occurring folate promote the growth of certain lactic acid-forming organism. A variety of different bacteria have been utilised in the assay of folate (Hawkes and Villota 1989). Recently *L casei* ATCC 7469 has been preferred because it responds to mono, di and tri-glutamate forms of folates which are the most common products after conjugase treatment of higher glutamate forms of folate (Tamura and others 1972; Hawkers and Villota 1989).

The organism shows a reduced response to polyglutamate forms of folate with more than three glutamate (Tamura and others 1972) and so conjugase treatments are incorporated into the procedure. As a result, the microbiological analysis for folates is applicable to most food matrices and vitamin supplements. The minimum detectable level on a wet basis is 2.5 μg/kg for folic acid. The minimum detection level assumes that the lowest practical sample dilution factor is 1:100 (Eitenmiller and Landen 1999). According to Gregory (1984), in a study of analysis of naturally occurring folates in selected foods, the folate content determined by microbiological assay was frequently greater than that determined by HPLC analysis of the same extract. For the current research, the microbiological assay was considered for analysis of folates.

The AOAC method has been used over a long period of time but has been the subject of intensive study during the past five years. There have been various reasons for this including the increasing evidence that folate has broader nutritional and health implications. The move to fortification of foods with folate and needs to monitor compliance with regulatory requirements has also prompted some of the research in this area. The issues that have been addressed are:

- The effectiveness of various conjugase treatments (Rader and others 1998).
- Procedures to enhance the use of the bacterial cultures by cryoprotective treatments and storage to maintain constant growth-response curves in day-to-day assay

(Grossowicz and others 1981; Wilson and Horne 1982; Tamura 1990; Shrestha and others 2000).

- The release of folates from food matrices by various enzymatic treatments.
- The impact of various pH conditions during assay (Kirsch and Chen 1984; Aiso and Tamura 1998).

10.3 Laboratory assessment of *Lactobacillus casei* for analysis of folates by the microbiological assay

Basically this microbial assay is based on the requirement of nutritional substances such as minerals, amino acids and vitamins for microorganism to grow. The growth response is limited by the nutrient supplied. The growth of the test organism in the sample assay solution is compared with that in a standard solution.

Equivalent responses of the test organism at different concentration levels of the assay solution and at corresponding levels of the standard solution are fundamental criteria of assay validity (Angyal 1996). The degree of utilisation of folates by *L casei* is dependent on the time of incubation, therefore the first step here was to study the rate of growth of the organism under the conditions of the assay. The growth curve was monitored to determine the time required for the culture to reach a plateau.

The cultures *L casei* subsp. Rhamnosus (ATCC 7469) used in this study were obtained from three different sources: Ms Lata Masih, University of Melbourne, Gilbert Chandler; from the culture resources of Victoria University, School of Life Sciences & Technology (originally sourced from the Australian Starter Culture Research Center ASCC 1521 ≅ ATCC 7469); and from the Department of Microbiology, the University of New South Wales, Sydney.

Initially, inocula for the traditional microbiological assay were prepared using serial transfer of the organism. The methods are based on those described by Angyal (1996) and presented in Section 6.10.2. In order to assess the suitability of the bacteria and to determine the appropriate period of incubation, the growth curve was established. For this, the organisms were first inoculated to *L casei* medium at a level of 10 percent.

However, this was found to give very high initial absorbance values and the solutions sampled during growth had to be diluted prior to measurement. Hence the rate of inoculation adopted for further assessment was 1 percent. The inoculated media were incubated at 37 °C for 24 h. On an hourly basis, samples were taken and absorbance determined. Various wavelengths have been reported to be suitable for this purpose and for the analyses reported here 540 nm was used. This experiment was repeated a number of times and typical results are presented in Figure 10.1. The resultant curves were very similar with a plateau being reached after a period of 18–20 h of incubation.

From the curves it is concluded that no further growth occurred after 18-20 h and therefore this period was suitable for assays in which folate contents of samples were being measured. This is similar to the findings of other groups using this assay system for folate analysis (Rader and others 1998; Shrestha and others 2000). In order to further assess the growth of the bacterium, samples of incubation mixtures were also subjected to analysis for plate counts. For this purpose, duplicate samples were taken every two hours. Each sample was 1.0 mL and this was mixed with agar solution prior to incubation at 37 °C for 24 h. Colonies were counted and the results calculated according to the method of the International Standards Organisation (Harrigan 1998). The growth data are presented in Figures 10.2 and 10.3. It is noted that the plate count results are expressed as colony forming units and are presented as a function of both absorbance and time. The absorbance values may include viable cells as well as dead bacteria. The appearance of the colonies on the cultured plates is shown in Figure 10.4.

The growth characteristics of the bacterium (Figures 10.1, 10.2 and 10.3) show the expected pattern with a logarithmic phase followed by the stationary phase. In terms of the assay conditions to be used in the analysis of folate, the results confirm the normal growth characteristics of the bacteria and show that incubation for 18 to 20 h covered the period of active growth. Therefore 20 h was selected as a suitable time and was used for all of the analyses reported here.

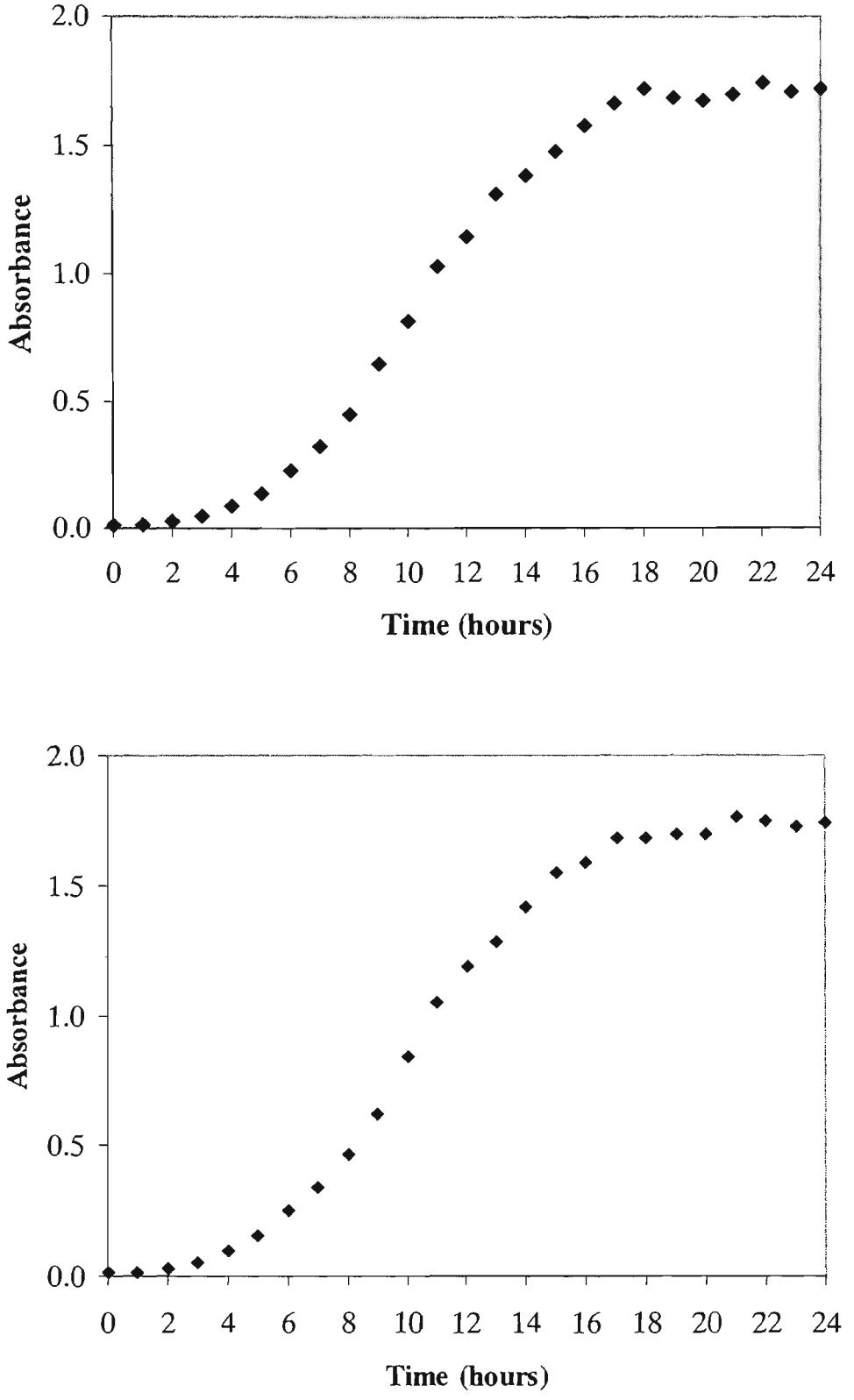


Figure 10.1 **Two typical growth curves obtained when cultures of *Lactobacillus casei* were grown in commercial *Lactobacillus casei* medium**
Note Absorbances were measured at 540 nm

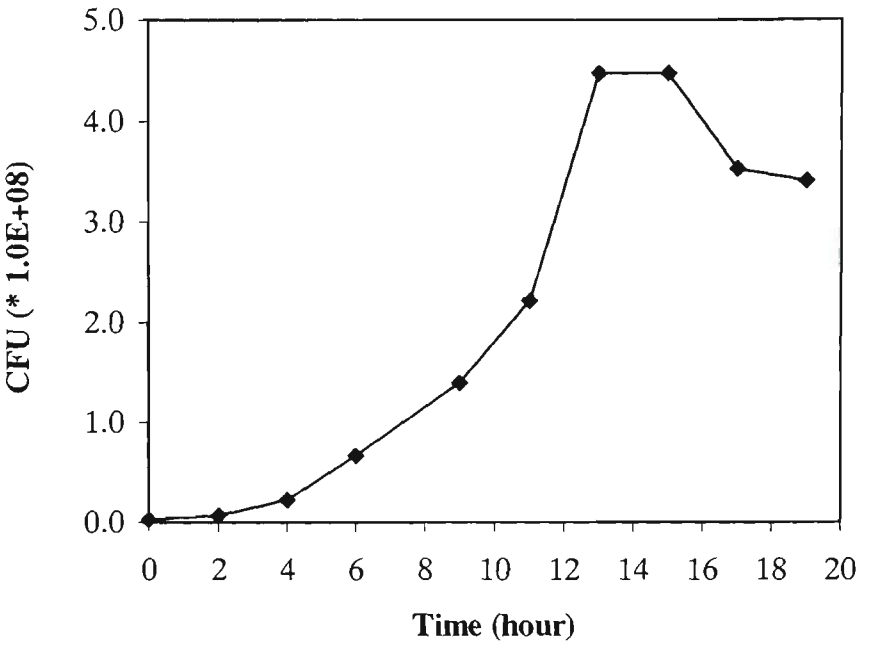


Figure 10.2 **The number of colony forming units (CFU) at various stages of incubation of inoculum with *Lactobacillus casei* medium**

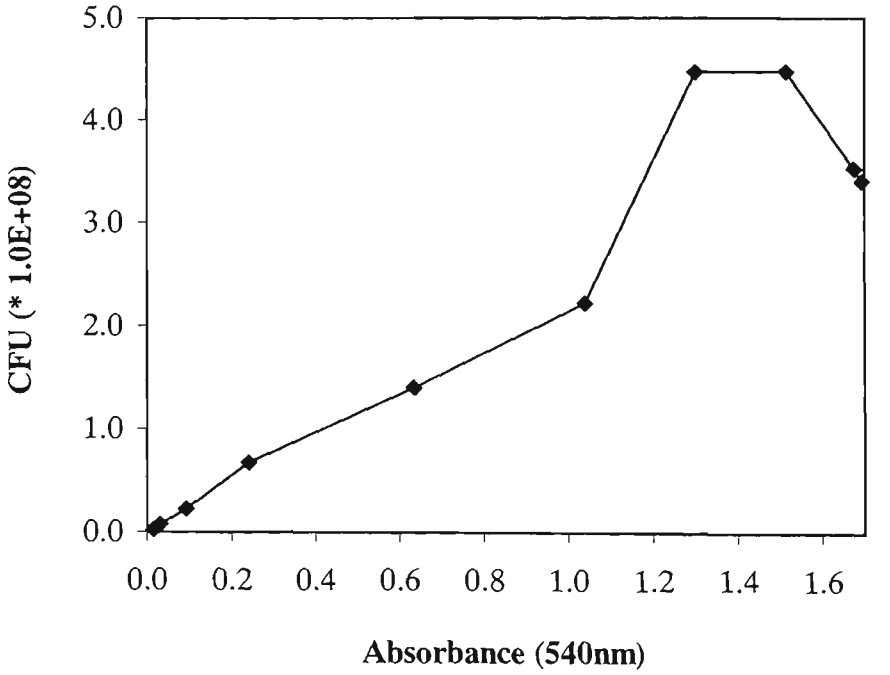


Figure 10.3 **The number of colony forming units (CFU) plotted as a function of absorbance for during the incubation of inoculum with *Lactobacillus casei* medium**

Note Absorbances were measured at 540 nm

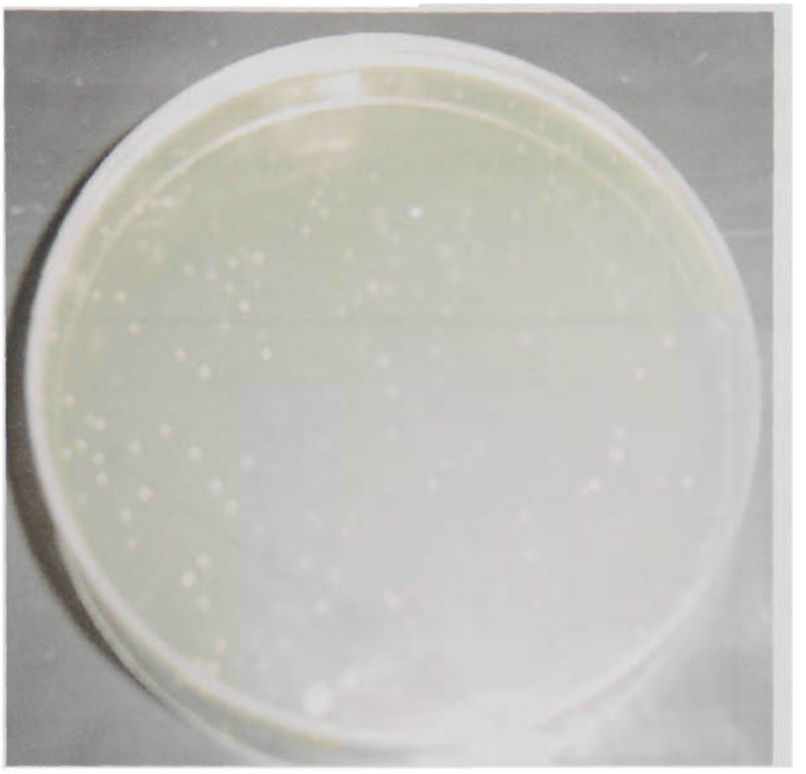


Figure 10.4 The appearance of bacterial colonies obtained when samples were taken during the incubation of *Lactobacillus casei* in broth culture and grown on plate cultures with the AOAC agar medium

In setting up a microbiological method, it is possible that the test organism may respond too vigorously due to the basal medium containing a substance that causes excessive growth of the test organism (Angyal 1996). Another issue is that insufficient cells of the organism for the assay might be used for inoculation. Therefore, in the published method used as the basis of folate measurement for this investigation (Angyal 1996) a further preliminary assessment recommended is the calibration of the spectrophotometer. The purpose of this step is to establish the absorbance value corresponding to a known dry weight of bacterial cells. The significance of this step is that subsequently a simple absorbance reading can be used to ensure that a suitable quantity and the same amount can be reproducibly applied throughout the investigation.

In order to calibrate the spectrophotometer used in this investigation, the procedure originally described by Angyal (1996) was followed (see Section 6.10.3). Samples of culture were taken at specified dilutions to give units of mg of dried cell weight and the absorbance values determined. The results are presented in Figure 10.5. According to

Angyal (1996), the response at 5 mL level of the standard will be equivalent to a dried cell weight of ≥1.25 mg/tube. Therefore for all subsequent analyses, it was necessary to ensure that this level of bacteria was used. Thus in preparing for each batch of assays, the bacterial culture was diluted to give the appropriate absorbance established here.

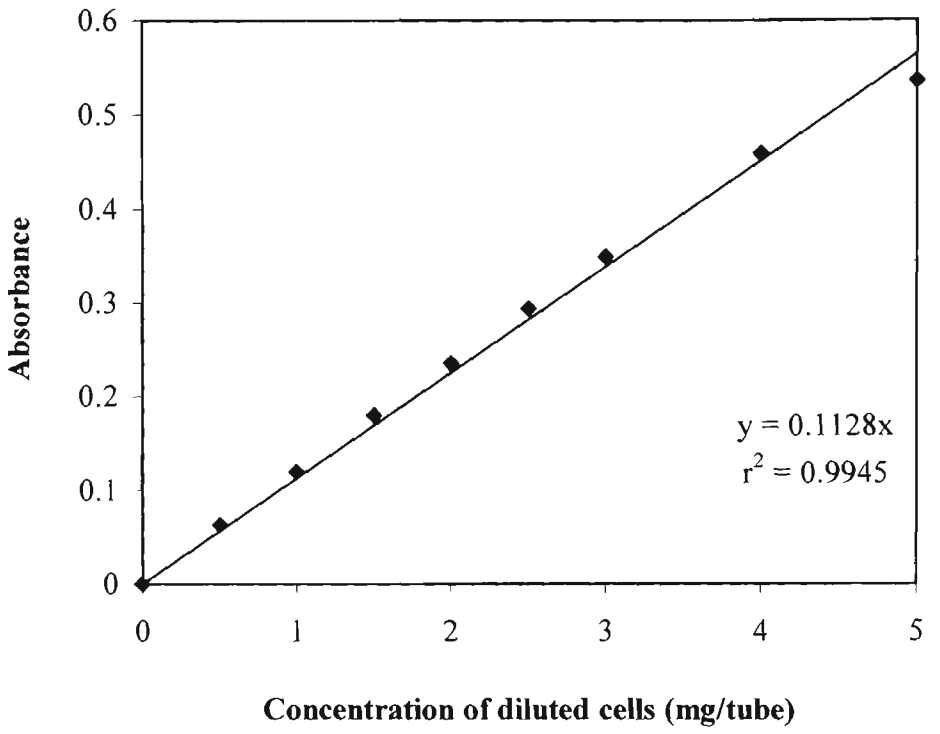


Figure 10.5 Relationship between number of bacteria and absorbance found during the calibration of the spectrophotometer
Note Absorbances were measured at 540 nm

Another issue considered in the establishment of the microbiological method was the maintenance of the bacterial cultures over time. In earlier published methods, including the AOAC procedure, cultures were stored at 4 °C and serial dilutions were prepared on a regular ongoing basis. More recently, it has been shown that glycerol can be used as a cryoprotectant (Wilson and Horne 1982; Shrestha and others 2000) so that cultures can be stored in the frozen state (-20 °C). This offers a convenient approach for day to day routine assay purposes and so was adopted here.

The bacterial cultures were used for folate assays over a number of months and where doubts arose over the efficacy of the culture, samples were assessed microscopically with gram staining to facilitate the monitoring. A photograph showing the typical

appearance of the bacteria is shown in Figure 10.6. In addition, samples were plated out at suitable dilutions and the resultant colonies inspected for uniformity of visual appearance. If any contamination of the culture was detected then fresh preparations were made by culturing from plate colonies identified as *L casei* on the basis of colony morphology.

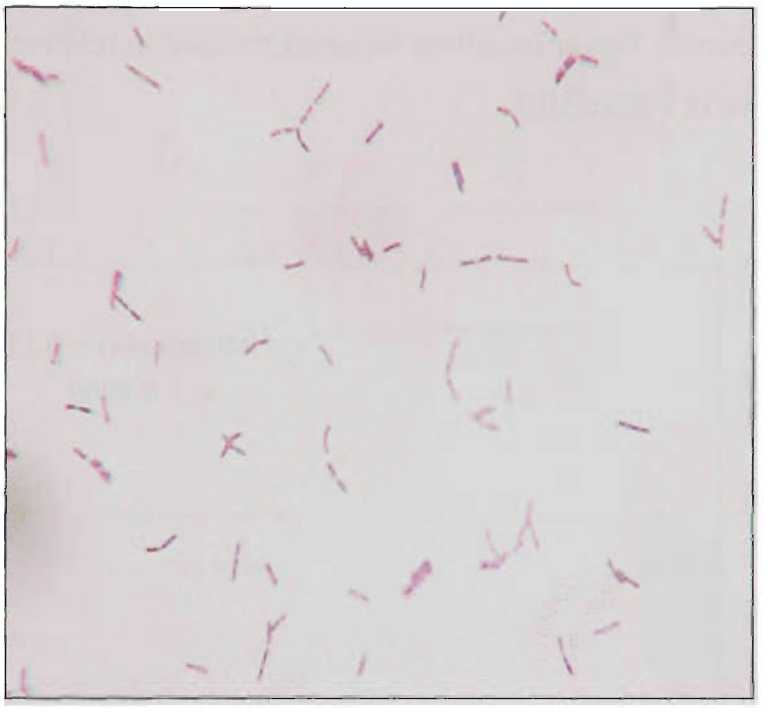


Figure 10.6 A typical culture of bacteria *Lactobacillus casei* used in the assay of folate observed under the microscope
Note The culture was prepared by Gram staining and observed at 100-fold magnification

For sub-samples of the cryoprotected culture which had been stored for periods longer than eight weeks it was found that larger volumes were needed in order to ensure the required level of absorbance for inoculation. When this occurred the culture was regrown and subsamples stored after addition of cryoprotectant.

Three separate sources of the bacterial strain were used in the initial phases of setting up the folate method. For these different sources, variations were found in the level of turbidity for blank analyses in which no source of folate was added. Overall, the three

sources showed similar growth characteristics. For the analyses the culture procured from the University of New South Wales, Sydney was used.

10.4 The establishment of standard curves for folate analysis

For the preparation of standard curves, the first issue was to determine a suitable dilution of the glycerol cryoprotected culture for the assay. For this purpose different volumes of this culture were diluted with sterilised saline solution and these were used to obtain standard curves. Lower inoculum volumes resulted in relatively flat curves and an example is shown in Figure 10.7.

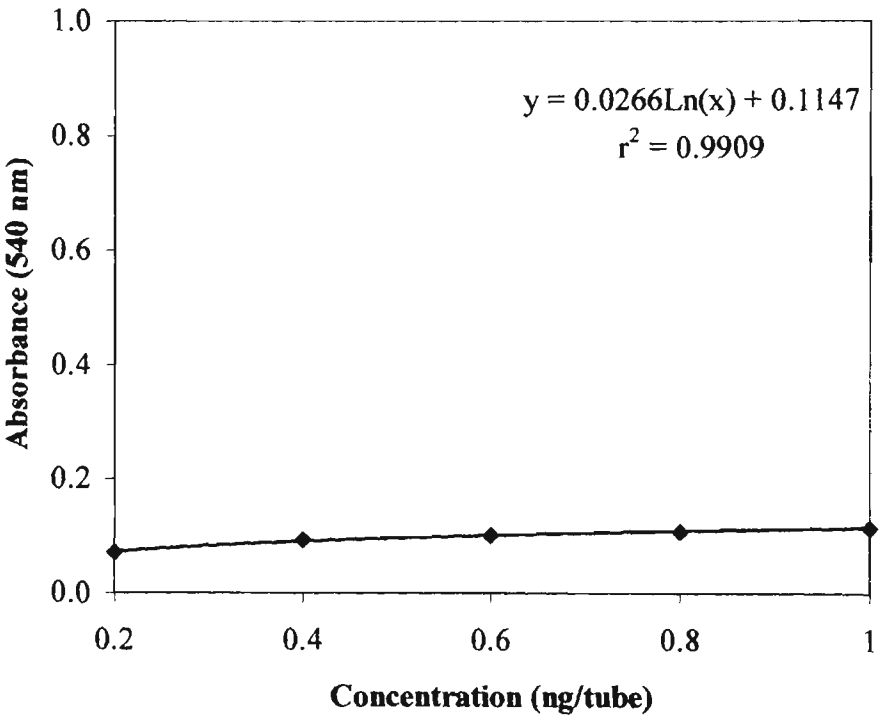
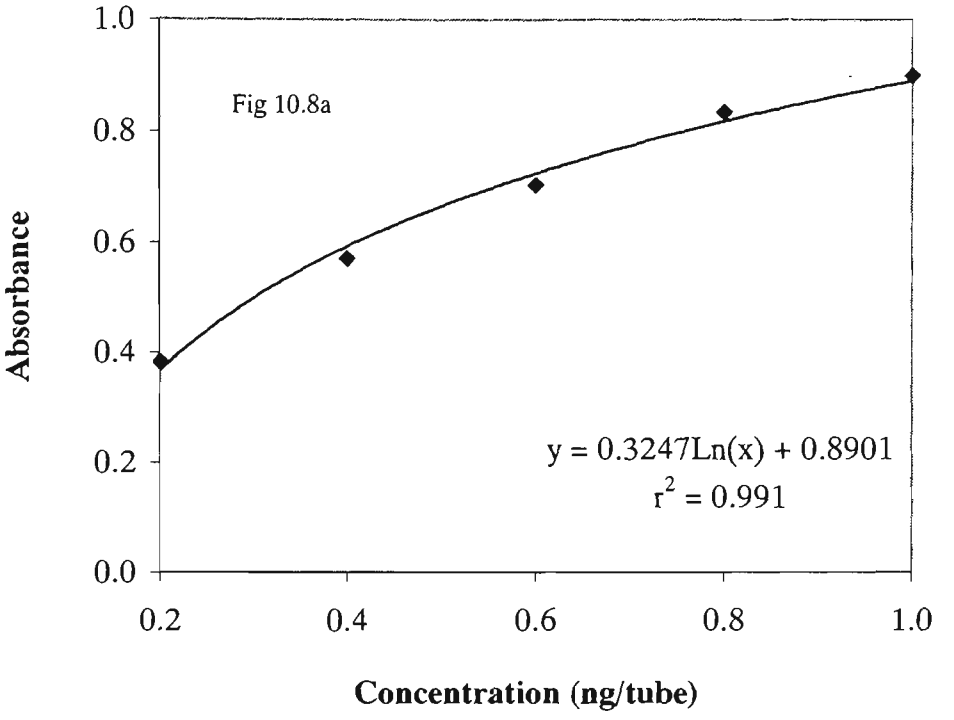


Figure 10.7 An example of a standard curve obtained in preliminary studies showing a relatively flat growth response to folate
Note Cryoprotected culture diluted to give an absorbance of 0.012 at 540nm

Larger volumes of culture were compared and some of these are presented in Figure 10.8. The curves which were most useful for quantitation were those in which the diluted culture had an absorbance of 0.085 and so this was used throughout the studies reported here.



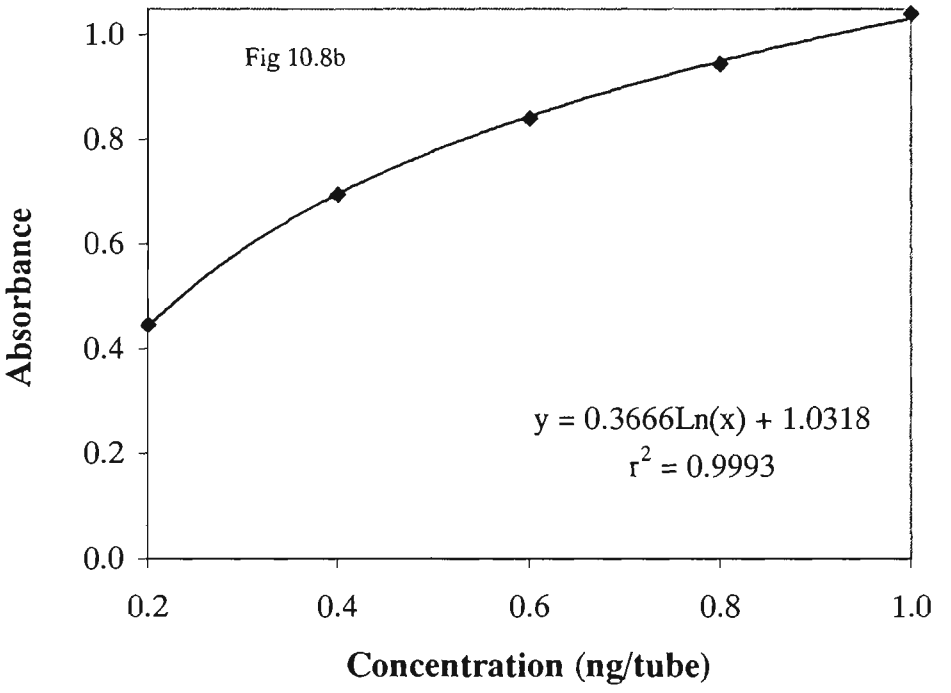


Figure 10.8 Examples of standard curves obtained when higher levels of cryoprotected culture were used in preparation of inoculum for folate assay. a) and b) corresponding to diluted culture absorbances of 0.050 and 0.085 respectively

Note Absorbances were measured at 540 nm

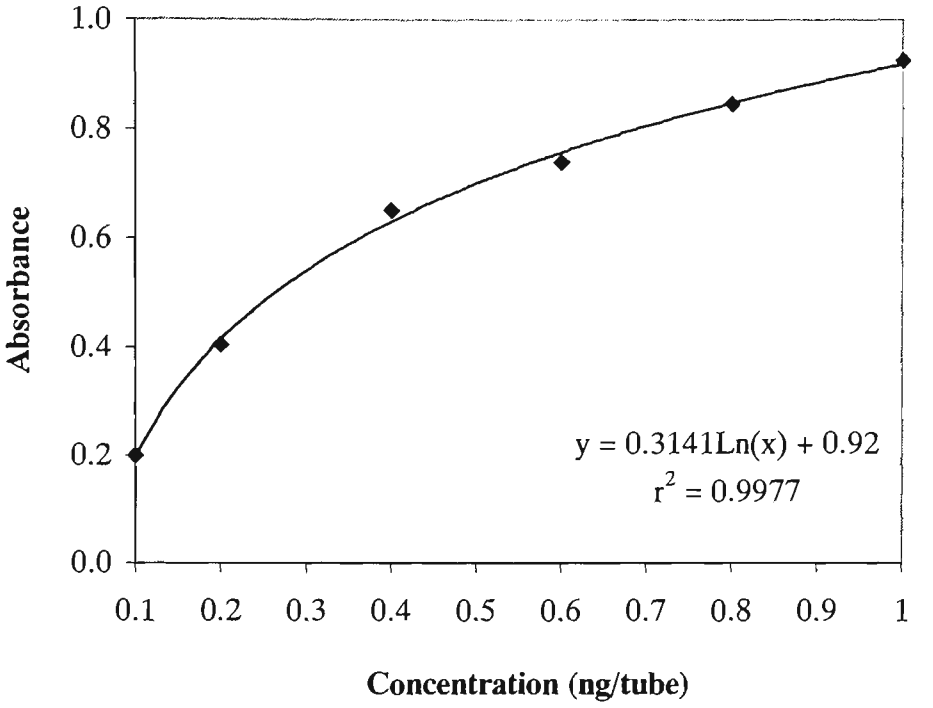
For calibration, the purest available form of folic acid was used after drying. The working standard of folic acid was prepared at a concentration of 1 ng/mL and used to construct a six-point standard curve with a concentration range of 0.1–1.0 ng/tube. Tubes were inoculated with the culture and incubated for a period of 18 hrs. For each tube, multiple absorbance readings were taken and the average values were plotted as a standard concentration response curve. Typical calibration curves are presented in Figure 10.9.

In order to calculate results of unknown samples, various regression analyses were performed. In virtually every case, logarithmic trendlines were found to yield correlation values in the range r^2 = 0.98–0.99 and these represented a better fit than linear and other regression models. Therefore throughout this study the results for standards run with each batch of analyses were plotted and the logarithmic trendline obtained and used for the calculation of the levels of folate in flour and noodle samples. It is also noted that relatively consistent standard curves were obtained over a period of a number of months.

10.5 The application of varying extraction conditions including enzyme treatments for measurement of folate in noodle samples

Many variations in the basic procedure for extracting folates have been reported. These include various heat/autoclaving treatments, deconjugation procedures as well as other enzymatic approaches to release of folate from the food matrix. As there is such a large number of possible combinations of treatment conditions and parameters, it was decided that a practical approach was to investigate two procedures published relatively recently and which appear to have given reasonable results when applied to wheat flour samples. In assessing enzyme treatments it is also noted that the conjugase used here was from chicken and was prepared from a chicken pancreas product as described in Section 6.10.1.

Accordingly an experiment was designed to directly compare the results for a noodle sample when these two methods were applied (Section 6.10.4 and 6.10.5). Both methods entailed the use of autoclaving, conjugase treatment as well as the use of other enzymes, although quite different conditions are used in the two different procedures.



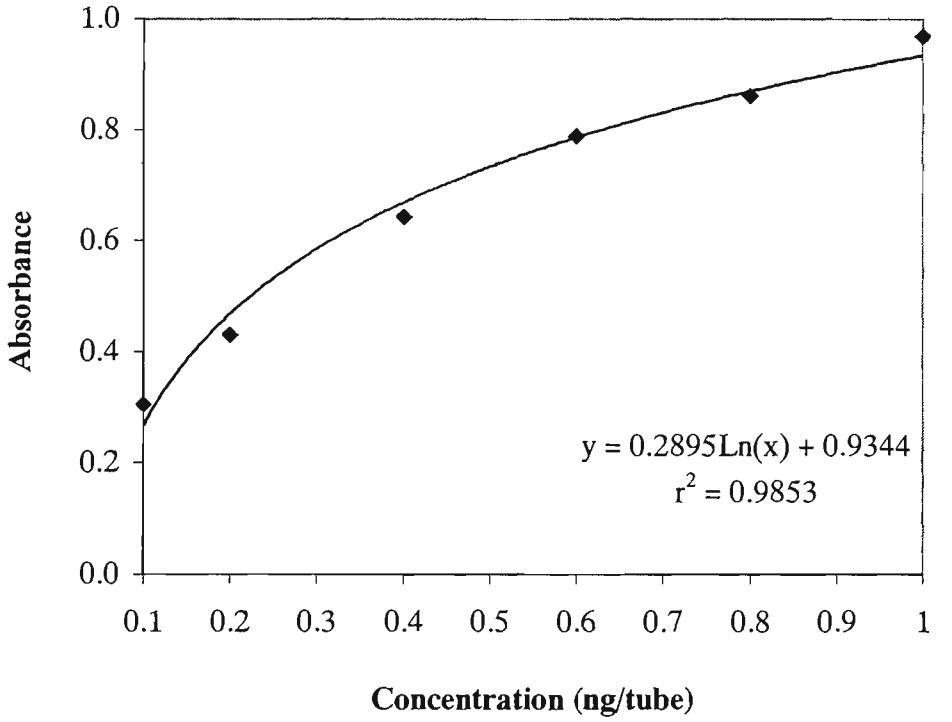


Figure 10.9 Typical standard curves for folates measured using the microbiological assay demonstrating the variability commonly observed from batch to batch
Note Absorbances were measured at 540 nm

The sample used was a commercial instant noodle sample and the results are presented in Table 10.1.

Table 10.1 A comparison of two published approaches for extraction and assay of folate using a commercial noodle sample

	Folate content
Method of Angyal (1996)	129 ± 7
Method of Shrestha and others (2000)	173 ± 21

Notes 1 Results are presented as mean ± sd
2 Units for folate contents are μg/kg expressed on a dry weight basis
3 For each method four extracts were prepared separately and each measured following the protocol described in Chapter 6 corresponding to twelve individual determinations

The differences are significant and this indicates that one or more of the variations in the conditions between the methods has resulted in the second method giving higher values. In comparing the results (Table 10.1) some of the significant differences in the methods were considered. One of these related to autoclaving conditions. In the experiment, samples were autoclaved at two different combinations of time and temperature: 121 °C for 15 min and 105 °C for 10 min. The higher folate level was found at lower autoclave temperatures. This is similar to the results of Shrestha and others (2000) where autoclave and cold extraction were compared. In the literature the only samples which are analysed without the use of any autoclaving treatment are those where a high level of fortification obviates the need for any breakdown of the food matrix. In the case of noodle samples with high levels of starch it is likely that the gelatinisation of this component is required and hence no further consideration was given to this issue.

Another possible explanation for differences (Table 10.1) is the level of conjugase added. Previously Pedersen (1988) found that increasing the amount of conjugase from either hog kidney or chicken pancreas could maximise the deconjugation of folates in extracts of peas and potatoes. In the comparison carried out here the method of Angyal incorporates much higher levels of conjugase. Therefore the results observed for the

noodle sample tested here do not indicate that the level of this enzyme is limiting the level of folate measured. It is possible that the levels of conjugase needed for an effective assay are higher for other sample types. In addition the level of conjugase needed may be influenced by the action of the other enzymes used to break down the food matrix. It has been recommended that in adapting folate methods to specific sample types different combinations of treatments should be trialled and that ultimately the procedure giving the highest results should be adopted (Tamura 1998).

Another way in which some of the published methods vary is in the use of amylase and protease preparations. It is noted that, the use of chicken conjugase without any other enzymes to extract folate from the samples was not specifically considered. The reason for this was that the samples in this study contained high levels of starch and so the use of protease and α-amylase was considered necessary in order to breakdown the food matrix. To evaluate the usefulness of enzyme treatments a comparison was carried out using the commercial noodle sample. The objective of this experiment was to determine if variations in the treatment significantly affected the level of folate extracted from and measured in noodle samples. In addition, the establishment of the optimum conditions for the extraction of folate in cereal-based products was required. For this experiment, three different extraction techniques were used. In each case this entailed autoclaving of the sample and this was followed by either extraction without enzyme; treatment with protease followed by α-amylase; and with all three enzymes. In the scientific literature (for example Martin and others 1990; Angyal 1996; Pfeiffer and others 1997; Rader and others 1998; Tamura 1998; Shrestha and others 2000; DeVries and others 2001) this latter treatment is referred to as the tri-enzyme method and this terminology has been adopted here. The results are presented in Table 10.2.

The results showed that samples treated without enzyme as a control had lower folate levels compared to those treated with protease followed by α-amylase. The results indicated that the use of protease and α-amylase might help to release the natural folate that was trapped or bound to the matrices of protein or starch. These findings were similar to those of other researchers who have also demonstrated that protein and carbohydrates in foods can bind and adsorb folates. Therefore treatment of high protein products with protease and conjugase resulted in significant increases in measurable

folate (DeSouza and Eitenmiller 1990; Martin and others 1990; Shrestha and others 2000). Studies by Cerna and Kas (1983), Pedersen (1988) and DeSouza and Eitenmiller (1990) also indicated that in certain types of food, α-amylase used in combination with conjugase had a strong effect on the level of folate measured.

Table 10.2 A comparison of enzyme treatments for extraction and assay of folate from a commercial noodle sample

Treatment	Folate content
Without any addition of enzyme	77 ± 13
Protease and α-amylase	120 ± 13
Tri-enzyme	173 ± 21

Notes 1 Results are presented as mean ± sd
2 Units for folate contents are μg/kg expressed on a dry weight basis
3 For each method four extracts were prepared separately and each measured following the protocol described in Chapter 6 corresponding to twelve individual determinations

The results in Table 10.2 indicate that polyglutamate forms of folate in noodles contribute to the dietary intake. When treated with tri-enzyme the folate level was significantly higher than for the other enzymatic treatment. Therefore, incubation with conjugase enzyme is a necessary step in the analysis of noodle samples. This also indicated that the use of conjugase treatment is important for microbiological assay as the rate of growth of *L casei* is highly dependent upon the pteroyglutamyl chain length (Tamura and others 1972, 1997; Hawkes and others 1989; Martin and others 1990). A recent study also showed that the use of tri-enzyme treatment is essential for obtaining the maximum possible values of food folate content when using HPLC analysis (Pfeiffer and others 1997). The results obtained in the current study show the value of tri-enzyme treatment for noodle samples and so this was adopted for all further analyses of folate.

The contribution of the polyglutamate folate to the human diet has been subject of considerable controversy over the years, but recent work has demonstrated that

polyglutamates ingested at normal dietary concentrations, have a similar availability to the monoglutamate form (Tamura and Stokstad 1973; Phillips and Wright 1982).

Another issue investigated here was the importance of blank incubations. A blank was set up in which conjugase was analysed without addition of any folate source, either from a food sample or a standard solution. This was necessary as it has previously been reported that some of these enzyme preparations contain low but measurable levels of folate (DeSouza and Eitenmiller 1990). Some other workers have suggested that the blank can be ignored (Martin and others 1990). It was found here that the chicken conjugase preparations contained sufficiently high levels of folate particularly in comparison with the levels of folate found in unfortified flours, that conjugase only blanks were required. Accordingly these were used in parallel with all sample assays throughout these studies.

10.6 The repeatability of analytical results obtained in the analysis of samples and reference materials

In order to check on the recovery, a known amount of a folic acid solution was added to a variety of samples before extraction and the assay was carried out using the conditions established in the preliminary phases of this work. In addition, a standard was analysed with each set of samples (Section 6.10.6). The typical results obtained are presented in Table 10.3.

Table 10.3 Recovery of added folic acid from selected samples using tri-enzyme extraction

Sample	Recovery (%)
Flour	112 - 115
Commercial noodles	108
AACC reference sample	110 - 126
Folic acid only (standard)	96 – 101

Note Results are presented as percentage values for multiple analyses carried out on each of five separate days

The value found for the standard folic acid indicates that all of the material was recovered when subjected to the extraction procedure including autoclaving followed by enzyme treatment procedures. When spiked samples were tested recovery values exceeded 100 percent and were relatively high in some cases. The results for noodle samples consistently gave good recoveries. Therefore the next comparisons made were between the expected values of reference materials and those found experimentally.

An AACC reference sample was originally used in collaborative trials and was supplied with analytical data from nine laboratories which had participated in that survey. For the study reported here, the AACC sample was chosen as it was a grain-based matrix. The AACC reference sample was used to compare the folate found in this study to the folate levels provided with the samples to ensure the validity of the method (Table 10.4).

Table 10.4 Analysis of AACC reference sample by using tri-enzyme extraction

Tri-enzyme	Without enzymes	Proportion free (%)
17 ± 1	15 ± 1	88.2

Notes
1 Results are presented as mean ± sd for multiple analyses carried out on each of five separate days
2 Units for folate contents are mg/kg expressed on an as is basis
3 The values provided with the AACC reference sample were mean: 13.95 and range: 11.60-16.20 mg/kg (Table 6.2)

The analysed values for the AACC reference sample were well above the mean value and also at the high end of the range of values found by the different laboratories originally analysing the sample (Table 6.2). The results obtained here for the reference sample are interpreted as indicating greater recovery of folate from the sample using the latest tri-enzyme procedure. Previously Rader and co-workers have compared results obtained using their tri-enzyme method with those provided with a number of AACC reference materials. They found variations with some of their results being higher and others lower than mean values for the samples. The tri-enzyme method adopted here appears to give significantly higher results than the method (Angyal 1996) used by Rader's group. This can clearly be seen in Table 10.1 where the values for a commercial

noodle sample were approximately 33 percent higher than those obtained with the method used by Angyal and Rader. It is also noted that the standard deviation figures obtained here (Table 10.4) are similar to those reported in the work of others including Rader and co-workers (1998, 2000) and are regarded as representing normal and acceptable variation. The data for samples analysed without the addition of any enzyme treatments have been included in Table 10.4. They demonstrate that under the conditions used here, the bulk of the folate appeared to be readily assayable requiring neither deconjugation nor breakdown of the food matrix. This might be expected as the AACC reference sample is known to be a fortified breakfast cereal. These results will be further discussed with results for noodles in subsequent sections of this chapter.

10.7 Analyses of flour samples

Wheat flour is the basic ingredient used to make noodle products and samples of the flour used in this study were prepared and analysed using the procedures established and applied to noodle samples in the previous section. The purpose was firstly to establish the level in the flour as a basis for further studies of fortification and the evaluation of the potential of these products to act as a vehicle for fortification. A further purpose was to establish the variability of results obtained with the method. The particular flour chosen for analysis was that selected for preparation of laboratory noodles in this study. The results of the tri-enzyme procedure along with those for flour samples analysed without enzyme treatment are shown in Table 10.5.

Table 10.5 Analysis of flour samples using tri-enzyme extraction

Tri-enzyme	Without enzyme	Proportion free (%)
182 ± 8	138 ± 12	75.8

Notes
1 Results are presented as mean ± sd (n = 7)
2 Units for folate contents are μg/kg expressed on a dry weight basis
3 The proportion value is calculated as the ratio of folate content in no enzyme treatment to the tri-enzyme value and expressed as a percentage

The results indicate good repeatability for the tri-enzyme method with a coefficient of variability of 4.6 percent. Comparing the folate levels found in this study with published

values it is emphasised that there is considerable variation in the literature. In a study of 63 commercially milled flours from US and Canada the mean folate content was found to be 190 μg/kg dry weight basis (Ranhotra and Keagy 1995). The range of values found for that survey was 160-210 μg/kg. In a more recent study it was found that a laboratory milled flour sample from Western Australian wheat contained 410 ± 44 μg/kg also expressed on a dry weight basis (Arcot and others 2002c). The extent to which the analysis and sample preparation methods might have impacted on the variability reported is unclear. Similarly variations determined genetically may account for some of the apparent differences. A further factor which is expected to influence folate levels in flour is the milling procedure and extraction rate achieved as the folate level in bran and pollard is typically much higher (Arcot and others 2002c).

The data presented in Table 10.5 also demonstrates that not all of the folate is readily measured if enzymatic treatment is omitted. The relative proportion found in the non treated extracts of flour is lower than that observed for the AACC reference sample (Table 10.4).

10.8 Fortification of flour and noodle samples

Here it has been confirmed that the native folate content in flour is at a relatively low level. One of the purposes of the current study has been to assess the suitability of noodles as a potential vehicle for fortification. In planning this phase of the study consideration was given to selection of appropriate levels of fortification. Within current Australian regulatory standards (Food Standards ANZ 2002) it is permissible to fortify a range of food products with folic acid. In addition, there is no designated limit on the amount that can be added to flour or flour-based foods. In labelling of fortified flour, the maximum level which can be claimed for the addition of folic acid is 100 μg per 35 g flour (equivalent to 2.86 mg folic acid/kg flour). This level is based upon current RDI values and represent up to 50 percent of the RDI for folates. Consideration of US requirements (Rader and others 2000) indicates that similar levels of addition are utilised to those described for Australia. Thus in designing experiments to study folate fortification of Asian noodles the level referred to in the standards was used. For comparison purposes levels of addition were also studied in which levels below and also

exceeding the value of 2.86 mg/kg flour were run along with controls of unfortified flour.

Another issue in the fortification of flours and noodles is the chemical form of folate which might be added. From the literature (NHMRC 1995; Ranhotra and Keagy 1995) it is known that folic acid is the major form of folate used for fortification. This is the form required in Australian regulations (Food Standards ANZ 2002) and it is known to be more stable than either of the two derivatives tetrahydrofolic acid or 5-methyltetrahydrofolic acid with respect to pH, temperature and moisture content (Hawkes and Villota 1989; Gregory 1989; Ball 1994; Eitenmiller and Landen 1999). For these reasons, folic acid has been used for fortification in this study.

Flour to which folic acid had been added was used to prepare white salted noodles. This style of noodles was chosen initially as the results obtained with riboflavin indicated greater stability in the noodles which did not include alkaline salt. The prepared noodles were dried for 24 h at 40 °C and then cooked to the optimum cooking time. The folate contents were determined and the results are shown in Table 10.6.

Table 10.6 Analysis of folate contents of cooked white salted noodles following fortification with different levels of folic acid

Fortification level	Folate level following fortification	Tri-enzyme analysis		Without enzyme	
		After cooking	Proportion lost	After cooking	Proportion lost
0	182	139 ± 18	23	102 ± 10	44
1430	1612	774 ± 97	52	403 ± 59	75
2860	3042	1703 ± 188	44	761 ± 47	75
4000	4182	2391 ± 170	43	1869 ± 197	55

Notes
1 Folate results are presented as mean ± sd
2 Units for folate contents are μg/kg expressed on a dry weight basis
3 The levels of fortification correspond to 0, 25, 50 and 70% RDI per serving, respectively based upon current Australian regulations (Food Standards ANZ 2002)
4 The proportional values are calculated as the ratio of folate content after cooking to that prior to cooking and expressed as percentages

The proportion of folate lost during cooking of noodles was found to lie in the range of 23-52 percent. The loss for the control noodles was consistently found to be lower than for the fortified noodles. For those noodles to which folic acid was added the proportional losses did not appear to vary greatly. The losses observed were around 45 percent and even at relatively high levels of fortification the losses were similar. Effectively, for this style of noodles, fortification with folate appears to be both practical and effective because the addition of the vitamin results in increased levels in the product and therefore would contribute to dietary folate intake.

There are a number of possible ways that folate might be lost and these include leaching of folate into the cooking water. Alternatively the folate molecules may be subject to chemical degradation due to the high temperatures, the pH of the cooking medium or related to the time of cooking. In relation to the results obtained here the same cooking time was used in all cases. It is not clear in which way losses have occurred. However, in the case of most noodles, the product is cooked in boiling water and the water then discarded. Therefore if losses are occurring by leaching then this material will not be available to the consumer of the noodles.

There have been a variety of studies reporting losses in folate in foods and earlier studies were reviewed in 1989 (Gregory 1989). The results typically indicate good retention of folate during storage of foods. The use of heat during processing did not appear to cause large decreases in folate contents. The greatest losses were observed during boiling and the evidence is that here the losses occur due to leaching rather than by any other mechanism.

Foods based on cereal grain have also been investigated and for breakfast cereals it appears that the method of manufacture and point of addition of folate critically affect the retention (Hawkes and Villota 1989). Losses have been found in the range of 39-79 percent for popped cereals and 18-22 percent for extruded cereals. A study of folate added at levels of 1-5 μg/g of wheat flour showed only small losses after one year of storage at 120 °F (49 °C), while losses of native folate were 40 percent after 12 weeks storage. The losses of folate when bread was baked averaged 11 percent whereas

endogenous folate decreased by 31 percent during baking (Keagy and others 1975; Osseyi and others 2001; Arcot and others 2002c).

Folate like many water-soluble vitamins is subject to leaching from foods into surrounding cooking water, in addition to chemical modes of degradation (Gregory 1989; Eitenmiller and Landen 1999). Losses of folate have been reported in boiled foods (Hurdle 1968). The boiling of vegetables causes quite variable losses of folate (Leichter 1978) and from 22-84 percent of the initial folate is typically recovered in the cooking water. The blanching of spinach resulted in a decrease of 33 percent of total folate (Chen and others 1983), 22 percent in asparagus and 84 percent loss in cauliflower (Leichter 1980). The reasons for the differences between these vegetables are not apparent. In addition, the sum of folate retained in the cooked vegetable plus that of the cooking water was nearly equivalent to the total folate of the raw vegetable in most cases. This indicated that aqueous extraction is frequently responsible for the losses of folate during cooking, rather than oxidation or thermal degradation (Leichter 1978).

In considering the results of stability and retention studies reported in the literature it is noted that most of the data was obtained using analytical methodology which may underestimate the folate values. The results obtained in the current study (particularly in Table 10.2) show that when samples were subjected to different preparation and extraction treatments then quite different results were obtained. The recent advances in use of enzymes for deconjugation and also extraction of folate potentially limit the value of published data in this area. The differences observed here between the apparent folate contents measured with and without enzyme treatments indicate that for different noodle samples and for varying treatments the proportion of folate recovered in untreated extracts shows considerable variation.

The results from this study indicated that the loss of folate in fortified noodles was double compared to the control. This might be due to the folate content in the control being low. Another issue is that the native folate in the flour may be present in molecular forms which are less readily leached or released from the food matrix during the boiling stage. Although almost half of the amount of fortified folate appeared to have been lost after the sample was cooked to an optimum time, the results clearly

indicted the potential of fortification to enhance the nutrient intake of this vitamin. The fortification of folate in noodle samples to a level of 50 percent RDI improved the level of folate in cooked noodles to a level of at least 25 percent RDI.

When the broad issue of folate fortification of foods was reviewed (NHMRC 1995), it was concluded that further studies of retention are required. Pasta was specifically identified as a product requiring study, particularly the effect of cooking on pasta and noodles which have been fortified with folate. The next stages of the current study were therefore designed to further investigate the levels of folate in commercial noodle samples representing the common styles available from retail outlets.

For the subsequent studies of Asian noodles, folate analyses have been carried out using the procedure based on Angyal (1996) and validated in earlier stages of this chapter. It is noted that each sample has been analysed using the tri-enzyme as well as the no enzyme extraction. This reflects common practice and the term total folate is used to describe the values obtained by the tri-enzyme approach and free folate to refer to the data for the conjugase only treatment.

10.9 The folate contents of commercial noodles

A number of commercial noodles were selected for this preliminary phase of the study. The basis of selection was to include as wide a range of wheat flour noodles as practicable. The samples were purchased at retail outlets and were chosen to include the three common styles of Asian noodles: white salted, yellow alkaline and instant noodles. Samples manufactured in as many different countries as possible were obtained and, in addition, samples were selected to encompass dry, fresh, aseptically packed and fried forms where these were readily available. Using the noodles in the form as they were purchased, the folate levels and the product pH were determined using the procedures already outlined. Again as the moisture contents of the noodles varied widely, all folate data was recalculated and expressed on a dry weight basis to facilitate direct comparisons. When the results were tabulated and compared with product information it became obvious that some of the samples could not clearly be categorised into one of the three styles (white salted, yellow alkaline and instant noodles). There were various reasons for this, including the pH values being

inconsistent with the declared ingredients for particular samples. In some instances, the products had a yellow appearance whilst having pH values below 7.0 and no declared colorants or other ingredients which might explain the visual characteristics. Accordingly, some samples are presented in a category designated as miscellaneous where the noodles do not clearly belong to one of the other three styles. The results for the commercial samples are presented in Tables 10.7–10.10.

Table 10.7 Folate contents and pH values of commercial white salted noodles

	pH	Total folate	Free folate	Proportion free
AOI	4.2	86 ± 11	74 ± 11	86.4
Myojo	3.9	89 ± 4	59 ± 7	65.9
Jona	5.9	70 ± 5	61 ± 6	87.3
Bea suey sormen	5.7	150 ± 19	136 ± 16	90.5
Trident udon	4.5	87 ± 3	59 ± 6	68.0
Shang hai	5.7	145 ± 13	130 ± 9	89.4
Kanto	5.7	95 ± 12	87 ± 13	91.2
Average	5.1	103	87	82.7

Notes 1 Results are presented as mean ± sd
2 Units for folate contents are μg/kg expressed on a dry weight basis
3 The proportion values are calculated as the ratio of free to total folate values and expressed as percentages

For white salted noodles, the range of pH values was from 4.2-5.7 and these are consistent with literature values for this style (Moss and others 1986, 1987). Overall the folate contents varied between 70-145 μg/kg. It is noted that for each of the samples which were purchased in the aseptically packaged form (AOI, Myojo and Trident) relatively little variation was found in folate contents (86-89 μg/kg) whereas in dried noodles greater variation was found. The folate levels do not appear to be directly related to the pH value. In addition, in those samples which had been manufactured in Japan and were aseptically packaged the folate levels were similar to other white noodles. For these samples it is likely that at least a few months had elapsed from the time of manufacture, encompassing sea transportation, prior to purchase. The fact that

folate levels in these samples were similar to other samples indicates that there is no gradual decline over time at the higher moisture contents in these products.

Table 10.8 Folate contents and pH values of commercial yellow alkaline noodles

	pH	Total folate	Free folate	Proportion free
Gold star	9.3	393 ± 34	358 ± 48	91.1
Taing Hokkien	9.1	110 ± 12	86 ± 9	72.9
Mi ga	9.7	178 ± 27	114 ± 10	64.2
Golden horse	10.3	92 ± 12	63 ± 8	67.9
Longevity	8.3	212 ± 37	170 ± 22	80.4
Average	9.34	197	158	75.3

Notes 1 Results are presented as mean ± sd
2 Units for folate contents are μg/kg expressed on a dry weight basis
3 The proportion values are calculated as the ratio of free to total folate values and expressed as percentages

For yellow alkaline noodles, the range of pH values was from 8.3-10.3. These results were expected and are consistent with the incorporation of alkaline salts in the formulation of each product. Overall, folate levels found in commercial yellow alkaline noodles varied between 92-393 μg/kg. The highest value found is for a product containing egg as a substantial ingredient and egg is known to contain folate (USDA ARS 2002). However the other product for which egg was declared as an ingredient contained lower folate content (178 μg/kg). The remaining noodles show variable results and the folate levels do not appear to relate directly to the pH values of the products. The two samples of alkaline noodles which were fresh, had high moisture contents and required storage under refrigeration do not show any evidence of reduced folate contents. This indicates that neither pH nor higher water activities are likely to impact on the stability of folate in these products, at least during storage.

Table 10.9 Folate contents and pH values of commercial instant noodles

Instant noodles	pH	Total folate	Free folate	Proportion free
Heinz	8.2	99 ± 11	55 ± 3	59.8
Trident Authentic	6.9	146 ± 7	85 ± 7	58.2
Nissin DR	7.3	98 ± 9	48 ± 3	49.0
Marketta	6.4	76 ± 6	50 ± 4	65.9
Pancit CF	6.8	151 ± 8	89 ± 5	59.0
Pancit Kalamansi	7.5	236 ± 18	92 ± 4	38.6
Nissin JAS	6.7	129 ± 10	55 ± 4	42.6
Sapporo Ichiban	6.5	876 ± 65	848 ± 74	96.8
Maggi	7.0	115 ± 12	72 ± 9	62.8
Average	7.03	214	154	59.0

Notes 1 Results are presented as mean ± sd
2 Units for folate contents are μg/kg expressed on a dry weight basis
3 The proportion values are calculated as the ratio of free to total folate values and expressed as percentages

Table 10.10 Folate contents and pH values of miscellaneous commercial noodles

	pH	Total folate	Free folate	Proportion free
Kantong Singapore	4.0	115 ± 6	92 ± 6	80.3
Wokka	4.2	53 ± 5	50 ± 3	95.8
Koyamaseimen	6.6	99 ± 13	86 ± 13	86.4
Taing udon	7.7	69 ± 4	58 ± 6	84.2
Fantastic	6.8	95 ± 8	64 ± 8	67.2
Average	5.86	86.	70	82.8

Notes 1 Results are presented as mean ± sd
2 Units for folate contents are μg/kg expressed on a dry weight basis
3 The proportion values are calculated as the ratio of free to total folate values and expressed as percentages

For instant noodles, the range of pH values was from 6.4-8.2. This is consistent with the incorporation of alkaline salts in these products. Overall, folate found in instant noodles varied between 76-876 μg/kg. The two highest values were found for the two products in which the labelling indicated the use of fortified flours. However, the ingredient listings for these two did not specifically declare that folate had been added. There is no correlation between the level of folate and product pH for the instant noodle products.

The miscellaneous category of noodles (Table 10.10) includes a variety of products for which the visual appearance or pH values were not characteristic of any of the three styles described above. The range of pH values was from 4.0-7.7 with the incorporation of low levels of alkaline salts in some samples. The folate analyses showed a range of 53-115 μg/kg which is within the range found for most of the samples for the three other styles (Tables 10.7-10.9).

In comparing the pH of three styles of noodles, the white salted had low pH, followed by instant noodles and yellow alkaline noodles. Although these noodle samples covered a wide range of pH (from pH 3 to pH 10) there was no clear pattern of folate found in different style of noodles relating to pH. Some of the higher folate levels observed may have arisen due to the incorporation of egg as an ingredient in the formulation of some products. In some cases of the instant noodles, fortification may account for the relatively high folate contents in two of the samples. However it is not possible to draw clear conclusions. Overall the folate contents of the noodles fell in the range of 53 to 212 μg/kg for the samples where egg or vitamin addition was indicated. It is noted that the unfortified white salted noodles prepared in this study (182 μg/kg - Table 10.6) fell within the range found for the commercial products.

In order to assess the potential contribution of noodles to folate intakes the figures obtained here were used to calculate the proportion of the RDI provided by the various samples. These calculated values are presented in Table 10.11.

Table 10.11 A comparison of the amount of noodles required to provide the RDI and the folate supplied in a serving of various commercial noodles

	Weight of noodle (kg)	Folate in one serving	Proportion of RDI in one serving (%)
White salted noodles			
AOI	2.33	3.01	1.51
Myojo	2.25	3.12	1.56
Jona	2.86	2.45	1.23
Bea suey sormen	1.33	5.25	2.63
Trident udon	2.30	3.05	1.52
Shang hai	1.38	5.08	2.54
Kanto	2.11	3.33	1.66
Yellow alkaline noodles			
Gold star	0.51	13.76	6.88
Taing Hokkien	1.82	3.85	1.93
Mi ga	1.12	6.23	3.12
Golden horse	2.17	3.22	1.61
Longevity	0.94	7.42	3.71
Instant noodles			
Heinz	2.02	3.47	1.73
Trident Authentic	1.37	5.11	2.56
Nissin DR	2.04	3.43	1.72
Marketta	2.63	2.66	1.33
Pancit CF	1.32	5.29	2.64
Pancit Kalamansi	0.85	8.26	4.13
Nissin JAS	1.55	4.52	2.26
Sapporo Ichiban	0.23	30.6	15.33
Maggi	1.74	4.03	2.01

Notes 1 Note that these data are calculated for uncooked noodles and do not take into account any losses during cooking

2 The folate contents of one serving are calculated on the basis of a serving of noodles being 35 g of product in the as is (as purchased) form immediately prior to cooking and expressed in units of μg

In relation to the availability of folates from noodles in the diet, a further issue is the losses which might occur during the cooking of the products. Therefore some of the commercial samples described here were cooked and the stability of folate assessed during the cooking process.

10.10 Investigation of the impact of cooking on the stability of folate in commercial noodles

In order to study the impact of cooking on the various styles of noodles some of the samples used in the previous experiment were used. For this the specific cooking instructions from the individual packages were first followed and the samples then checked as to whether they were fully cooked. For those with some remaining uncooked core, the noodles were further boiled until the optimum cooking time for the particular sample. The cooking times and the losses are summarised in Table 10.12. The results for folate contents for each of the noodles are presented in Figures 10.10-10.16

Table 10.12 Cooking times and corresponding losses of total folate for selected commercial noodles

Noodle description	Cooking time	Relative loss
White salted		
AOI	2.0	21
Shanghai	7.25	30
Yellow alkaline		
Golden Horse	3.0	27
Gold star	0.25	14.9
Instant noodles		
Nissin DR	3.0	6.1
Marketta	3.0	3.8
Miscellaneous		
Wokka	3.0	9.1

Notes 1 Cooking times are those found to be optimum for the particular sample and are in minutes
2 Relative losses are expressed as percentage values and have been calculated from total folate contents which were first adjusted to a dry weight basis

For both white salted and yellow alkaline noodles two different brands were chosen so that one was in the fresh form and the other dried. In each case the fresh form required much shorter cooking times. For the two brands of instant noodles, the cooking times were intermediate as the products require re-hydration, but the drying by deep-frying has already resulted in partial cooking of the product. The results for folate contents are also presented in Figures 10.10–10.12 with the losses found in white and yellow noodles being in the range from 15–36 percent. The results show that the losses of folate during cooking were dependent on the cooking time. In the case of yellow alkaline noodles, the typical fresh style (Gold star) required only 25 s of cooking but this still resulted in a 15 percent loss. Overall, the lowest loss was measured in cooking of instant noodles.

The noodles purchased for this evaluation were manufactured in a variety of different countries. It is difficult to reach conclusions as the products were originally prepared from different types of flours, possibly from wheats grown under varying environmental conditions and having diverse genetic backgrounds. Therefore the folate contents of the flours used to make these noodles might be expected to show at least some variability. The noodles analysed here were classified according to the pH, regardless of the noodle colour. In general, the product pH did not appear to be a good indicator of the relative stability of folate during boiling. From the measurements of folate in commercial noodles cooking time was a significant factor influencing the loss of folate. In addition, for instant noodles the relative losses appeared to be low.

Whilst there is relatively published data with which to compare the results for Asian noodles, a summary is presented in Table 10.13. Most of the products analysed, including the egg noodles, have durum-based ingredients (compare with Table 3.2 and Section 3.3). The influence of cooking of pasta on folate is very variable although the relative losses are similar to those found for the three styles of Asian noodles which ranged between 4 - 30 percent (Table 10.12). The losses calculated for Japanese noodles from US data were much higher than those found for the two different brands measured in the current study.

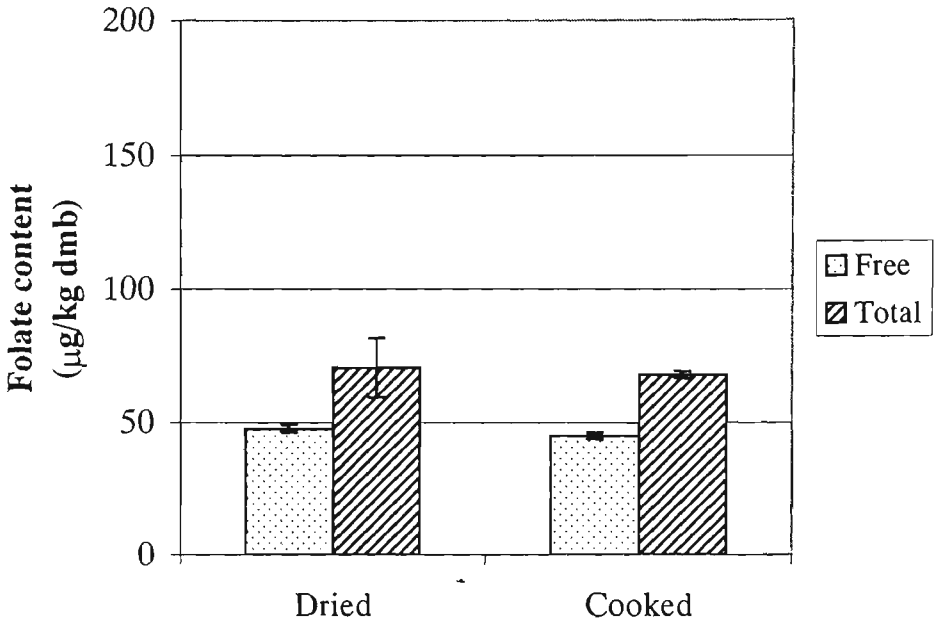


Figure 10.10 Folate contents of dried and cooked white salted noodles (AOI brand)

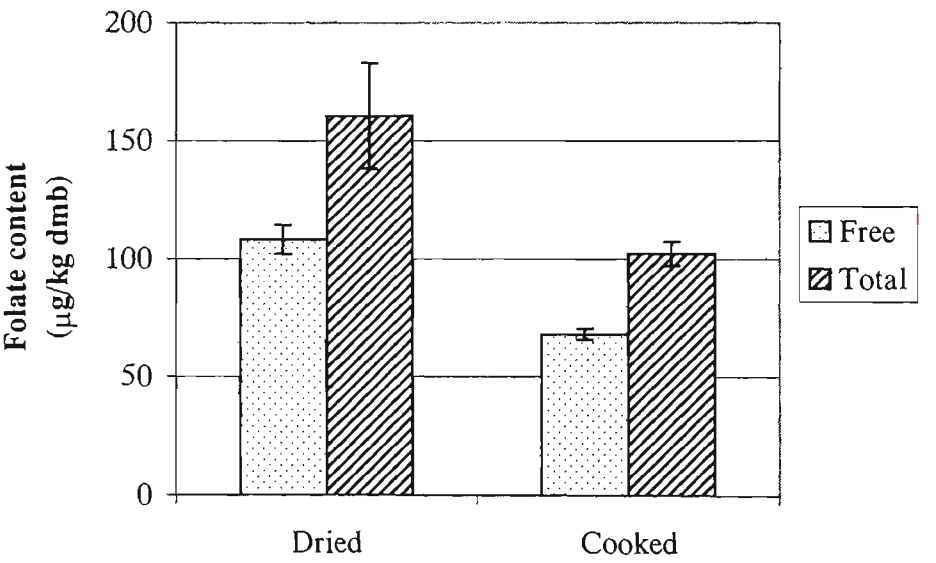


Figure 10.11 Folate contents of dried and cooked white salted noodles (Shanghai brand)

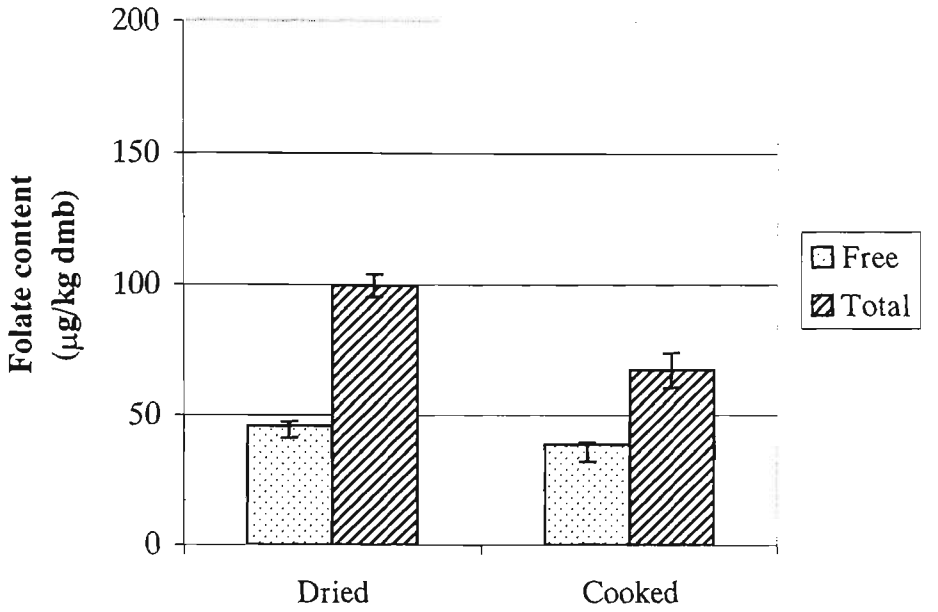


Figure 10.12 Folate contents of dried and cooked yellow alkaline noodles, (Golden horse brand)

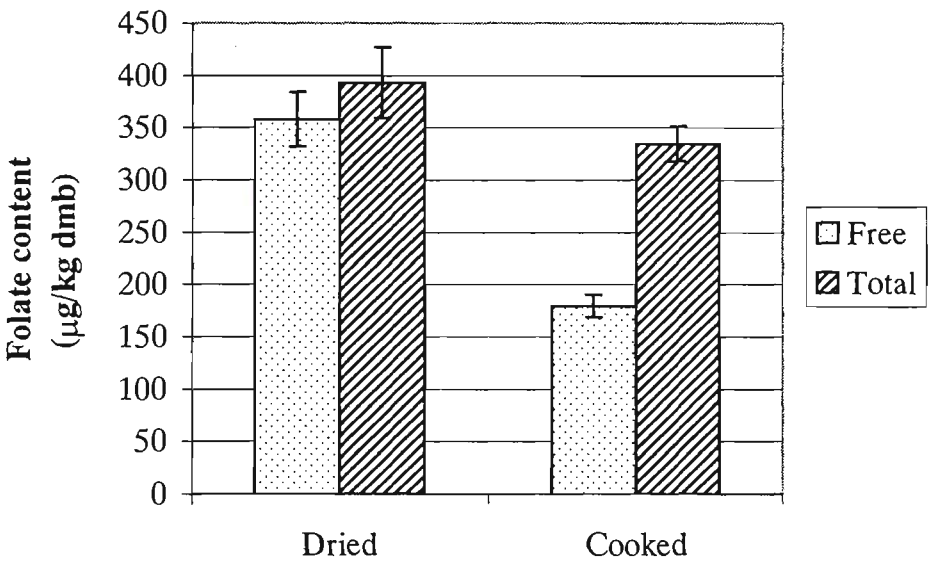


Figure 10.13 Folate contents of dried and cooked yellow alkaline noodles, (Gold star brand)

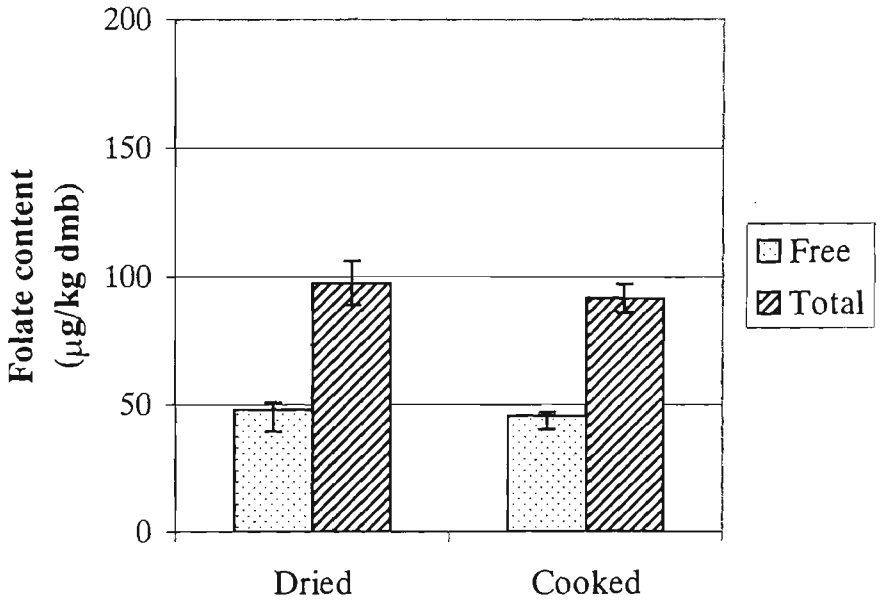


Figure 10.14 Folate contents of dried and cooked instant noodles (Nissin Demae Ramen brand)

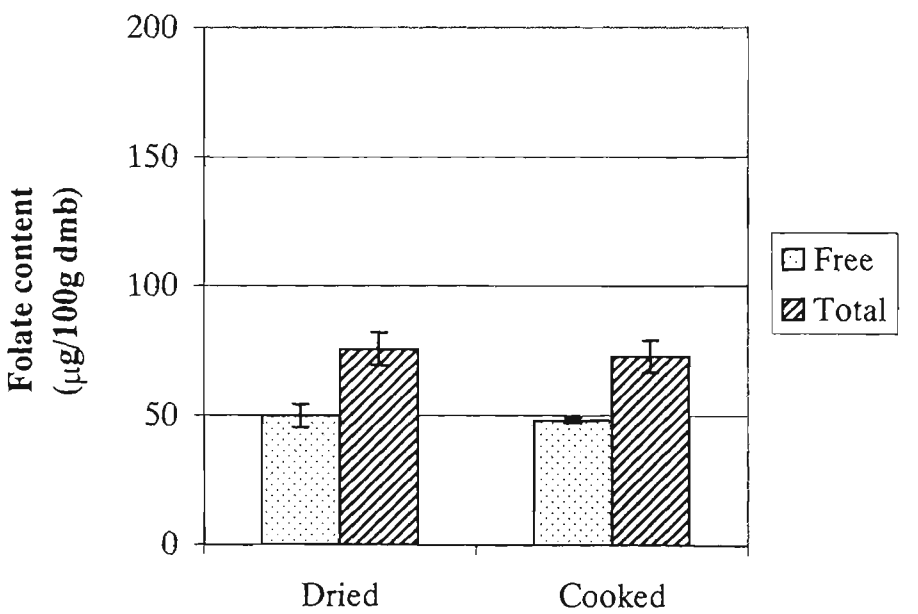


Figure 10.15 Folate contents of dried and cooked instant noodles (Marketta brand)

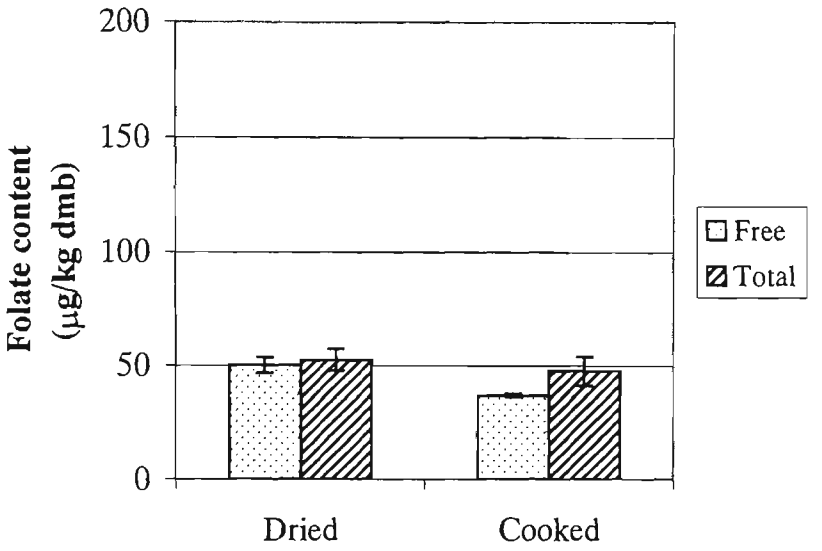


Figure 10.16 Folate contents of dried and cooked miscellaneous noodles (Wokka brand)

Table 10.13 A comparison of total folate contents and cooking losses for pasta and noodle products

	Folate content	Folate loss (%)	Reference
Spaghetti	0.24 (0.13-0.31)	21(12-46)	Ranhotra and others 1985
Egg noodles	0.26 (0.21-0.37)	21(1-42)	
Macaroni	0.19 (0.13-0.23)	23 (19-34)	
Enriched macaroni	0.12 (0.11-0.13)	nd	Douglass and Matthews 1982
Enriched noodles	0.16 (0.09-0.25)	nd	
Enriched pasta (four different products)	0.253-0.257	19.7-20.0	USDA ARS 2002
Noodles, Japanese, soba	0.064	59.7	
Noodles, Japanese, somen	0.015	59.6	

Notes
1 Vitamin contents are for dried noodles and are expressed in units of mg/kg on an as is basis
2 Loss values are the proportion of total folate measured in the dried noodle sample which was lost during cooking and are expressed as a percentage
3 Where necessary loss values have been calculated from data in the original references by first expressing folate contents on a dry matter basis
4 nd indicates not determined

The relative contribution of the commercial noodles to daily intakes of folate were calculated and are presented in Table 10.14. In each case the levels of folate in each serving are relatively low and none of the styles of noodles could be regarded as good sources of folate. The factors contributing to the variations found between the different samples of commercial noodles remains unclear. In addition to the possible contributions of non-flour ingredients and fortification, the differences might be due to variable folate levels in the wheat flours used, the steps and conditions of processing applied as well as sample handling and storage conditions. In order to further evaluate the significance of some of these variables, in the next stage of this study samples of each style of noodle were prepared and studied under controlled conditions in the laboratory.

Table 10.14 The weight of noodles containing the RDI amount of folate and the calculated amount of folate in a typical serving of each noodle sample RDI for commercial noodles before and after cooking

	Before cooking		After cooking	
	Weight of noodle	**Amount of folate in one serving**	**Weight of noodle**	**Amount of folate in one serving**
AOI	2.33	3.01	2.94	2.38
Shanghai	1.38	5.08	1.96	3.57
Golden Horse	2.17	3.22	2.99	2.35
Gold star	0.51	13.76	0.60	11.73
Nissin Demae Ramen	2.04	3.43	2.17	3.22
Marketta	2.63	2.66	2.74	2.56
Wokka	3.77	1.86	4.17	1.68

Notes 1 For weight of noodle in kg, units of folate contents are the amount of noodles (kg) needed to provide 200 ug folate per day

2 For serving size, units of folate contents are μg/35g of noodles expressed on an as purchased weight basis

10.11 The stability of folic acid during the processing of three styles of noodles prepared in the laboratory

Samples of the three styles of noodles were prepared in the laboratory. For this it was decided to use only one flour in order to facilitate direct comparisons between the styles of noodles and the processing conditions. In addition, all of the samples were fortified to the same extent. From the data in Table 10.6 the losses found for white salted noodles were similar regardless of the level of fortification and so the level of fortification selected was that corresponding to 50 percent RDI (Food Standards ANZ 2002). Samples were taken at the various stages of processing and folate contents analysed. The results are shown in Figures 10.17 - 10.19.

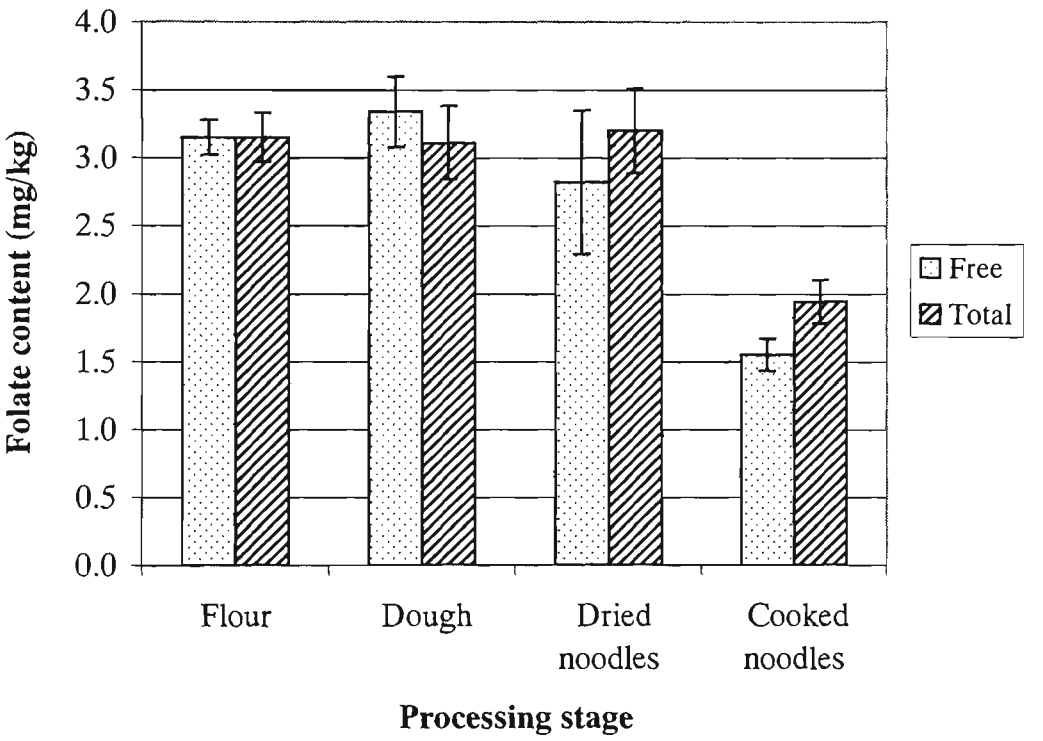


Figure 10.17 Folate content in white salted noodles during different stages of processing

Note Folate contents are presented as mean ± sd expressed in units of mg/kg on a dry weight basis

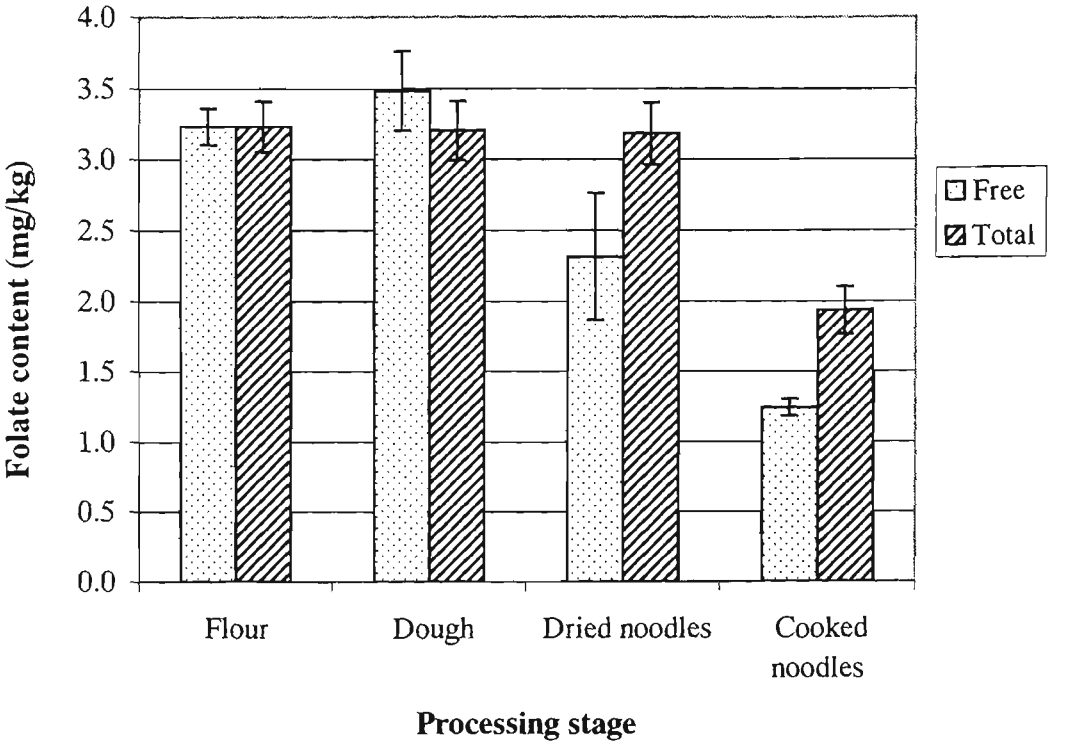


Figure 10.18 The folate content in yellow alkaline noodles during different stages of processing

Note Folate contents are presented as mean ± sd expressed in units of mg/kg on a dry weight basis

The patterns of results observed for white salted and yellow alkaline noodles are very similar. No significant decrease in total folate content occurred during the processing from fortified flour to the dried product. The step of drying at 40 °C had no impact on total folate contents. As the two styles of noodles were made using the same conditions, as well as the same flour and folate fortification level, it can be concluded that up until the cooking step, pH is not influencing the retention of folate in these products.

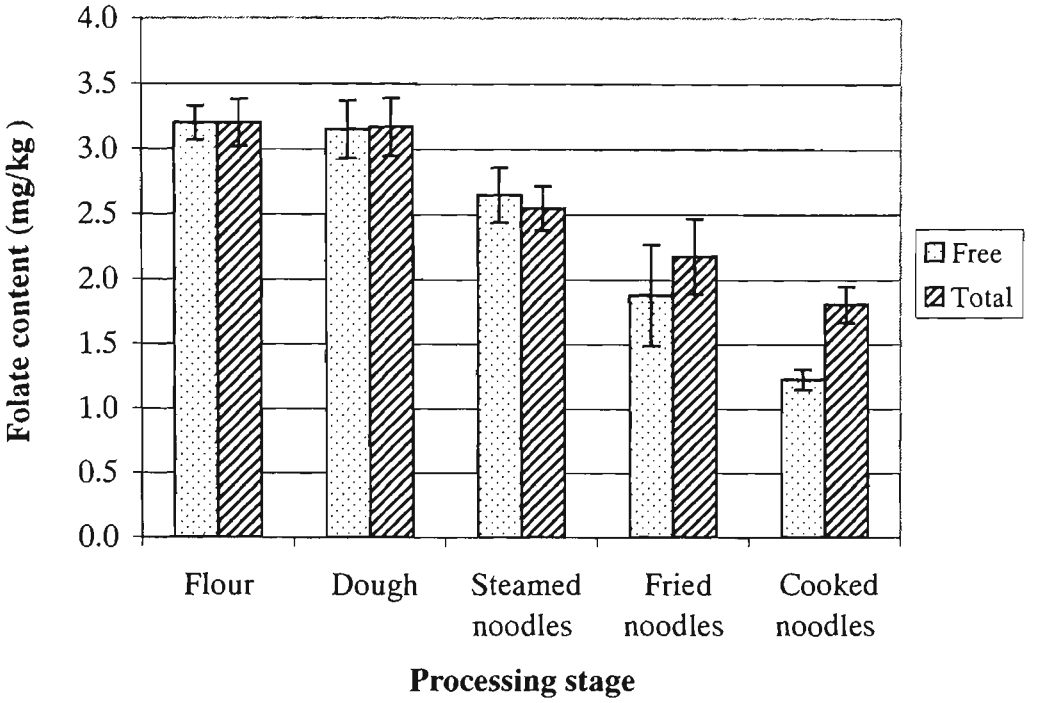


Figure 10.19 Folate content in instant noodles during different stages of processing

Note Folate contents are presented as mean ± sd expressed in units of mg/kg on a dry weight basis

Relatively large losses occurred when dried white and yellow noodles were cooked in boiling water with 38 percent losses in white salted noodles and 40 percent in yellow alkaline noodles. The results obtained here are similar to that found earlier (Table 10.6) for white salted noodles when varying fortification levels were considered. It is noted that the cooking times for the two noodle styles were chosen such that uncooked core was no longer observed. Thus the times were 3.50 min for white and 6.10 min for yellow noodles. This indicates that pH may have been a factor during boiling. Folate appeared to be more stable in the yellow alkaline noodles than at the lower pH values of the white salted product because similar losses occurred despite the differing cooking times.

For instant noodles, the overall loss in total folate was 44 percent. This appears to be similar to those found for the other two styles (Figures 10.17 and 10.18). However the pattern is different with significant losses occurring both during steaming of the noodle strands (100 °C for 2 min) and also upon cooking (100 °C for 3 min). Whilst some loss appeared to occur during deep-frying this was not significant. The formulation of instant

noodles prepared in the laboratory included sufficient kansui to raise the pH of the deep-fried product to 7.4. Again these results indicate that this addition of alkaline salt did not increase the loss of folate in the sample (Figure 10.18) when compared with the other two styles.

10.12 Observations on the significance of free and total folate contents of noodles during processing

During the analysis of the commercial and laboratory noodles reported here, both free and total folate contents were measured. This approach reflects that used in many recent studies (Shrestha and others 2000; DeVries and others 2001; Arcot and others 2002a, 2002b, 2002c; Johnston and others 2002a, 2002b; Rader and others 1998, 2000). In the current study, one of the purposes of monitoring free folates during processing has been to evaluate the usefulness of this measurement.

In summarising the data, the proportion of total folate found to be free in flour was 76 percent (Table 10.5). Similar or even higher proportions were found in most of the commercial dried noodles (Tables 10.7-10.10) although the values for instant noodles were typically much lower, averaging 59 percent. The significance of these results is not immediately obvious, but consideration of the changes during processing may provide further insights. The changes for each of the three styles of noodles prepared in the laboratory (Figures 10.17- 10.19) show that effectively the free folate was not significantly lower than the total folate levels in the flours or during dough mixing. However, when further processing involving drying or heating caused the proportions of free folate to decline. For each style there was an effective decrease in the proportion of free folate. The most likely explanation of these observations is that during processing of the noodles some of the folates are becoming trapped within the food matrix and cannot be effectively extracted unless the sample is treated with amylase and protease. These enzymes used in the determination of total folate allow release of all folate molecules into solution for effective assay. This explanation would be consistent with the further observation or decreased proportions of free folate for the various commercial noodles which were analysed before and after cooking (Figures 10.10-10.16).

The results found here indicate that the total folates results are more useful. In terms of nutritionally availability, the release of folates from a food matrix, particularly one in which starch has gelatinised, will be similar to that occurring during analysis of total folate. On this basis it is suggested that the measurement of free folate is probably not particularly useful. It is therefore recommended that enzymatic digestion by amylase and protease should be considered for the analysis of folates in foods. For further studies of cereal-based foods including noodles, it is recommended that the tri-enzyme method be applied for total folate.

10.13 The potential contributions of Asian noodles to dietary folate intakes

In order to evaluate Asian noodles as a potential source of folate, the results for samples prepared in the laboratory were recalculated to facilitate comparisons. The amounts of noodles required to provide RDI amounts of folate were estimated and the resultant data are presented in Table 10.15.

In this comparison white salted noodles were used as the example of unfortified noodles. These were chosen for comparative purposes as similar relative losses were observed for white salted and yellow alkaline noodles (Figures 10.13 - 10.14). The data for the control sample can be directly compared to that presented for a similar treatment presented earlier (Table 10.6) although here these are expressed on a different basis. The relative losses were the same in the two experiments.

For the unfortified noodles, the amount of folate in the dried noodles represented a relatively small proportion of the amount of folate required on a daily basis. The losses during cooking further decrease the amount provided by the noodles so that a serving provides less than 3 percent of the RDI value. Asian noodles therefore are not a good source of folate in the diet. However the data also show that noodles can provide an effective means of enhancing folate intakes. The addition of folate at a rate equivalent to 50 percent RDI per serving resulted in cooked noodles retaining folate corresponding to one third of the RDI. All three styles of noodle were fortified to the same level and the results clearly demonstrate that all are equally effective as sources of folate and can be used to supply folate in the diet.

Table 10.15 A comparison of the amount of noodles required to provide the RDI and the folate supplied in a serving of various noodles prepared in the laboratory

	Before cooking			After cooking		
	Folate in one serving	**Weight of noodle (kg)**	**Proportion of RDI in one serving (%)**	**Folate in one serving**	**Weight of noodle (kg)**	**Proportion of RDI in one serving (%)**
Unfortified noodles	7.5	0.93	3.8	4.9	1.44	2.4
White salted	112.2	0.06	56.1	68.0	0.10	34.0
Yellow alkaline	111.6	0.06	55.8	67.7	0.10	33.9
Instant noodles	76.5	0.09	38.3	63.7	0.11	31.8

Notes
1 The unfortified sample used in this comparison was of white salted noodles prepared in the laboratory without incorporation of additional folate
2 In the calculation of this data only total folate values were used
3 The weight of noodle values are the amount of noodles (kg) required in order to provide the RDI value of 200 μg folate
4 The folate contents of one serving are calculated on the basis of a serving of noodles being 35 g of product in the as is or as purchased form immediately prior to cooking, and expressed in units of μg

Within the Australian regulatory context, the levels of folate found in the experimental noodles after fortification would enable the products to be considered as good sources of the nutrient. Claims to that effect would then be permitted because the vitamin level provides at least 25 percent of the RDI per serve.

10.14 General discussion of folate contents, stability and fortification in Asian noodles

The microbiological method was chosen for this study as it is reported to measure all forms of folate when used in conjunction with a conjugase treatment step. This method is well established and widely adopted as the AOAC procedure. However, for sample

preparation, a number of recent reports indicate that the use of additional enzymes enhance extraction of folate for analysis.

In the current study the conditions for extraction of folate from flour and noodles were evaluated and it was found that:

- The use of tri-enzyme treatments gave significantly higher content values.
- The pH and specific conditions of enzyme treatment influenced the final results obtained.

It is concluded that the approach of Shrestha and others (2000) used in conjunction with chicken conjugase treatment gave the best results for measurement of Asian noodle products. The results presented here demonstrate that in applying well established methods for vitamin analysis, it is essential to consider extraction procedures effective for the specific food products being analysed.

For the analysis procedure adopted here for noodles samples it was found that the use of appropriate blanks was important. Enzyme only blanks were needed as the commercial preparation contained readily measurable levels of folate. With the use of a suitable number of replicates, it was possible to obtain reasonably reliable data. The validity of the procedure was evaluated using the AACC reference sample as well as flour and noodle samples. The results for the reference sample were consistently at the higher end of the range originally obtained in the AACC collaborative survey (17 ± 1 compared to a range of 11.60 – 16.20, Table 6.2).

During the current study, analyses were routinely carried out for extracts designated as free and total folate. The results obtained indicated that the amount of free folate tended to decline during processing of Asian noodles. This is interpreted as indicating that folate became bound chemically or was entrapped in the food matrix as a result of the elevated temperatures used during processing. The greatest decreases in free folate were observed for instant noodles where higher temperatures are used in preparation. It is concluded that the measurement of free folate is not a particularly useful procedure for studies such as that reported here.

Using the validated procedure for extraction and analysis, the folate contents of selected commercial noodle samples were measured and it was found that

- Higher folate levels were typically found in yellow alkaline samples compared to white salted and instant noodles.
- The pH value of the noodles did not appear to influence the level of folate in the dried products.

When samples of some of the noodles were cooked and folate levels analysed, it was found that:

- There was no clear pattern of folate losses. In addition, the relative losses for different brands were not related to the cooking time of the particular noodles analysed (Table 10.12).
- Again the product pH following cooking did not appear to impact on the loss of folate.

Samples of the three styles of noodles were prepared in the laboratory and the levels of folate analysed at each stage of processing. The results are summarised in Table 10.16 and these show that there were no losses of folate levels in white salted and yellow alkaline noodles during processing from the initial step of fortification of flour to the stage of drying of the noodles. For these noodles, the only significant losses occurred subsequently when the dried products were cooked and the folate contents decreased regardless of the alkaline or acid conditions in the noodles. In contrast, for instant noodles, losses of folate appeared to occur at each step during processing and including the ultimate boiling of the product. Thermal processing conditions including steaming (2 min) and frying (45 s) during manufacture of instant noodles as well as boiling for each of the different styles had an adverse influence on the stability of folate. Overall it appeared that losses were primarily associated with any step involving higher temperatures. Thus it is concluded that the drying temperature at 40 °C did not affect the stability of folate.

Table 10.16 Relative losses of total folate during processing of laboratory noodles

Noodle style	Processing stage			Folate loss	Cumulative folate loss
White salted	Flour	→	dough	1.3	1.3
	Dough	→	dried noodles	0	1.3
	Dried noodles	→	cooked noodles	40	41.3
Yellow alkaline	Flour	→	dough	0.93	0.93
	Dough	→	dried noodles	0.63	1.55
	Dried noodle	→	cooked noodles	39.3	40.9
Instant	Flour	→	dough	0.94	0.94
	Dough	→	steamed noodles	19.4	20.3
	Steamed	→	fried noodles	11.6	31.9
	Fried noodles	→	cooked noodles	11.6	43.4

Note All values are relative losses of folate expressed as percentages and calculated in comparison with the levels originally present in the flour after fortification
Based on the data presented in Figures 10.13-10.15

In comparing the overall losses of folate for the three styles of noodles prepared under carefully controlled conditions, the same overall losses were found for each. Thus after the three styles of noodles were boiled to their individual optimum cooking times, the total losses were 40 percent. In a further set of experiments, white salted noodles were fortified to different extents and the relative losses compared. Fortified noodles showed higher relative losses than unfortified. However when folate was added at levels of up to 70 percent RDI per serving, the relative losses were around 40 percent and the vitamin retained in the cooked noodles was approximately 42 percent of the RDI per serving. This demonstrates that noodles fortified with folate could be considered as a good source of this important micronutrient. It is concluded that each of the styles of Asian noodles can be used effectively for fortification purposes in order to enhance dietary intakes of folate.

Chapter 11

The measurement and stability of thiamin in three different styles of Asian noodles

The purpose of this chapter is to describe and discuss the results obtained during the analysis of Asian noodles for thiamin. These encompass the validation of the analytical method, along with results for commercial noodles and products prepared in the laboratory.

11.1 Introduction

Among the vitamins, thiamin was one of the earliest to be discovered. One of its roles is in carbohydrate metabolism and there continues to be concerns about the adequacy of intakes in many countries. Cereal grain foods are recognised as a good source of thiamin and, in some countries, have been chosen as staple foods suitable for fortification in order to ensure that dietary supplies are satisfactory. However, it is one of the least stable of the vitamins and accordingly the aim of this phase of the study has been to establish the levels of thiamin in Asian noodles along with its stability during processing and storage. In addition, the suitability and potential of noodles to enhance dietary intakes through fortification has been investigated.

11.2 Selection of a suitable method for thiamin assay

Based upon a comprehensive review of the literature (see section 4.3), various optional methods for analysis of thiamin were considered, including the internationally accepted standard methods of the AOAC (1990d), AACC (1994a) and ICC (1990), in addition to newer HPLC procedures published during the past decade.

The AOAC procedure and many using HPLC are based upon the conversion of thiamin to thiochrome which is more readily measured due to its fluorescent properties. In an earlier phase of this study, the use of various HPLC methods was evaluated. In further preliminary work, it was found that the requirement for pre-column oxidation of sample

extracts for HPLC meant that the latter approach gave no advantages for the foods of interest here. In addition, the sensitivity of the AOAC method was advantageous in the analysis of at least some noodle samples. The AOAC method is accepted internationally and offers the advantage of being well established and reliable. It can be carried out rapidly, economically and consequently is applicable to routine determination of thiamin (Ellefson 1985b). Precision is known to be generally very good for this chemical procedure.

Within the AOAC standards, there are two separate fluorimetric procedures (method numbers 942.23 and 953.17 (AOAC 1990i, 1990d respectively) which can be used for the determination of thiamin in food products. However, the latter method was chosen for this study as it has been widely applied in determining the thiamin contents in food products including those of cereal origin where the amounts of bound thiamin and thiamin pyrophosphate are not significant (AOAC 1990d). For the fluorimetric method the enzyme digestion (942.23C) and Bio-Rex 70 purification (942.23D) steps are eliminated. This, in turn, enhances throughput and reliability of the results (Eitenmiller and Landen 1995).

In the fluorimetric procedure the food is extracted in dilute acid which stabilises thiamin and also effects complete solubilisation of various forms of the vitamin present. Subsequently, thiamin is oxidised by potassium ferricyanide in the presence of strong alkali to yield thiochrome (as described in section 4.3). The measurement is based on solubility of thiochrome in 2-methyl-1-propanol and the yield is very constant under standardised conditions (Ellefson 1985b). The procedure results in the concentration of the thiochrome and, in the presence of alcohol, fluorescence intensifies. Hence, extraction into 2-methyl-1-propanol not only functions in separation of the thiochrome from interfering substances but also increases the sensitivity of the assay (Gubler 1991).

11.3 The establishment of the fluorimetric method for thiamin analysis

In setting up the AOAC procedure for thiamin, it was necessary to select a suitable slit width value from those available (slits 3, 5 and 10). Firstly, slit 10 showed very high readings (>1000) indicating concentrations that were too high to measure. In the case of fluorescence measurements, the concentration of sample must be kept low enough to

avoid inner filter effects, while being high enough to provide an adequate supply of photons to the detector (Harris 1996). Therefore, slit 10 was found to be unsuitable for determination of thiamin contents in this case. In order to select the optimum conditions for assay, a series of cereal samples were analysed for thiamin contents and fluorescence readings taken for slit settings 3 and 5. The resulting thiamin content data showed virtually no difference between the two slits. Setting 5 was selected for all further experiments on the basis that this gave results having less variability than those for slit 3. This is consistent with the general principle that as wide an excitation slit as possible should be selected in order to ensure that sufficient light reaches the sample (Harris 1996).

11.4 Reliability of thiamin analyses

It has been reported that the reproducibility of results for the thiochrome procedure may be a problem unless the analyst is careful and adopts good technique (Ellefson 1985b). Therefore preliminary studies were performed to establish the reliability of the assay used. Two approaches were used (Section 6.11) and the results for these are presented in Table 11.1. Firstly, two different commercial flour samples were analysed repeatedly over a period of five weeks. In addition, the chemical reference (quinine sulphate solution) recommended in the standard procedures was routinely analysed whenever the fluorimeter was used for thiamin testing.

The results show that there was considerable difference in the thiamin contents between the two flours and this issue is further addressed later in Section 11.6. There was also some variation from day to day for each flour. The variability was greater for the Continental Farina which had the lower thiamin level. The coefficient of variability value for both flours can be compared with published data. In one study of the fluorimetric procedure, yeast, rusk and flour samples gave coefficients in the range of 4.2 to 6.5 percent (Brubacher and others 1985d). In another study a coefficient of 3.7 percent has been reported (ICC 1990). In the ICC standard, it was also noted that a study found that coefficients of up to 13 percent are commonly found for vitamin determinations.

Table 11.1 The results of repeatability studies for analyses for thiamin in flour samples and fluorescence readings of the quinine sulphate standard

Description	Thiamin analyses of flour		Fluorescence of quinine sulphate
	Continental	Bakers	
Time span of analyses	5 weeks	5 weeks	2 months
Number of replicates	21	21	36
Mean value	2.06	11.55	54.5
Range of results	1.89-2.28	10.99-12.22	52.3-57.6
Standard deviation	0.12	0.26	1.50
Coefficient of variability (%)	5.80	2.25	2.74

Note Thiamin values are presented as mg/kg on a dry weight basis

The coefficient found for fluorescence readings of the quinine sulphate solution demonstrate the consistent performance of the fluorimeter. It was concluded that the level of repeatability of the method is within reasonable and normal levels. A further series of analyses was then carried out using the AACC reference sample for which analytical results were already available. The results are presented in Table 11.2 and show that the variability was acceptable and that the mean result found here is very close to that found in the original AACC collaborative survey involving 14 laboratories (Table 6.2). On the basis of these studies (Tables 11.1 and 11.2) it was concluded that the procedure was valid for the current investigation. Hence it was used for all further studies on thiamin in Asian noodles reported here.

Table 11.2 The results of repeatability studies for thiamin analyses of the AACC reference sample

Description	Thiamin analyses
Time span of analyses	3 weeks
Number of replicates	12
Mean value	71.8
Range of results	63.7-78.7
Reference values	69.3 (60.6-82.8)
Standard deviation	6.5
Coefficient of variability (%)	9.03

Notes 1 Thiamin values are presented in units of mg/kg and expressed on an as is moisture basis
2 Reference values are from Table 6.2 presented as mean (range)

11.5 Analysis of commercial noodle samples

After selecting a suitable analytical method for thiamin and establishing the repeatability of the procedure, it was applied in a study of a series of noodles obtained from retail sources. The noodles selected all had wheat flour as the primary ingredient. For each of the major styles of Asian noodles, samples of five different brands were obtained from retail outlets, giving a total of fifteen samples. In addition, the selection was made to include noodles from different countries of origin and also having a variety of ingredients. The details of the fifteen samples are presented in Chapter 6, Tables 6.6–6.8.

The noodles selected were dried noodles as these are the most popular type of noodle in Asian countries. Prior to analysis, a commercial grinder was used to prepare the dried noodles. Each was tested for moisture content, thiamin content and pH value and the results are presented in Tables 11.3 and 11.4.

The decision to measure pH was based upon the fact that different noodles are expected to vary in pH depending on ingredients, particularly alkaline salts, used in yellow alkaline and to a lesser extent in instant noodle manufacture (Moss and others 1987).

The procedure used for measurement of pH involved mixing a sample with water and measuring the supernatant as described in the AACC Standard (AACC 1994b).

Each noodle sample was also analysed after cooking. In selecting conditions for cooking each sample, a number of possibilities were considered including cooking all for the same length of time, cooking according to recommendations on the labels and cooking until optimum cooking time had been reached. It is noted that no suggested cooking times were provided for the yellow alkaline noodles studied in this phase. For the latter type, the optimum time was assessed and used. For the other samples, each was firstly cooked for the period of time suggested. A noodle strand was then evaluated and for those which were not fully cooked, the boiling was continued with monitoring up to the point where cooking was found to be complete. It is noted that, for a number of the samples, the optimum times determined and used here did not reflect those recommended on the packaging.

11.5.1 Moisture analyses

The moisture contents of the dried and cooked noodle samples were analysed primarily to allow recalculation of thiamin data to a constant moisture basis. However, the results are also interesting as they show a range of values for the different samples (Table 11.3). The dried white salted and yellow alkaline noodles had moistures in the range of 12.1 to 15.7 percent. This contrasted with the instant samples which ranged from 7.1 to 9.6 percent, resulting from the greater loss of moisture during deep-frying. Samples of all three types absorbed large amounts of water upon cooking although the final moisture values for instant noodles tended to be lower. This may reflect the presence of oil absorbed during the drying stage of the manufacturing process.

Table 11.3 Moisture contents of selected commercial noodle samples in the dried form and following cooking

Sample description	Uncooked	Cooked
White salted noodles		
Dragons	13.1 ± 0.21	79.5 ± 0.42
Enriched	13.4 ± 0.15	80.2 ± 0.07
Thin Kanto	15.5 ± 0.3	80.6 ± 0.99
Tomoshiraga	15.3 ± 0.1	82.5 ± 0.61
Yellow alkaline noodles		
Song Long	13.2 ± 0.5	80.2 ± 1.63
Vegetarian	15.2 ± 0.16	85.0 ± 0.27
Mai Hong	13.5 ± 0.17	81.8 ± 0.42
Nouilles	15.7 ± 0.19	81.1 ± 0.64
Fantastic	12.1 ± 0.28	81.4 ± 0.99
Instant noodles		
Ma Ma	7.1 ± 0.21	78.4 ± 0.27
Ma Ma Mien	9.5 ± 0.14	77.2 ± 0.59
Trident	9.3 ± 0.42	78.9 ± 0.57
Wai Wai	9.6 ± 0.14	78.9 ± 0.58
Indomie	9.1 ± 0.07	79.5 ± 0.34

Note Results are presented as mean ± sd and are expressed in units of g per 100g

Table 11.4 Thiamin contents and pH values of commercial Asian noodle samples in the dried form and following cooking

Sample/ recommended cooking time	pH		Thiamin content		Relative loss of thiamin (%)
	Before cooking	After cooking	Before cooking	After cooking	
White salted noodles					
Dragons (2 min)	5.88	6.27	1.55 ± 0.07	0.49 ± 0.03	68.4
Enriched (3 min)	5.83	6.22	0.81 ± 0.05	0.25 ± 0.03	69.1
Thin Kanto (3 min)	5.71	6.17	0.76 ± 0.07	0.41 ± 0.05	46.1
Tomoshiraga somen (3 min)	5.65	6.19	1.28 ± 0.09	0.46 ± 0.04	64.1
Average	**5.76**	**6.28**	**1.36**	**0.51**	**61.7**
Yellow alkaline noodles					
Mai Hong	7.85	6.95	0.28 ± 0.05	0.12 ± 0.06	57.1
Fantastic	10.36	9.62	0.36 ± 0.04	0.21 ± 0.03	41.7
Song Long	5.55	6.26	1.76 ± 0.06	0.66 ± 0.04	62.5
Nouilles Chinois	4.86	5.59	0.69 ± 0.04	0.27 ± 0.04	60.9
Vegetarian	9.89	8.81	0.11 ± 0.02	0.08 ± 0.02	27.3
Average	**7.70**	**7.45**	**0.64**	**0.27**	**49.9**
Instant noodles					
Mama (3 min)	6.39	7.66	0.83 ± 0.15	0.28 ± 0.02	66.3
Indomie (3 min)	6.62	7.22	1.46 ± 0.08	0.29 ± 0.04	80.1
Ma Ma Mien (3-4 min)	6.22	7.02	0.84 ± 0.05	0.26 ± 0.03	69.1
Trident (2 min)	6.77	7.78	1.55 ± 0.07	0.45 ± 0.04	70.4
Wai Wai (2 min)	6.12	7.18	0.66 ± 0.09	0.19 ± 0.03	71.2
Average	**6.42**	**7.37**	**1.06**	**0.29**	**71.4**

Notes 1 Cooking times were those recommended on product labels - no times were provided for the alkaline noodles

2 Thiamin results are presented as mean ± sd and expressed in units of mg/kg on a dry weight basis

11.5.2 Thiamin contents of commercial white salted noodles

The results obtained for white salted noodles (Table 11.4) showed thiamin levels in the range of 0.76–2.41 mg/kg prior to cooking. The lowest thiamin was measured in Thin Kanto noodles although this product had the same ingredients. The average value for the five noodle samples was 1.36 mg/kg.

The ingredients listed in Table 6.5 show that each of the brands of white salted noodles are basically made from a mixture of flour, water and salt. The quantity of salt may be adjusted according to noodle type, market requirements and climate. For example, dry noodles require more salt (3 percent) than boiled noodles (2 percent) and less salt is required in winter than other seasons. The volume of water is also adjusted for flour quality, type of mixer and climate (Nagao 1996).

These variations in ingredients and methods of noodle making probably explain the differences in thiamin contents found for commercial white salted noodles. These may also result from the noodles being made from flours having diverse origins. The proportions of ingredients in the noodle formulation and the processing conditions varied according to the type of product. From the ingredients listed, white salted noodles contained normal salt that did not influence the pH or noodle colour. A creamy white appearance was observed for these samples. The pH levels of these samples was tested before and after cooking. For this the standard procedure described in Section 6.4.2 was followed. pH values of the samples were found to range between 5.65–5.88 before cooking.

Noodle samples were cooked in the same way as might be done by a consumer in the home. During cooking, the noodles absorbed water and the level of water absorption depended on the size of noodle strands and this varied between to brands studied. The pH of cooked noodle samples was found to be in the range of 6.17-6.55. These values again showed relatively little variation in pH levels of commercial white salted samples either before or after cooking. There was a significant effect on thiamin contents of the samples during cooking. It was found that thiamin losses on cooking of the different dried noodles varied from 46.1-69.1 percent. There was some variation between the samples and the average loss was 62 percent.

11.5.3 Thiamin contents of commercial yellow alkaline noodles

The results for yellow alkaline noodles (Table 11.4) showed variations between the samples. Different ingredients were declared (Table 6.6) and the levels of thiamin ranged from 0.11 to 1.76 mg/kg with an average value of 0.64 mg/kg which is lower than that found for white salted style noodles. Of the different brands of yellow alkaline noodles, only one brand declared alkaline salts as an ingredient (Mai Hong). Others did not clearly declare type of salt was used (Fantastic, Nouilles Chinois), or even completely lacked any declaration (Song Long). It is noted that the reference to water ash for the Vegetarian brand probably implies alkaline salts.

The pH levels of these samples varied from 4.86 to 10.36 before cooking and 5.59 to 9.62 after cooking. This level of variation was much higher than expected. These variations also affected the colour of noodles, with a range from bright to dark-dull yellow appearance. It is known that there is a relation between flour quality and alkaline salt which changes the pH levels as well as the brightness and yellowness in yellow alkaline noodles (Huang 1996a). Normally, the pH of alkaline noodles would be expected to be in the range of 9-11 and this typically produces a yellow colour. However, there were three out of five samples with different yellow coloration which measured as being slightly acidic (Song Long, pH 5.55 and Nouilles Chinois, pH 4.86) as well as neutral level (Mai Hong, pH 7.85). Mai Hong clearly declared the artificial colour additive sunset yellow in the ingredients, the others did not. It is possible that the yellow appearance of other samples may have been due to a colorant additive although these were not listed as ingredients.

Even though the five samples were yellow alkaline style noodles, the data shows much greater variation in these products than that found for white salted noodles. From the pH levels measured (Table 11.4) and the colour observed visually, it is likely that the quality of wheat flour, the amount of kansui, and the processing methods varied from brand to brand.

From the results, there appears to be a relationship between the thiamin contents and pH levels of the yellow alkaline noodle samples. It is known that thiamin is rapidly destroyed in neutral and alkaline solutions (Gregory 1996). For three yellow alkaline

noodle samples before cooking: Fantastic (pH 10.36), Mai Hong (pH 7.85) and Vegetarian (pH 9.89) thiamin was measured at very low amounts: 0.36, 0.28, and 0.11 mg/kg respectively. On the other hand, thiamin contents were much higher in the other two samples which were slightly acidic: Song Long 1.76 mg/kg, and Nouilles Chinois, 0.69 mg/kg. Based on the pH measured of Song Long and Nouilles Chinois noodles, it is suggested that there was no alkaline salt present in the formulations and the salt listed on the labels was common salt only. The yellow colour could be a result of incorporation of an additive colorant. The reason may be that in recent years, consumers continue to demand products having yellow colour but prefer lower levels of alkaline salt (Kubomura 1998).

After cooking, the two acidic samples also retained a higher thiamin contents than others and the highest relative thiamin losses were found. As a result of the instability of thiamin at elevated temperatures, losses occurred in all samples. From the results, it appears that the higher the thiamin content, the greater the thiamin loss.

11.5.4 Thiamin contents of commercial instant noodles

Many brands of instant noodles are available in the marketplace and five different brands were selected for the current study. According to the ingredients listed on the labels, only Indomie specifically declared the use of alkaline salt (mixture of potassium carbonate and sodium polyphosphate), the others simply listed 'salt' in the ingredients. The results (Table 11.4) show the pH values of instant noodles did not vary very much and were in the range of 6.12-6.77 before cooking and 7.02-7.78 after. From the data, it can be concluded that all these instant noodles incorporated alkaline salt at low levels and this increased the pH of noodles one unit as compared to the pH levels of white salted noodles containing common salt only (refer to Table 11.4 and Table 6.7).

In dried form (before cooking), the observed colour of the instant noodles varied from bright to dark yellow. This was probably a result of the additional processing steps of steaming and frying where frying time and temperature are critical control points influencing product quality. Dark yellow colour in some samples may be caused by browning reactions during frying. Other factors influencing colour include quality of flour and oil as well as the use of alkaline salt as ingredients.

The results demonstrate that the pH, ingredients and processing methods of instant noodles influence the thiamin contents. Relative thiamin losses were found to vary in the range of 66.3–80.1 percent. These were the highest values in comparison with those for white salted and yellow alkaline noodles.

11.5.5 Discussion of thiamin data for commercial noodle samples

In considering these results it is noted firstly that the packaging of the samples did not contain any nutrient data with which the analytical results may be compared. Secondly, there is virtually no data in any food composition tables with which direct comparisons may be drawn. It is has been reported that noodles (chow mein) contains no thiamin (Briggs and Wahlqvist 1988). The only other data for Asian noodles which could be found are values for two samples of white salted noodles which were reported to contain 1.1 and 5.2 mg/kg (USDA ARS 2002). The lower of these values is similar to the results found here (Table 11.4). It is likely that the higher value reflects fortification of the flour used in manufacture. Whilst some data is also available for durum products, these are US values and also demonstrate fortification. These data are further discussed later in the context of a discussion of thiamin losses during cooking of Asian noodle products (see Section 11.9 and Table 11.10).

In comparing the pH levels of commercial noodle samples (Table 11.4) white salted noodles had the lowest pH levels before and after cooking, due to the use of common salt as an ingredient. In contrast, highest pH levels were found in the yellow alkaline style resulting from the addition of alkaline salt to produce the desired yellow colour. The average pH values of instant noodles were within the neutral pH range and were quite similar to each other. Generally, the results indicated that the variation of pH levels of different noodle styles was primarily due to the varying use of alkaline salts as ingredients. For the samples with alkaline pH values, it was found that the pH levels reduced one unit after cooking (refer to Table 11.4). This may be a result of leaching of some of the alkaline salts from the noodles during cooking.

The data comparing thiamin (Table 11.4) shows white salted noodles to have higher levels although there is clearly variation between individual samples for each style of

product. During exposure of noodles to heat (cooking process), losses of thiamin occurred for all three styles. The average retention of thiamin after cooking ranged from 0.51, 0.27 and 0.29 mg/kg for white salted, yellow alkaline and instant styles respectively. Due to its water solubility, losses of thiamin from noodles may be a result of leaching of during cooking. Higher thiamin contents remained after cooking for samples having the higher levels before cooking (refer to Table 11.4).

Although variations occurred between the styles of noodles, the most obvious conclusion is that the average loss of thiamin during cooking is high for all styles. This result might be expected, as it is known that boiling and heat can cause losses of thiamin (Holland and others 1991). There are two possible explanations for the apparent losses of thiamin during boiling of the noodle samples. Firstly chemical breakdown of the thiamin molecule may occur due to the heating. Secondly leaching and release of thiamin from the noodle into the boiling water might occur. The results obtained do not clearly identify whether one or both of these have occurred.

The results for the noodles clearly show that the thiamin content of the five white salted samples varied quite markedly. The stability of the thiamin in these noodles was similar as demonstrated by the proportional losses observed. The level of variation observed for the instant noodles was also quite high. Less variation was found for yellow alkaline noodles and this probably reflects the relatively low thiamin contents of the samples. It is concluded that considerable variation exists in the potential contribution of different brands of Asian noodles to dietary thiamin consumption. In addition, even for five samples of white salted noodles, there is considerable variation. Similar variability was seen for the five instant noodle samples. In the case of the yellow alkaline noodles, formulation and pH varied widely and these factors may at least partly explain the differences in thiamin contents and stabilities measured for these samples.

In attempting to explain the variations observed here, it is noted that the original thiamin contents of the flours used in noodle manufacture may have varied. This could partly explain some of the results. In addition, differences in processing of the samples may have been important. In order to investigate these issues, a laboratory study was developed in which Asian noodles were prepared under controlled conditions and using defined ingredients.

11.6 Measurement of thiamin in flour samples

It is known that a number of factors influence the potential use of wheat flours and these include variations in the milling extraction rates, inherent flour quality characteristics as well as the nutritional value (Ranhotra 1994; Kent-Jones and Amos 1967). Therefore, in this phase of the study, the first step was to look at the thiamin contents of commercial flour samples which might be used for further experimentation. The moisture contents and thiamin values of six commercial flour samples are presented in Table 11.5.

Table 11.5 Moisture and thiamin contents and pH levels of different types of commercially milled flours

Brand	Ingredients (as listed)	Thiamin content	Moisture content	pH
Continental Farina	Flour, vitamin (thiamin)	2.09 ± 0.12	12.1 ± 0.07	5.95
Maximus	Flour, vitamin (thiamin)	11.96 ± 0.26	13.1 ± 0.35	6.05
Defiance	Unbleached wheat flour	5.73 ± 0.21	13.6 ± 0.26	5.84
Mc.Alpin's	Wholemeal flour, baking powder	3.38 ± 0.14	11.7 ± 0.09	6.67
Milano	Flour, vitamin (thiamin)	13.86 ± 0.26	13.1 ± 0.11	5.92

Notes 1 Thiamin data are presented as mean ± sd and are expressed in units of mg/kg on a dry weight basis
2 Results of moisture content are presented as mean ± sd and are expressed in units of g/100g

The results highlight the variability in thiamin contents of commercial flour depending on whether fortification with thiamin had been carried out. In order to facilitate interpretation of these results, a further series of flours was also analysed. These samples were obtained from laboratory millings of wheat grown in different regions of Australia. These provide information on the inherent levels of thiamin present in flours that had not been fortified at all. The results for the laboratory-milled flours are presented in Table 11.6.

Table 11.6 Thiamin and moisture contents of flours milled in the laboratory from a variety of wheat types

Sample type	Port zone	Thiamin content	Moisture content
Australian Soft	Albany, WA	0.87 ± 0.03	13.6
US Hard Red Winter	Not known	1.18 ± 0.07	13.7
Australian Premium White	Port Adelaide, SA	1.83 ± 0.04	13.7
Australian Premium White	Brisbane, Qld	1.75 ± 0.04	13.8
Australian Premium White	Geelong, Vic	1.37 ± 0.08	14.6
Australian Prime Hard	Port Kembla, NSW	2.2 ± 0.4	13.8
Australian Hard	Newcastle, NSW	3.0 ± 0.1	13.7

Notes 1 Thiamin data are presented as mean ± sd and expressed in units of mg/kg and on a dry weight basis
2 Results of moisture content for these samples were kindly supplied by Agrifood Technology and are expressed in units of g/100g

Firstly the results show typical moisture contents as might be expected for milled flours (Table 11.6). The values have been used in calculating the corrected thiamin results. The results for commercial flours shown in Table 11.5 demonstrate that the pH of typical flours is similar to that of white salted noodles (Table 11.4). The only higher value was seen for the self-raising flour in which the addition of aerating agents has probably influenced the pH of the sample. In relation to the thiamin contents in commercial flours, there is considerable variation with the range being 2.09 to 13.9 mg/kg. Clearly the higher values reflect fortification of the flour at the mill. In Australia, since 1992 it has been a mandatory legal requirement that flours for breadmaking be fortified with thiamin (Lewis and others 2003). The minimum level is 6.4 mg/kg. Thus the breadmaking flour analysed here complies with the standard with a value well in excess of that required. Flours for purposes other than breadmaking are not required to be fortified. Of the samples analysed here (Table 11.5) the pizza flour appears to have been fortified and this is consistent with the declared ingredient list on the packaging. On the other had, the flour with the lowest thiamin level was Continental Farina (2.09 mg/kg) and this was labeled as having been fortified.

In order to provide further data for comparative purposes, the laboratory milled flours showed thiamin contents of 0.85-1.83 mg/kg. The flours had been milled to extraction rates of around 75 percent, from wheats grown in different Australian states. The original grain samples also included the full range of types available in Australia representing each of the primary commercial classes of wheat available. Therefore the range found here demonstrates the range of thiamin contents that might be encountered in straight-run flours which have not been fortified. In comparing the results for the laboratory and commercial flours, the latter are higher. The results lead to the conclusion that each of the commercial flours had been fortified to at least some extent at the flour mill.

The sample of US wheat was analysed as it was available to the study (Table 11.6). However, no conclusions can be drawn from the analysis of one individual sample. It has been reported in early studies that wheat from different countries may vary in thiamin levels (Kent-Jones and Amos 1967). Some of the early data also indicated that Australian wheats may have had higher thiamin. Some recent literature values are presented in Table 11.7. These demonstrate variability in the contents of thiamin in flours within limited geographical areas and also apparent differences for different areas. The extent to which fortification explains some of the data is not clear. The information available does indicate that wheats from different origins may vary in thiamin contents. There is no recent data to confirm this and further studies may be warranted to clarify these issues.

The likely impact of flour vitamin contents on levels in the resulting noodles is not readily predicted. Accordingly, two of the flours analysed for thiamin were then selected for further studies. Continental Farina was chosen for preparation of white salted noodles in the laboratory and Maximus for yellow alkaline and instant noodles. These choices were based upon the known characteristics of the flours and their suitabilities for the specific products (Crosbie and others 1998).

Table 11.7 Thiamin contents of wheaten flours reported in literature sources (white milled flours only)

Flour origin and description	Thiamin content	Reference
Australia, various samples (pre-fortification)	2.2–2.7	Saxelby and Brown 1980; English and others1990
Australian study with eight samples (after introduction of mandatory fortification)	4.0–8.5	Menz and others 1995
Europe, six types	0.6–5.6	Scherz and Senser 2000
UK, three types	3.0–3.2	Holland and others 1991
US, five types	6.74–8.92	USDA ARS 2002

Note Thiamin data are expressed in units of mg/kg on an as is basis

11.7 Laboratory studies of the influence of noodle processing on thiamin contents

The next objective was to study the influence of processing and the impact of processing parameters on thiamin losses. All three styles of noodles were prepared in the laboratory and samples were taken at each stage of processing and also following storage under various conditions. The moisture contents were measured and similar patterns were found to those tabulated in earlier stages of this project (Table 8.7). The specific results are not presented here, but were used in calculation of data obtained when thiamin was analysed. The thiamin contents of white salted and yellow alkaline noodles at each stage of processing were analysed and are shown in Figures 11.1–11.2.

The results show that relatively little loss occurred for white salted noodles at each step from flour to dough to the dried noodles. However, half of the thiamin was lost during the cooking of the dried prepared noodles. In the case of the yellow alkaline style noodles, there was clearly loss of thiamin at each stage of noodle processing, both during preparation prior to cooking and subsequently upon cooking.

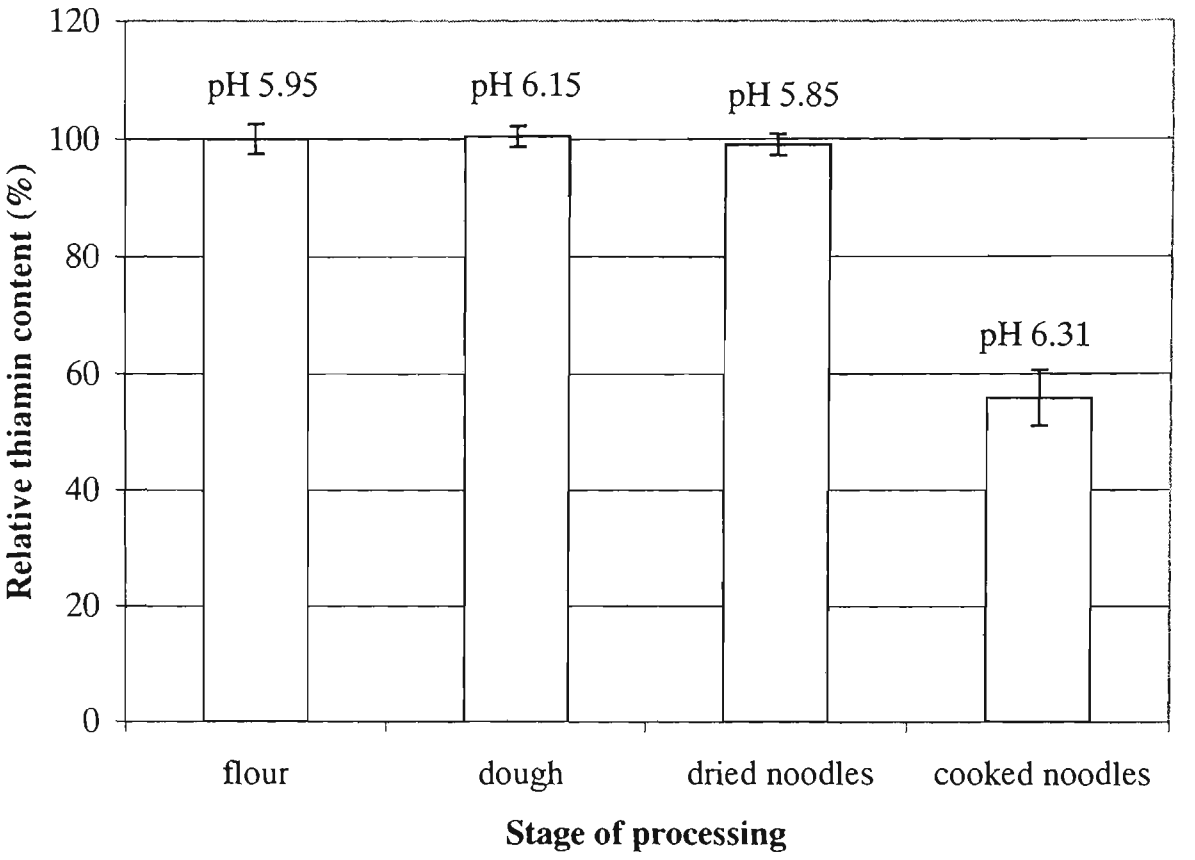


Figure 11.1 Relative thiamin contents and corresponding pH values at different stages during processing of white salted noodles

Note Thiamin values are means ± sd, expressed as a percentage of the amount present in the flour, calculated after all values had been adjusted to a dry weight basis

Instant noodles were also prepared in the laboratory using a process typical of current commercial practice and the results of thiamin analysis of samples taken at various stages are shown in Figure 11.3. The results indicate that relatively little thiamin was lost during dough preparation with some loss during the deep-frying step. As well as decomposition due to heating, losses of thiamin have to be considered through dissolution in water. Leaching into surrounding aqueous media may also be one of the causes contributing to the losses of thiamin during cooking.

The pH characteristics of the different noodle styles were measured to identify if there were any major differences during the processing which might relate to thiamin losses. These results are also presented in Figures 11.1-11.3. These pH data show that the typical values were slightly acidic for white salted and relatively alkaline for yellow alkaline noodles. The value for instant noodles was also alkaline (pH 7.41) consistent with the addition of the alkaline salts but at lower levels (0.1 percent) than those for

yellow alkaline noodles (1.0 percent). There were some changes in pH during cooking, particularly for yellow alkaline noodles where the pH dropped from 10.6 to 8.8. This may reflect leaching and loss of the salts into the boiling water.

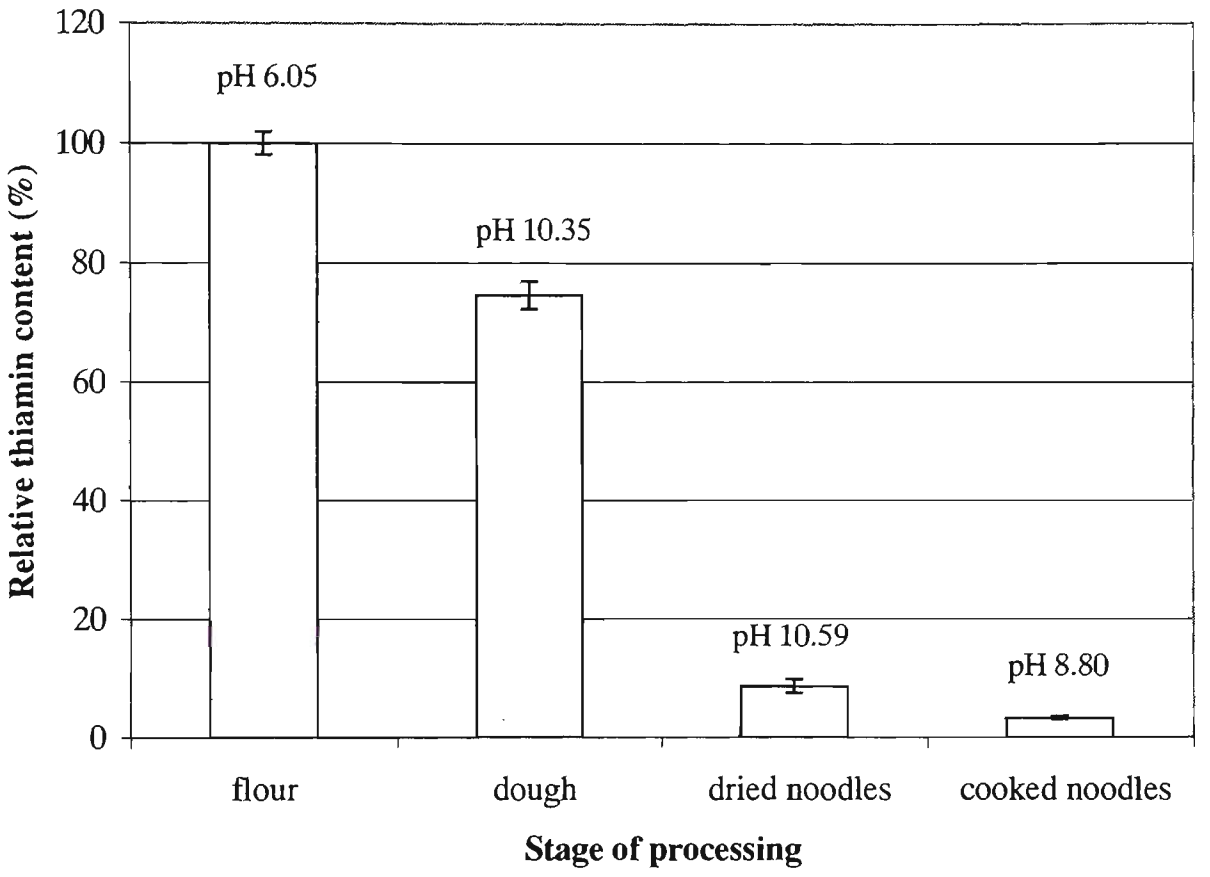


Figure 11.2 Relative thiamin contents and corresponding pH values at different stages during processing of yellow alkaline noodles

Note Thiamin values are presented as mean ± sd, expressed as a percentage of the amount present in the flour, calculated after all values had been adjusted to a dry weight basis

Results of the thiamin contents and pH studies provided differentiation among flours and noodles. In addition, the thiamin results can be directly related to pH parameters in both cooked and uncooked noodles. These demonstrate that the variation in ingredients and processing accounts at least partially for differences in thiamin levels of different noodle styles.

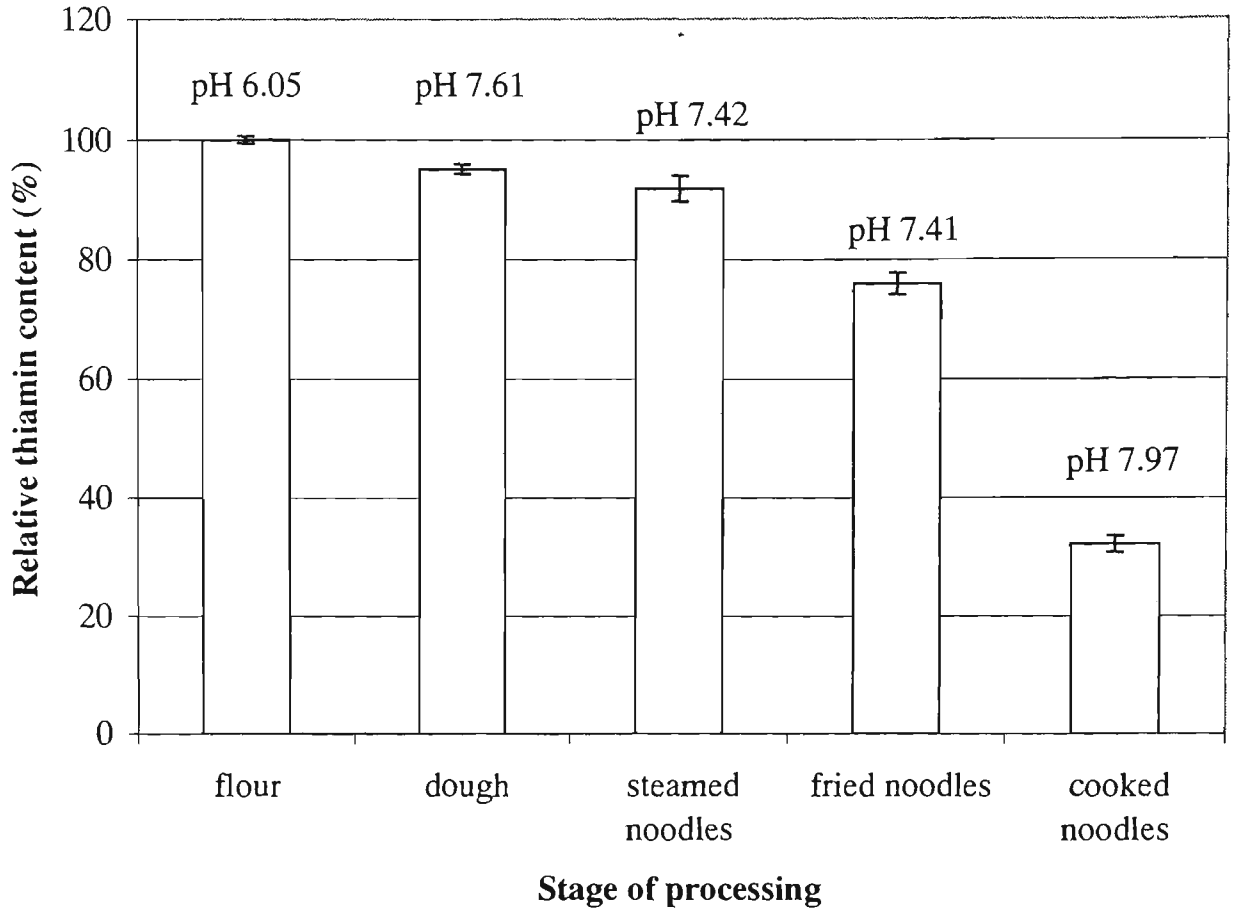


Figure 11.3 Relative thiamin contents and corresponding pH values at different stages during processing of instant noodles

Note Thiamin values are means ± sd, expressed as a percentage of the amount present in the flour, calculated after all values had been adjusted to a dry weight basis

11.8 Effect of cooking time on thiamin content of noodles prepared in the laboratory

The data for white salted, yellow alkaline and instant noodles (Figures 11.1-11.3) indicate that significant losses of thiamin have occurred during cooking for all three styles of noodles. A further series of experiments was designed to study the rates of loss of thiamin during the cooking process. In particular the optimum cooking times for different noodle styles were compared and the additional thiamin losses resulting from overcooking were assessed. Samples of each style were cooked for varying periods ranging up to 17 min. It is noted that this time frame was selected to extend well beyond the expected optimum cooking times. The noodle strands were taken out at one-minute intervals and immediately cooled to minimise further changes after sampling. Then, the moisture and thiamin contents of all samples before cooking and at each time during

cooking were measured. The results for moisture contents are shown in Table 11.8 and these show that the initial rates of moisture uptake were very high. Again, these data were used in the calculation of the thiamin results presented in Figures 11.4-11.6. It should be noted that in the figures different vertical scales have been used for each of the different styles of noodles. In addition, the amounts of thiamin found in the samples before cooking were used as the reference values to compare the relative losses. It is also noted that the cooking times presented may differ slightly from values reported in previous chapters. It is likely that this reflects the use of flours having different characteristics.

The results demonstrate that the heating and duration of overcooking significantly influenced thiamin levels in the noodle samples. In all three cases, a large reduction of thiamin occurred in the initial minute when noodles were first exposed to the boiling temperature. The rate declined until the optimum cooking time was reached and then it generally slowed further upon extended heating.

At the optimum cooking point, all the samples lost almost half of their respective initial thiamin contents. Yellow alkaline noodles had the greatest loss at the optimum cooking time (59.3 percent) as well as at the 16^{th} minute of cooking (78.0 percent). Instant noodles showed a similar pattern but to a lesser degree. This might be due to the affect of different amount of alkaline salts in the ingredient formulations of the two styles. White salted noodles show the lowest rates of loss among the samples tested.

Table 11.8 Moisture contents of laboratory noodle samples during cooking of the dried products

Cooking time (min)	White salted noodles	Yellow alkaline noodle	Instant noodle
0	9.8 ± 0.2	9.8 ± 0.07	10.4 ± 0.6
1	47.3 ± 0.1	45.1 ± 0.6	47.4 ± 0.7
2	55.8 ± 0.8	54.5 ± 0.7	49.8 ± 0.1
3	60.9 ± 0.4	57.3 ± 0.1	54.0 ± 0.4
4	63.1 ± 0.3	61.1 ± 0.6	58.8 ± 0.8
5	67.4 ± 0.8	63.8 ± 0.9	64.6 ± 0.4
6	68.4 ± 0.7	66.2 ± 0.9	65.7 ± 0.07
7	71.0 ± 0.21	66.4 ± 0.21	68.3 ± 0.9
8	71.8 ± 0.9	68.5 ± 0.5	69.2 ± 0.6
9	74.2 ± 0.9	71.0 ± 0.2	70.6 ± 0.6
10	75.7 ± 0.6	72.3 ± 0.7	72.0 ± 0.3
11	76.9 ± 0.6	73.8 ± 0.4	72.6 ± 0.9
12	77.0 ± 0.9	74.4 ± 0.3	74.5 ± 0.2
13	77.1 ± 0.7	74.5 ± 0.2	75.5 ± 0.2
14	78.9 ± 0.6	76.4 ± 0.2	75.7 ± 0.4
15	79.3 ± 0.3	76.5 ± 0.4	76.2 ±0.1
16	82.5 ± 0.3	77.2 ± 0.2	77.3 ±0.1
17	nd	78.4 ± 0.5	nd

Notes 1 Results are expressed as mean ± sd, in units of g/100g
2 The abbreviation nd indicates not determined

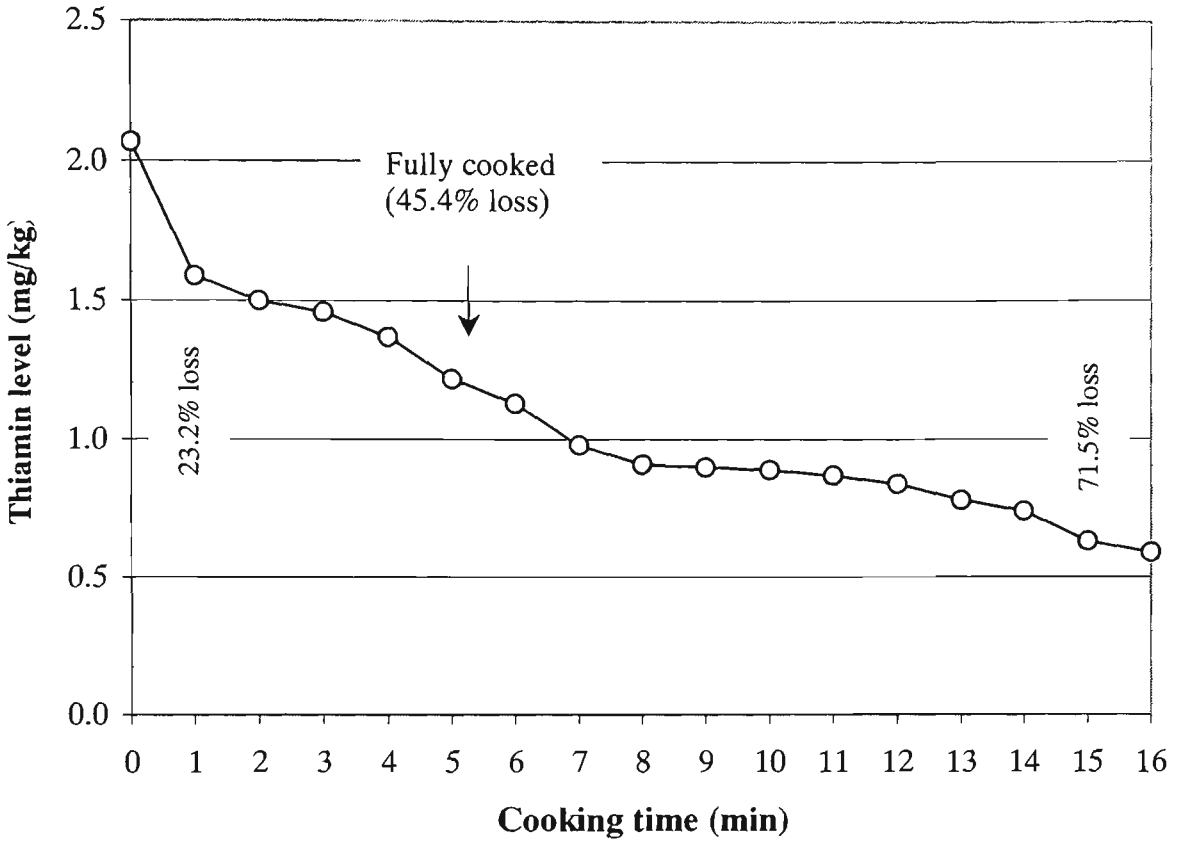


Figure 11.4 The time course of thiamin loss during cooking of white salted noodles

Note Thiamin contents are presented as mean values expressed in units of mg/kg on a dry weight basis

In this experiment, the optimum cooking time of noodles varied from style to style. These results were in accord with those reported by Moss and others (1986), who found that alkaline noodles had a longer cooking requirement than those with common salt. In addition, noodles made from high protein flours (yellow alkaline and instant styles) require longer cooking times than soft flours (white salted style). The results here indicated that the yellow alkaline noodles had a longer cooking time than the white salted style. Instant noodles had a short cooking time as the manufacturing process already included steaming and deep-frying steps which are effectively pre-cooking the product.

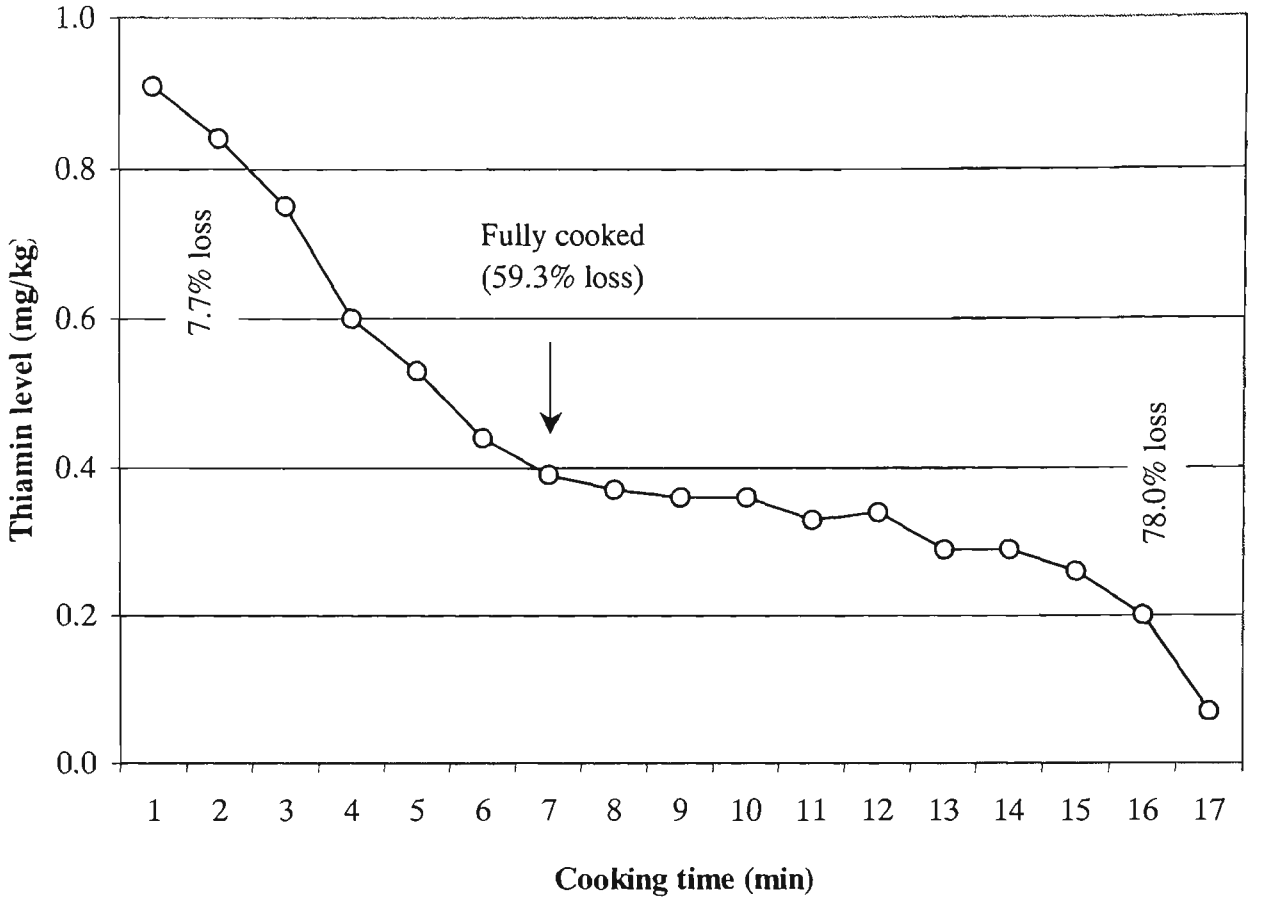


Figure 11.5 The time course of thiamin loss during cooking of yellow alkaline noodles

Note Thiamin contents are presented as mean values expressed in units of mg/kg on a dry weight basis

11.9 A comparison of the impact of cooking for commercial and laboratory noodle products

In the experimental phases of this study the thiamin contents and losses were studied in both commercial and laboratory samples. In order to further clarify the factors influencing thiamin stability in Asian noodles, the average results of the commercial samples have been compared with those for laboratory samples and the results shown in Table 11.9. It is noted that the relative loss for both commercial and laboratory noodles was calculated based on the thiamin contents of the final products (which is the same form as commercial products obtained from retail shops) and the cooked samples.

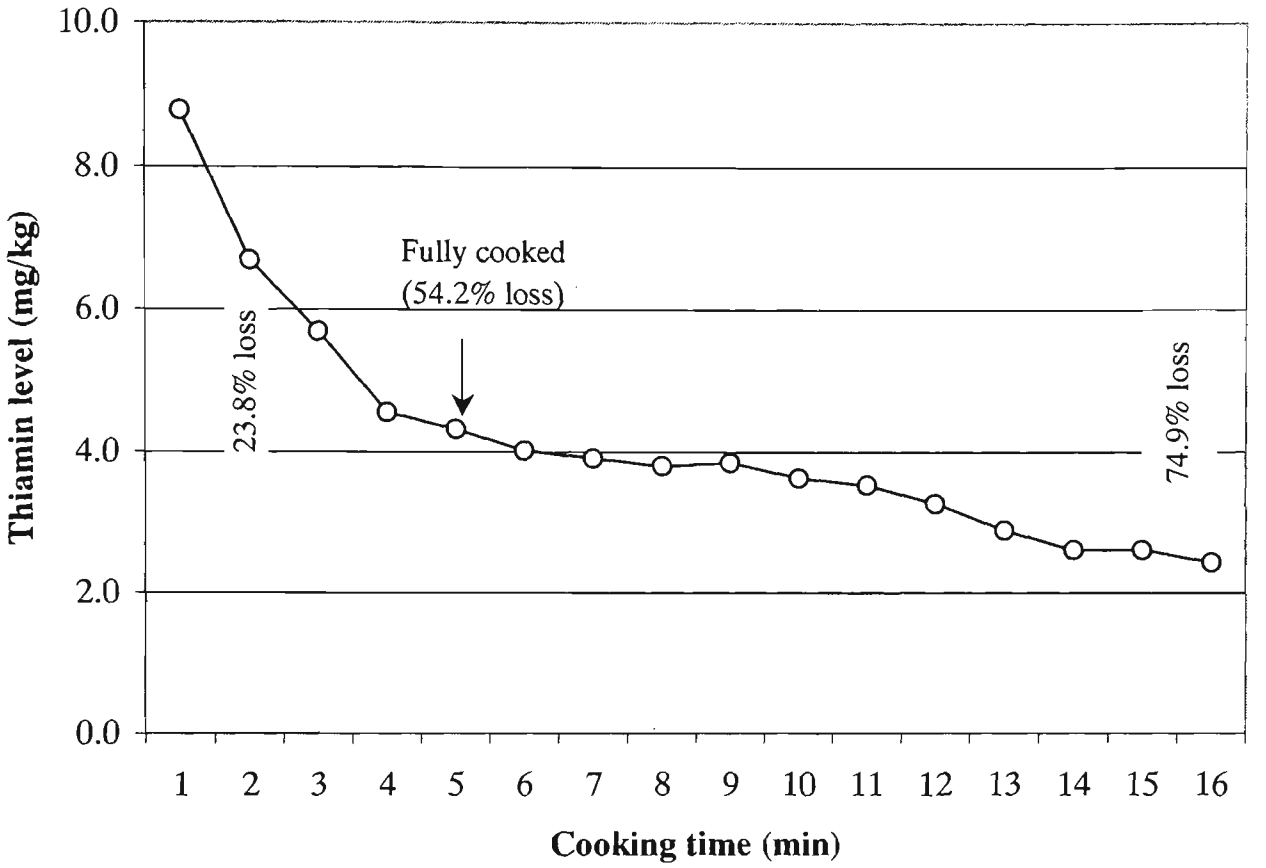


Figure 11.6 The time course of thiamin loss during cooking of instant noodles
Note Thiamin contents are presented as mean values expressed in units of mg/kg on a dry weight basis

The results show differences and these partly reflect the fact that considerable variation was seen between the five samples tested for each style of noodles. The variation seen for the five commercial yellow alkaline noodles was great and this can be explained by the fact that some of these had relatively low pH values (Table 11.4). The loss seen for the yellow alkaline noodles prepared in the laboratory was higher as these noodles incorporated alkaline salts. The laboratory samples were slightly more alkaline than any of the commercial noodles. Overall the differences reflect the fact that a number of factors may influence the ultimate thiamin content of the product ultimately eaten by the consumer.

In comparing the results for both commercial and laboratory noodles with published information, there is relatively little specific data useful for direct comparison. It is generally known that boiling can result in significant losses. For example, information given in the UK food composition tables (Holland and others 1991) shows that data was calculated on the basis of an expected average loss of 40 percent during boiling of

cereal foods. Changes in the thiamin levels during boiling in the current study for white salted noodles (43.2 percent from Table 11.9) was similar to the literature value. In the case of yellow alkaline noodles, only 5 percent was lost in boiling, as very little thiamin remained in the dried noodles, due to losses having occurred during earlier steps of processing.

Table 11.9 Comparison of relative thiamin losses during cooking for commercial and laboratory noodles

Noodle style	Commercial	Laboratory
White salted	61.7 (46.1 – 69.1)	43.6
Yellow alkaline	49.9 (27.3 – 62.5)	62.1
Instant	71.4 (66.3 – 80.1)	57.6

Notes 1 Values are the percentage of thiamin present in the dried product lost during cooking
2 Data for commercial noodles are from Table 11.4 presented as averages with ranges in parentheses
3 Data for laboratory noodles are averages values from those presented in Figures 11.1 - 11.3

Some data for the cooking of commercial durum products is summarised in Table 11.10. These were for products fortified in accordance with US regulatory requirements which explains the relatively high contents found. The relative loss values showed considerable variation for each category of product reported. In all cases cooking caused losses of 30–66 percent. The high values may reflect the relatively long cooking times normally required for durum pasta products. However the relatively low values for some products cannot be easily explained.

Although the reported levels of thiamin in commercial cereal-based products vary between 5.2-13.1 mg/kg, the average losses upon cooking of these samples were 44.9 percent. The losses during boiling of white salted noodles (Figure 11.1) appear to be consistent with the US values (44.2 percent, Table 11.11). Accordingly, it could be concluded that cooking of white salted noodles and cereal-based products, where no alkaline ingredients are incorporated, will result in approximately 40-50 percent loss of the original thiamin present in the dried form prior to cooking.

The high levels of loss might be related to the storage conditions, moisture content, temperature, cooking time as well as the amount of water used to cook the dry products (Farrer 1955; Dwivedi and Arnold 1973).

Table 11.10 Literature values for relative losses of thiamin during cooking of pasta products

	Thiamin content	Thiamin loss	Reference
Spaghetti	1.09	58	Watanabe and Ciacco 1990
Spaghetti	9.3-13.7	41.9-56.1	Ranhotra and others 1983
Egg noodles	10.0-12.9	43.4-57.9	
Macaroni	9.9-11.1	30.6-65.7	
Spaghetti	9.4-14.0	46 (43-48)	Ranhotra and others 1985
Egg noodles	9.0-11.5	42 (10-55)	
Macaroni	8.1-10.9	43 (37-50)	
Noodles, eggs enriched	11.7 ± 0.3	49.4	USDA ARS 2002
Noodles, eggs, spinach enriched	12.0 ± 0.65	34.9	
Noodles, Japanese, soba	5.2 ± 0.17	32.4	
Noodles, Japanese, somen	1.1 ± 0.04	44.0	
Spaghetti, enriched	11.5 ± 0.3	47.7	
Spaghetti, protein-fortified	13.1 ± 0.59	43.4	

Notes 1 Thiamin contents are expressed in units of mg/kg in the product as purchased
2 Thiamin loss values are expressed as the percentage loss from dried to cooked product

11.10 The relationship of colour and thiamin content of instant noodles

It is known that thiamin is generally unstable under heating conditions and during deep-frying higher temperatures are usually encountered. In the current study, oil temperatures of 150 °C, for 45 s were used to reflect typical commercial practice. These conditions appeared to result in losses corresponding to approximately 2 mg/kg of thiamin (Figure 11.3). It was also observed during this study that the visual appearance

of instant noodle samples varied considerably. In order to establish if there is a relationship between colour and thiamin contents, the colour characteristics of instant noodles were measured using the Minolta Chroma Meter (Section 6.4.3). The results and visual observations are presented in Table 11.11.

Table 11.11 The colour characteristics and thiamin contents of instant noodles

Sample type	Brand	Visual colour assessment	Colour parameter			Thiamin content
			L^*	a^*	b^*	
Commercial	Mama	Brownish	59.96	3.25	25.10	0.83
	Indomie	Yellowish	69.11	- 3.22	22.61	1.46
	Ma Ma Mien	Creamy	66.67	- 0.99	17.11	0.84
	Trident	Creamy	64.26	- 0.94	15.18	1.52
	Wai Wai	Brown	58.51	3.98	26.87	0.66
Laboratory		Creamy	62.15	- 1.80	17.69	9.18

Note Thiamin data are mean values expressed in units of mg/kg and originally presented in Table 11.4

The results firstly show considerable variation in visual appearance. In addition there is considerable variation in all three colour parameters with lighter appearance corresponding to lower L^*, a^* and b^* values. The thiamin content of the laboratory noodles was much higher, reflecting the fortification of the flour used for noodle preparation. The results for the commercial samples do show that the lowest thiamin content in the deep-fried noodles was found for the Wai Wai sample which showed the lowest L^* and highest a^* and b^* values. There does appear to be a relationship between colour and thiamin content. From the limited number of samples assessed it appears that darker colour indicates lower thiamin contents. This may be due to longer periods of deep-frying. Further work may be warranted to clarify this finding.

11.11 Further studies on the impact of processing variables and storage on thiamin contents of noodles

In order to extend the results showing losses in thiamin during processing (Figures 11.1-11.3), further experiments were carried out to clarify the impact of varying conditions during processing and storage of noodle products. Dried noodles of each style are often stored for extended periods prior to consumption. Hence the influence of storage on thiamin was investigated along with other processing variables.

11.11.1 The impact of drying temperature

For white salted and yellow alkaline styles of noodles, once the dough has been mixed and sheeted, the strands are cut and then dried. Regarding this, an experiment was set up to study the changes of thiamin in white salted and yellow alkaline noodles which were subjected to drying conditions for a longer period of time than normally applied. It is noted that parallel experiments for instant noodles were not appropriate as these are effectively dried during the deep-frying stage described in Figure 11.3.

Firstly, in order to assess the impact of drying of noodles at 40 °C in a dehydrator, both white salted and yellow alkaline noodle doughs were prepared. Samples were placed in the drier for extended periods of up to nine days. These were sub-sampled at one-day intervals and analysed for thiamin contents. The results are shown in Figure 11.7.

The data for thiamin contents following drying for the first day are consistent with the data already presented in Figures 11.1-11.2. White salted noodle doughs contained an average of 2.07 mg/kg thiamin. Drying at 40 °C dropped the moisture contents from 33.4 percent (dough) to 9.76 percent (dried noodles) and this was accompanied by virtually no loss in thiamin. Drying for periods of up to nine days resulted in no further losses. The minor variations observed reflected the inherent variability associated with the measurement of thiamin by the AOAC method and previously discussed in Section 11.4.

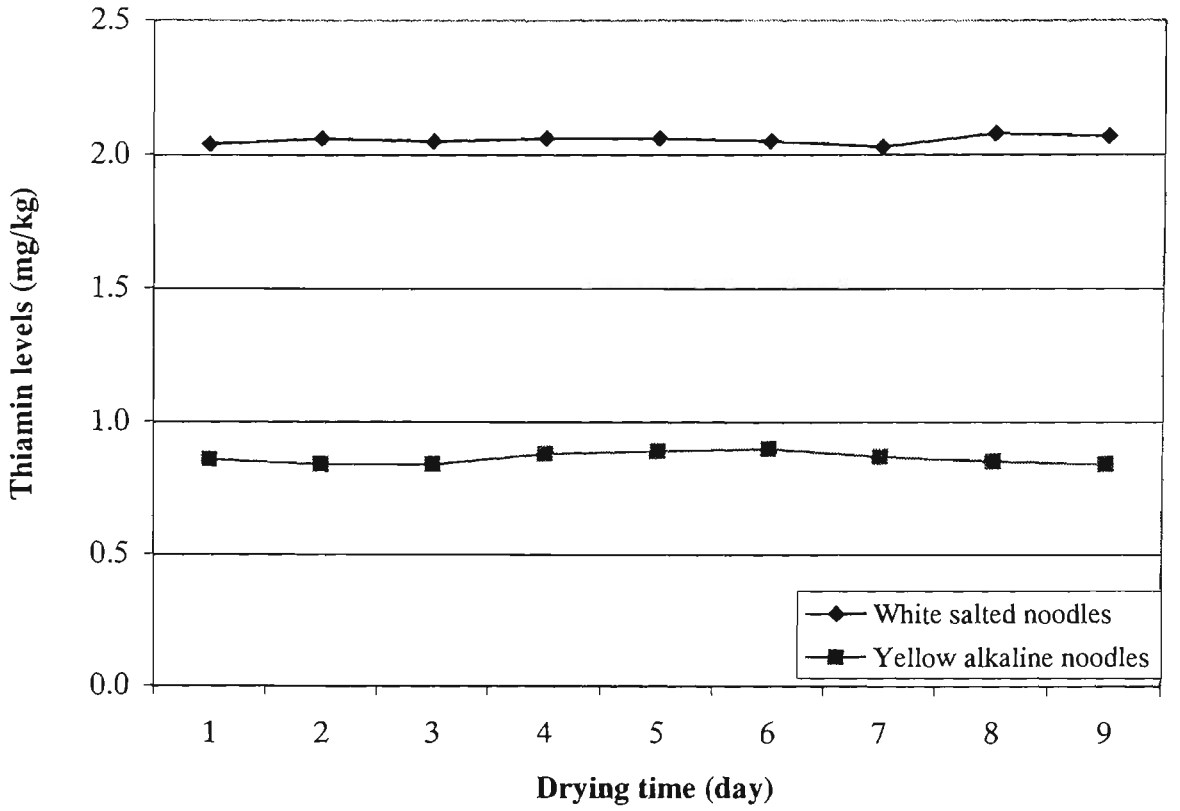


Figure 11.7 The influence of drying time at 40 °C on thiamin contents of noodles
Note Values are expressed on a dry weight basis

For yellow alkaline doughs there was considerable loss of thiamin due to the drying at 40 °C where the moisture dropped from 35.5 to 9.8 percent. During the first day, the average loss of thiamin was around 8.0 mg/kg with the level in the dried noodles at approximately 1.0 mg/kg. Again some minor variation in levels was observed upon extended drying at 40 °C for up to nine days. However, there was no clear trend of loss during extended dehydration and the variability again reflected minor variations due to the analysis procedure.

11.11.2 Effect of storage conditions on thiamin in fresh yellow alkaline noodles

The results in Figure 11.2 showed that for yellow alkaline noodles the pH of the dough was around pH 10.4. Considerable losses of thiamin occurred in the dough at both the mixing and the drying stages. An additional issue of relevance here is that yellow alkaline noodles may be either dried soon after dough preparation, or alternatively, are stored and sold in the fresh form. Such noodles may then be stored by a consumer prior

to cooking and eating. An experiment was conducted in order to compare the effect of storage of fresh and dried yellow alkaline noodles and the results are shown in Table 11.12. Again samples were analysed for moisture at all stages and the data in calculating thiamin data to a dry basis. Little difference was observed for those storage at room temperature and under refrigeration.

Table 11.12 The relative losses of thiamin in yellow alkaline noodles for three different storage conditions

Sample/processing treatment	Thiamin content	Relative loss
Flour	12.0 ± 0.3	-
Dough	9.1 ± 0.2	24.0
Noodles dried for one day at 40 °C	1.0 ± 0.1	91.6
Noodles stored fresh for one day at room temperature	0.9 ± 0.1	92.8
Noodles stored fresh for one day under refrigeration temperature	1.6 ± 0.1	86.9

Notes 1 Thiamin contents are presented as mean ± sd in units of mg/kg on a dry weight basis
2 Relative losses are expressed in comparison with the thiamin present in the flour as a percentage

The data show that the losses in thiamin for all three treatments were very high. Losses in fresh noodles stored at room temperature were similar to the noodles dried for one day at 40 °C. This probably reflects the influences of higher water activity and elevated temperatures respectively on the rate of destruction of thiamin (Gregory 1996). On the other hand, the use of refrigeration temperature appeared to slow the rate of loss. This is in accordance with findings for fruits and vegetables showing that they should be stored under refrigeration to avoid vitamin losses (Kramer 1982). However, for yellow alkaline noodles the calculated values of overall losses of thiamin in comparison with the levels originally present in the commercial flours showed that the total losses of thiamin were all very high. The storage conditions studied here have only a minor impact on the overall losses.

11.11.3 Effects of prolonged storage times

A further series of experiments was designed to investigate the influence of long-term storage of noodles. For this, samples of all three styles of noodles were prepared, dried and then stored for periods of one, two, three and four months. Thiamin contents were analysed in dried samples as well as in samples which had been cooked to the optimum point following storage. The results are presented in Figures 11.8-11.10.

In all cases, there were only minor variations seen in the thiamin contents for dried noodles (white salted and yellow alkaline) and fried noodles (instant) as well as for cooked products. It was concluded that prolonged storage periods do not adversely influence the ultimate thiamin status of any of the styles of noodles. These findings confirm the results expected. It has previously been shown that for other foods at ambient temperatures, thiamin exhibited excellent stability under conditions of low water activity (Gregory 1996).

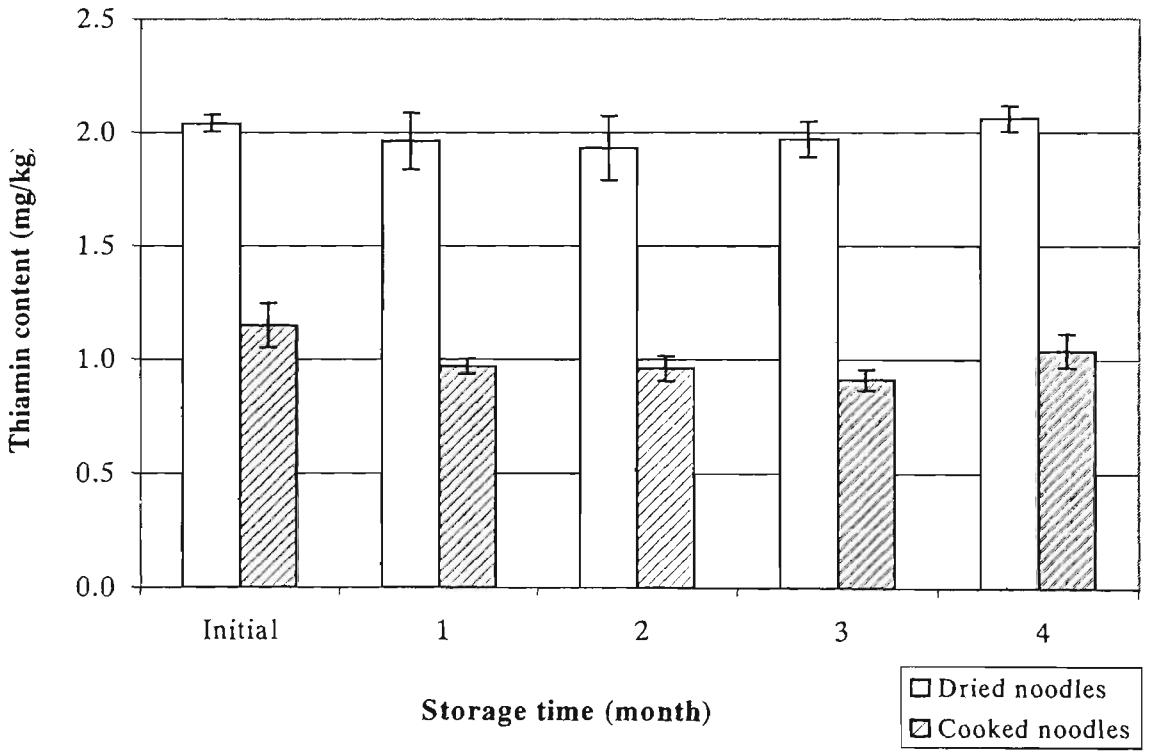


Figure 11.8 The impact of extended storage of dried white salted noodles upon thiamin contents

Note Values are mean ± sd, expressed on a dry weight basis

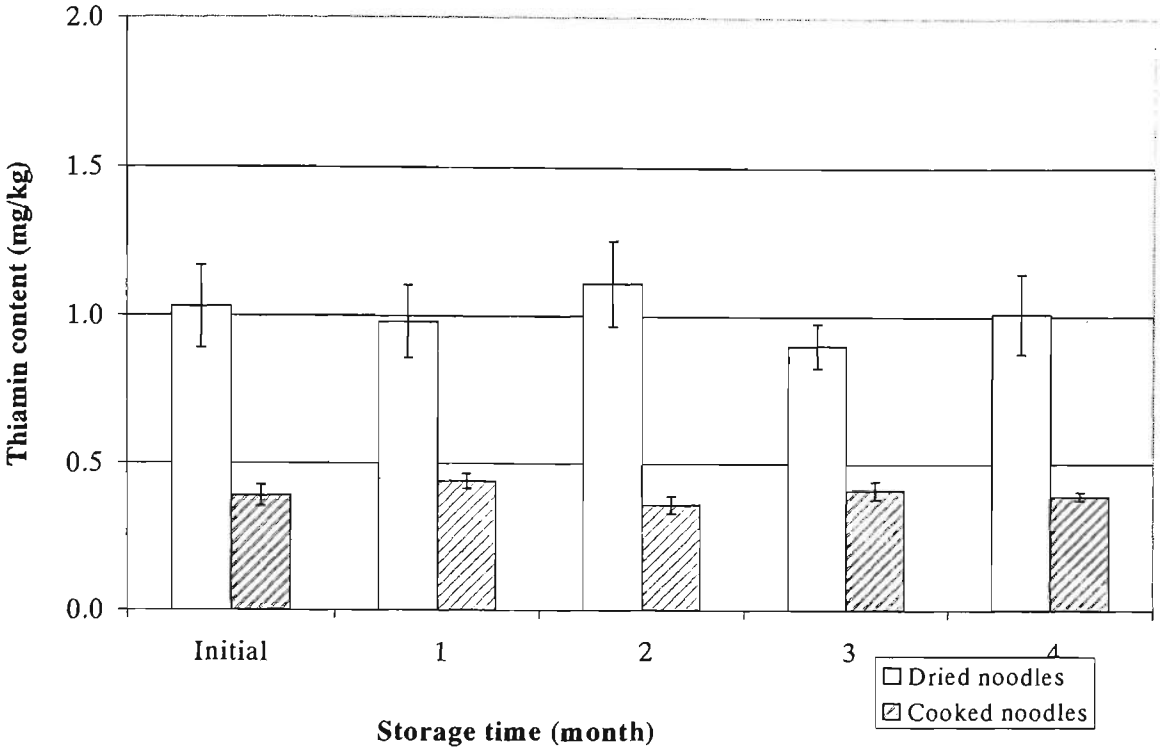


Figure 11.9 The impact of extended storage of dried yellow alkaline noodles upon thiamin contents

Note Values are mean ± sd, expressed on a dry weight basis

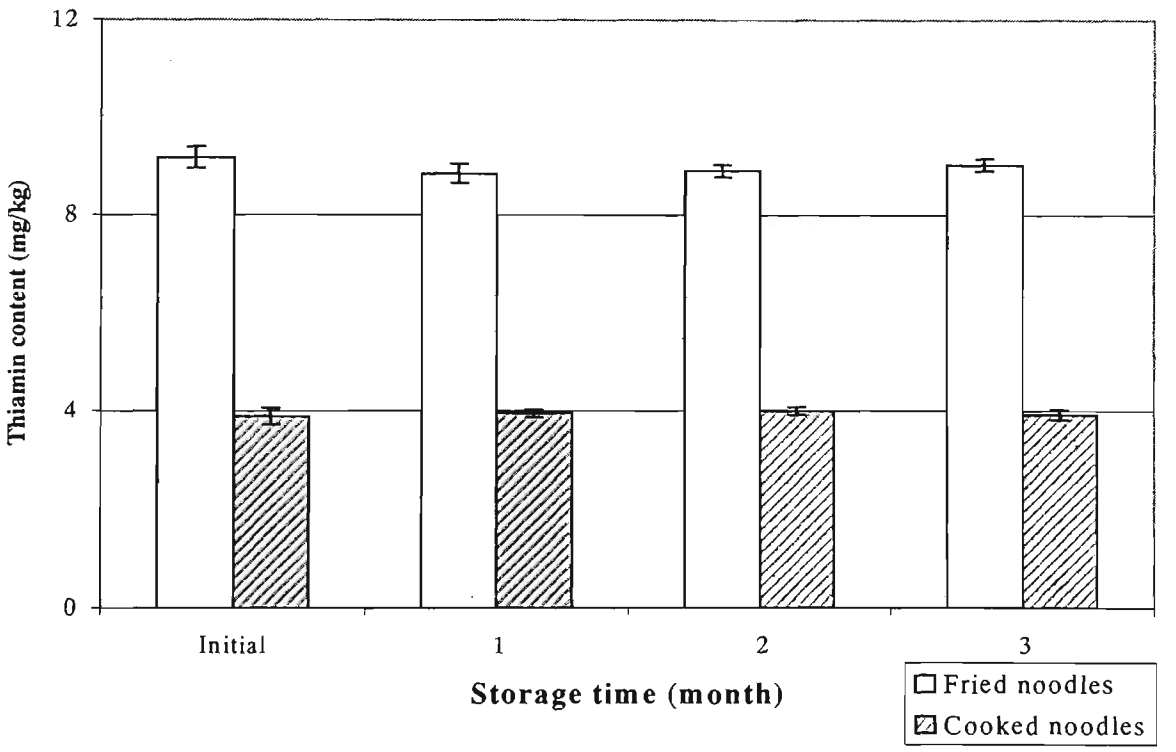


Figure 11.10 The impact of extended storage of fried instant noodles upon thiamin contents

Note Values are mean ± sd, expressed on a dry weight basis

11.12 General discussion of thiamin stability during processing of Asian noodles

In summarising the investigation of thiamin, initially the standard fluorimetric procedure was set up and shown to give satisfactory precision and accuracy when applied to reference samples as well as dried and cooked Asian noodles. A number of selected commercial noodle samples were analysed for thiamin contents. The losses of thiamin during cooking of these noodles were also studied. The results showed:

- Different brands of each style of noodles had quite a wide range of thiamin levels.
- Varying losses of thiamin occurred from sample to sample during boiling of the dried noodles.
- Noodle pH appeared to be directly related to the amount of alkaline salt added and this in turn influenced the thiamin stability.

It is concluded that although flour is regarded as a good source of thiamin, the measured contents of the vitamin in some of the noodle samples are relatively low. The values are lower than might have been expected and are well below those for other foods considered to be good sources of this vitamin.

In order to further study the thiamin contents of the three styles of noodles and the factors influencing the contents, noodles were made in the laboratory under controlled conditions. The results showed that:

- Cooking had a major impact for all noodle styles. For each, cooking to the individual optimum point reduced the thiamin content to almost half of that initially present in the dried noodle.
- Boiling for times beyond the optimum caused further declines in thiamin levels.
- The losses in preparation of white salted style noodles were low until the cooking of the dried noodles. The boiling resulted in a 43 percent decline in total thiamin level.
- The relative losses of thiamin were high for yellow alkaline noodles and losses occurred at each step in the process.
- For instant noodles losses also occurred at each step. The losses were lower than for yellow alkaline but greater than for white salted noodles.

In order to conveniently summarise the results obtained in the laboratory studies, the relative thiamin losses observed at each stage of the process are presented in Table 11.13. This shows the relative significance of each step in processing.

Table 11.13 Relative losses of thiamin during processing of laboratory noodles

Noodle style	Processing stage			Thiamin loss	Cumulative thiamin loss
White salted	Flour	→	dough	0.0	0.0
	Dough	→	dried noodles	1.0	1.0
	Dried noodles	→	cooked noodles	43.2	44.2
Yellow alkaline	Flour	→	dough	25.4	25.4
	Dough	→	dried noodles	66.0	91.4
	Dried noodle	→	cooked noodles	5.4	96.7
Instant	Flour	→	dough	4.9	4.9
	Dough	→	steamed noodles	3.2	8.1
	Steamed	→	fried noodles	15.9	24.0
	Fried noodles	→	cooked noodles	43.8	67.8

Notes All values are relative losses of thiamin expressed as a percentage and calculated in comparison with the levels originally present in the flour

In comparing the three styles of noodles, the thiamin lost for white salted noodles was primarily at the cooking stage with very little lost during processing. The data highlights the high relative losses of thiamin in the processing of yellow alkaline noodles prior to the cooking step. At the stage when the noodles were being cooked, most of the thiamin originally present had already been lost. The data for instant noodles shows a pattern of loss between those shown by the other two styles. Significant reductions in thiamin content occurred during initial processing as well as subsequent cooking.

Throughout these experiments the results indicated that the levels of thiamin loss depended on the amount of alkaline salt used in the formulation which in turn influenced the pH levels during processing. Yellow alkaline noodles were high in pH,

thus there was considerable loss of thiamin on processing. Instant noodles also had alkaline pH but much lower than yellow alkaline noodles, therefore, smaller losses. In contrast, the processing method and ingredients for white salted noodles results in thiamin being more stable due to the mildly acidic conditions.

The level of losses of thiamin after processing of spaghetti found in low treatment temperature (45 °C for 27 h) was 18 percent (Watanabe and Ciacco 1990), this was high compared to the loss of white salted noodles found in this study. The losses of white salted noodles during processing found in this study is lower than the losses of thiamin during processing of spaghetti dried at 45 °C for 27 h (18 percent) (Watanabe and Ciacco 1990).

Storage of noodles from one to four months under dark conditions and at ambient temperature did not result in any loss of thiamin. In addition the application of prolonged drying conditions for up to nine days did not adversely influence the ultimate thiamin status of the products.

The results also demonstrate that even with flour that has been fortified to levels much higher than the Australian minimum legal requirement, the amount of thiamin remaining in the product consumed is quite low. This is the case for both yellow alkaline and instant styles of noodles. It is concluded that fortification is unlikely to provide a solution to the problem of excessive losses in thiamin that occur in those Asian noodles having elevated pH due to the incorporation of alkaline salts in the formulation.

Chapter 12

General discussion and conclusions

The purpose of this chapter is to summarise the results obtained during the current study, draw final conclusions and make recommendations for further research into the B group vitamins in Asian noodles.

12.1 Introduction

In developing this project a survey of the literature indicated that B group vitamins can be relatively unstable. Different conditions are reported to result in losses of particular members of the B group vitamins. Available information indicates that losses can occur during processing of durum pasta products. The investigation here has focussed on Asian noodle products which are a major staple food globally and which potentially could be a significant dietary source of the B group vitamins. The issue of the vitamins naturally present in flour is explored along with the practicality of fortification of the three major styles of Asian noodles.

The results described in this thesis fall into four broad areas. These are:

1. Selection and validation of vitamin analysis procedures
2. Analysis of wheat flour samples
3. Investigation of selected commercial noodles
4. Studies of the stability of the vitamins in Asian noodles prepared in the laboratory under controlled conditions.

The results for each of these is now reviewed as a basis for presenting the primary conclusions of this project and a discussion of areas recommended for further research.

12.2 Selection and validation of vitamin analysis procedures

Whilst method development was not the primary emphasis of this project, considerable effort was made to select and validate suitable methods for vitamin analysis. Firstly, various published methods which had potential for simultaneous determination of up to six B group vitamins were evaluated. These were based upon HPLC using reversed-phase columns and some of the procedures provided good separations of standard vitamins. However when various extracts of flours were analysed on HPLC, it was not possible to obtain satisfactory separations of vitamin peaks from others present in chromatograms. The use of a variety of solid phase extraction procedures did not enhance the results nor did these effect concentration of extracts. The results for niacin and nicotinamide were the least satisfactory as these eluted along with many contaminant peaks in the earliest part of each of the chromatograms. Accordingly further studies focused on the remaining B group vitamins. In the ensuing evaluation of procedures for analysis of the vitamins of interest, each was considered separately.

For the measurement of the individual vitamins (B-6, riboflavin, folate and thiamin), one procedure was selected and investigated. Each was thoroughly evaluated in terms of the repeatability of results obtained with the AACC reference sample, flour samples, as well as noodle samples. In each case this necessitated the comparison of extraction procedures and the results clearly show that sample extraction and treatment protocols are very important to the reliable determination of each of the vitamins studied. In most cases the results obtained for the vitamins in the AACC reference sample were close to the mean value supplied with the sample. For folate the mean value was on the very top end of the range of results provided from the AACC participating laboratories.

One of the conclusions from the evaluation of the vitamin analyses was that the published procedures required adaptation and optimization in order to obtain the most useful results for the particular samples being studied here. As an example of the significance of extraction procedures, for folate, comparisons were made between various procedures including that adopted and recommended very recently by the FDA for total folate of foods and widely applied to the analysis of food samples in the context of regulatory activities. The procedure adopted here gave consistently higher values than those obtained with the FDA method.

Another conclusion from the results obtained for folates during processing of Asian noodles concerns the usefulness of the measurement of free folate. This is measured using the conjugase enzyme preparation but without the amylase or protease which are applied in the measurement of total folate contents. The data reported here indicates that free folate contents decrease as a result of heating noodles during processing or cooking. This probably reflects the reactivity of the folates at higher temperature or their entrapment in the food matrix as a result of starch gelatinisation and gelation. It is concluded that the measurement of free folate is not useful for flour-based samples. The significance of this conclusion for the analysis of foods generally is not clear. Globally there is considerable interest in the folate contents of foods, the role of folate in health, the adequacy of intakes as well as the level of fortification that should be regulated. Accordingly it is recommended that the general usefulness of free folate analysis should be further investigated.

Whilst there is an ongoing requirement to review and refine analytical methods, the procedures adopted for the current study gave reasonably reliable results and facilitated the thorough investigation of vitamin contents and stability reported here.

12.3 Analysis of wheat flour samples

A series of wheat flour samples were analysed for the B-group vitamins during the course of this study. The purpose of these analyses was three-fold: firstly to ensure the validity of the extraction and analytical methods for flour and flour-based foods; secondly to allow comparison with published data for flour, and thirdly as a basis of comparison for the various noodle products made from the flours during the subsequent phases of the study.

For determination of B-group vitamins, samples of flours were measured using the methods validated in the current study. Some of the results obtained here were similar to values in published sources including various food composition tables. In some cases it was difficult to interpret the data as some of the literature values are for flours which have been fortified for at least some of the vitamins of interest here.

In considering the significance of the results for flours and their application in noodlemaking, it is likely that a proportion of the variability in data reflects the differences in milling extraction rates. It is known that each of the vitamins tends to be concentrated in the outer layers of the grain. In addition, it is known that lower flour extraction rates are typically preferred for noodle making.

Further studies of vitamin contents in relation to flour milling may be warranted because the current analytical methods as well as extraction procedures (especially for folates) have been shown here to provide higher results for flours than those used in some of the classical investigations in this area. From the results of the current study, the levels of vitamins were typically quite low. It might be expected that the levels of the vitamins in Asian noodles would also be similar. Therefore in the next phase, various commercial noodles were analysed.

12.4 Investigation of selected commercial noodles

A range of commercial Asian noodles were purchased from retail outlets in Melbourne and these were chosen to include white salted, yellow alkaline and instant noodles. It is noted here that different ranges of noodle samples were used at various stages of the study. This partly reflects the changing availability of some of the specific brands over the period of the study. A second issue here is that a variety of samples were purchased and in some cases it proved difficult to clearly categorise the samples. Accordingly it was decided to designate some as miscellaneous Asian noodles.

In reference to the selection of the commercial noodles, the purpose was not to undertake a comprehensive survey of all the noodles available. Rather, a limited number of samples were procured in order to provide an indication of the range of vitamin contents which might be expected in each of the product styles. The approach was basically random. However, efforts were made to include samples from as many different countries as practically possible. This was partly to incorporate variations in factors such as the geographic and genetic origins of wheat used, the organoleptic preferences of the consumers, the ingredient formulations as well as the processing conditions applied during manufacture.

In preliminary studies it was found that the levels of vitamin B-6 and riboflavin in wheat flours and commercial samples of noodles were relatively low and accordingly more detailed studies of commercial noodle samples were restricted to analysis of folate and thiamin. For these, samples were analysed for pH and vitamin content both before and after cooking. The findings were that:

- The appearance and pH level of some samples were not consistent with the listing of ingredients declared in the labeling and packaging of the product. This therefore required a reconsideration of the categorization that been attempted. The vitamin content results were interpreted in relation to the measured pH values of the samples.
- For yellow alkaline noodles the pH values varied widely and the appearance of some products may have resulted from the presence of undeclared colorants.
- Considerable variation was observed in the levels of each of the vitamins in the noodles. For total folate the range was 53–393 μg/kg and for thiamin 0.11–1.76 mg/kg (dry weight basis).
- Overall, those yellow alkaline noodles having high pH readings had lower thiamin and higher total folate contents than the other styles of noodles.
- Interpreting the data for the commercial products was difficult because the results indicated that some of the noodles might have been made from fortified flours.
- When the impact of cooking on vitamin levels was investigated there appeared to be no clear pattern or trend although significant losses were observed in virtually all samples tested in this way.

The wide variations found in levels of folate and thiamin clearly demonstrate the problems which might be encountered during the sampling and analysis of food samples. This is particularly relevant in the development of food composition tables and databases. Whilst there has been a rapid expansion in the availability of such information over recent decades, clearly caution should be exercised by those preparing as well as those using these sources of information.

From the results for commercial noodle samples, it was difficult to draw conclusions regarding the factors which might have been responsible for the vitamin contents which were found. It is likely that the results were ultimately due the combination of a number

of factors. The extent to which the various noodles were manufactured from fortified flours was unclear, as was the impact of other ingredients. Although limited in number, the results for flours reported in this study also indicate that these vary in vitamin contents. Some of the relevant factors may be the impact of environment, genetic variability or possibly milling parameters, or a combination of these.

Accordingly, in order to further investigate the factors determining vitamin contents and stability in these products, three styles of Asian noodles were prepared in the laboratory under controlled conditions.

12.5 Studies of the stability of the vitamins in Asian noodles prepared in the laboratory under controlled conditions

Initially, procedures for preparation of each of the three main styles of Asian noodles in the laboratory were set up and used under controlled conditions. These were selected to reflect formulations and processes widely used in commercial manufacture. It must, however, be recognized that considerable variation can be found in the practices adopted between different countries, between specific regions within countries and even individual manufacturers.

In this study, noodles were made from commercial flours and in many of the experiments the formulation included additional vitamin added for fortification purposes. It is noted that the fortification levels were selected following consideration of current Australian regulations which in turn refer to Australian RDI values for the particular vitamins. The issue of particular serving sizes was also considered and here the amount of noodle in either the dry or the cooked form, equivalent to 35g of the dried product on an as purchased basis was used.

It is clearly recognized that the RDI values adopted in different countries vary significantly and that these may also change as they are subject to periodic review. Recently there has been considerable debate in the scientific literature regarding recommended levels of folate for fortification and some of those being considered are much higher than current Australian RDI values.

It is noted that during the course of this research various flours were used that that some variations were found from batch to batch. In all cases appropriate precautions were taken and blanks used to ensure the validity of the results. The primary purposes of this phase of the study have been to assess the effects of ingredients and processing conditions, the relative importance of processing and cooking as contributors to losses of the vitamins, along with the impact of storage, and the potential of particular styles of noodles to act as vehicles for fortification.

The overall losses in the four B group vitamins were measured in terms of the amount present in the final cooked product as it is consumed, in comparison with the total levels originally incorporated into the formulation in the flour or added through fortification. The results for the four vitamins are compared and summarised in Table 12.1.

Table 12.1 A comparison of total relative losses of B group vitamins from initial ingredients to cooked product for three styles of Asian noodles

	PN	Riboflavin	Folate	Thiamin
White salted	57.3	52.9	41.3	44.2
Yellow alkaline	62.2	71.1	40.9	96.7
Instant	65.7	51.6	43.4	67.8

Notes 1 Based upon data presented in Tables 8.10, 9.14, 10.15 and 11.14
2 Relative losses are expressed as percentages compared to the total level of the vitamin in the ingredient formulation

The losses of all of the vitamins are high in each of the styles of Asian noodle. The vitamin demonstrating the greatest stability is folate for which the losses were essentially the same in all three styles. The overall losses for PN were higher but again similar for each style. The highest losses observed were for riboflavin and thiamin in yellow alkaline products. In order to provide a clearer understanding of the losses, samples were also analysed representing noodles at each stage of processing and the data are summarised in Tables 12.2 and 12.3.

These data show that not only were all total losses high, in most cases there were significant losses during cooking. However there are quite different patterns when

cooking and processing are compared. In some cases there was virtually no loss during noodle processing (folate in white salted and yellow alkaline as well as thiamin in white salted). In contrast there were very high losses of thiamin in yellow alkaline noodles during processing.

Table 12.2 A comparison of relative losses of B group vitamins during processing and cooking of three styles of Asian noodles

		PN	Riboflavin	Folate	Thiamin
White salted	Processing	21.7	27.1	1.3	1.0
	Cooking	35.7	25.8	40.0	43.2
Yellow alkaline	Processing	20.3	36.4	1.6	91.4
	Cooking	41.9	37.7	39.3	5.4
Instant	Processing	42.7	38.4	31.9	24.0
	Cooking	23.1	13.2	11.6	43.8

Notes 1 Based upon data presented in Tables 8.10, 9.14, 10.15 and 11.14
2 Relative losses are expressed as percentages compared to the levels of the vitamin in the ingredient formulation

When the cumulative losses are compared, further differences in the patterns are highlighted. Firstly, the losses during dough mixing are surprisingly high in some cases. Thus 20% of PN is lost in mixing of all styles and, for yellow alkaline noodles, both riboflavin and thiamin were lost at this stage. The latter observations can be explained by the relatively high pH of the yellow alkaline noodles and the known instability of both riboflavin and thiamin under these conditions. The similar level of losses for PN for each of the styles indicates that pH is not the primary factor and the findings for PN during dough mixing cannot be readily explained from the known characteristics of this vitamin.

Table 12.3 A comparison of cumulative relative losses of B group vitamins during individual processing steps and cooking of three styles of Asian noodles

Noodle style/processing stage			PN	Riboflavin	Folate	Thiamin
White salted						
Flour	→	dough	21.7	8.6	1.3	0.0
Dough	→	dried	21.7	27.1	1.3	1.0
Dried	→	cooked	57.3	52.9	41.3	44.2
Yellow alkaline						
Flour	→	dough	20.3	11.9	0.93	25.4
Dough	→	dried	20.3	36.4	1.55	91.4
Dried	→	cooked	62.2	71.1	40.9	96.7
Instant						
Flour	→	dough	21.7	11.3	0.94	4.9
Dough	→	steamed	23.1	13.9	20.3	8.1
Steamed	→	fried	42.7	38.4	31.9	24.0
Fried	→	cooked	65.7	51.6	43.4	67.8

Notes 1 Based upon data presented in Tables 8.10, 9.14, 10.15 and 11.14
2 All values are cumulative data and represent the relative losses compared to the levels of the vitamin in the ingredient formulation and are expressed as percentages

White salted and yellow alkaline noodles are often dried after preparation and for this step vitamin losses were typically low (Table 12.3). However, the results for riboflavin were relatively high for both styles of noodles. This indicates that pH was not the primary reason for the losses in riboflavin. It appears that in combination with the moisture present in the dough, the temperature of 40 °C may have been sufficiently high to have impacted on riboflavin. This effect was not seen for thiamin which was stable during the drying of white salted noodles. The very high loss in thiamin in yellow alkaline noodles is attributed to the high pH of this product. These results are consistent with the relatively low contents found in commercial samples which had the higher pH values.

The losses in the vitamins at each stage of processing for instant noodles do not show a strong impact of pH except in the case of thiamin where overall losses were intermediate between those of the other two styles. The losses during steaming were generally low, although the time period was two minutes. This is unexpected particularly as the boiling of each style of noodles was associated with high losses. For folate the loss of 20% is quite high and cannot be readily explained. The losses during the deep-frying of instant noodles were relatively high, despite the short period of 45 sec, and these are attributed to the use of a temperature of 150 °C.

Although some analyses were carried out to investigate the levels of vitamins which had leached into cooking water, in most cases the levels were low and further studies would be required to fully explore the relative importance of leaching and chemical degradation of the vitamins lost from Asian noodles.

Cooking dried processed noodles of each style resulted in significant losses for each of the vitamins in dried noodles. In the case of folate and thiamin these results largely confirm those found for commercial noodles. When samples of noodles were cooked for varying times including for periods well beyond the optimum point the thiamin content continued to decline. Ideally noodles should be cooked for periods as short as possible to minimize the losses.

In assessing the factors which might be used to explain the results for each vitamin, there is a considerable body of literature concerning the stability including summaries such as that presented in Chapter 2 (Table 2.2). Many of the results reported from the current study are consistent with existing knowledge. However, the pattern for folate provides an interesting comparison with published information. Typically folates are regarded as relatively unstable under most conditions known to impact on retention of vitamin compounds in foods. It has also been reported that different forms of folate vary in stability characteristics (Eitenmiller and Landen 1999). The findings here for Asian noodles highlight the difficulties in using previous findings for the prediction of vitamin stability and demonstrate that direct studies of products under laboratory conditions can have considerable value.

The primary conclusions from these studies of noodle processing are that the overall losses of each vitamin are high and that the patterns of loss are different for individual vitamins and relate also to the style of Asian noodles.

12.6 Studies on the storage of Asian noodles prepared in the laboratory

Based upon the impact of dough mixing and drying of the cut noodle sheets by heating at 40 °C (Table 12.3) it was decided to further investigate the loss of selected vitamins during short term storage. The earlier results indicated that levels of folate as well as PN were not adversely influenced by drying at 40 °C. Therefore thiamin and riboflavin were selected for study due to the relatively high losses found during dough handling and the significance of product pH. These vitamins were measured following storage for one day and the results are summarised in Table 12.4. The relative losses further confirm the adverse influence of high pH values on both vitamins. The results showed:

- High losses were found for thiamin in yellow alkaline noodles in all cases.
- In contrast, relatively low losses were found for riboflavin. The losses were higher in yellow alkaline noodles than white salted noodles.
- Refrigeration temperatures did result in a minor enhancement in the stability of thiamin and riboflavin.
- Drying at 40 °C accelerated the loss of riboflavin in both cases. However, losses from white salted were always lower than those for yellow alkaline noodles.

When noodles containing fortification levels of thiamin and riboflavin were stored for periods longer than one day, it appeared that the moisture levels were important. There was a clear trend of riboflavin destruction when yellow alkaline noodles were stored at room temperature for up to 96 h. Similarly, there was a reduction in riboflavin level during drying yellow alkaline noodles at 40 °C. However, no loss took place after 28 h of drying. The results indicated that the level of riboflavin content in the fortified noodles was relate to the pH and moisture content . Once an equilibrium moisture level was reached during drying, no further loss in these vitamins was observed.

Table 12.4 A comparison of relative losses of thiamin and riboflavin in white salted and yellow alkaline noodles during short term storage

Storage condition	Thiamin	Riboflavin	
	Yellow alkaline	White salted	Yellow alkaline
Room temperature	92.8	9.3	16.6
Refrigeration temperature	86.9	6.6	15.2
Drying at 40 °C	91.4	27.1	36.4

Notes 1 Based upon data presented in Tables 9.14 and 11.14
2 Relative losses are expressed as percentages compared to the levels of the vitamin in the ingredient formulation
3 Losses of thiamin in white salted noodles were not directly measured as the overall losses during processing were less than 1% (Table 12.2)

The reduction of thiamin content was also related to the pH, moisture level and thermal conditions. When the three styles of dried product were stored for up to 4 months no loss in thiamin levels were observed.

12.7 Major conclusions

The final conclusions of this study are summarized here:

1. In the selection and validation of suitable methods for analysis of Asian noodles, it is important to ensure that suitable sample preparation and extraction procedures are applied for each of the individual vitamins.

2. The contents of the B group vitamins in wheat flours were found to vary, but the levels were relatively low except where fortification had occurred.

3. When a selection of commercial Asian noodles was analysed, the amounts of folate and thiamin were found to vary widely. Yellow alkaline noodles were typically lower in thiamin and higher in folate than the samples of white salted and instant products. When samples of each style of the commercial noodles were cooked significant losses of the vitamins were found.

4. The analysis of commercial samples clearly demonstrates the variations that can occur in the development of food composition tables and databases. It is recommended strongly that considerable caution needs to be exercised in the selection and analysis of samples as well as the presentation, interpretation and application of such data.

5. Based upon studies of Asian noodles made under laboratory conditions it was found that for vitamin B-6, riboflavin, folate and thiamin, the total losses from the levels in the ingredient formulation to the product as consumed were at least 40% and ranged up to 97% in the case of thiamin in yellow alkaline noodles.

6. The investigation of laboratory noodles also shows that different factors cause losses for each of the four different B group vitamins. The specific factors identified are summarized in Table 12.5.

Table 12.5 Summary of main factors influencing loss of each of the B group vitamin in Asian noodles

Vitamin	Main factors causing losses
Vitamin B-6	Losses during dough mixing and during high temperature treatments. Not impacted by pH
Riboflavin	Some loss on dough mixing, significant losses on drying at 40 °C. Losses during high temperature treatments. Each of these loss effects is increased at high pH
Folate	Only affected by high temperatures with little impact of pH
Thiamin	Losses at any stage where high pH or high temperature applies

7. Although losses in the B group vitamins during storage of noodles was not investigated fully, from the results obtained here, the losses upon storage appear to be relatively small in comparison with the overall losses in processing and cooking.

8. If Asian noodles were to be fortified with the B group vitamins studied here, the relatively high losses of the vitamins would require the addition of substantial

overages for each of the vitamins and also for each of the styles of noodles. In the cases of thiamin and riboflavin in yellow alkaline noodles as well as PN in all styles, fortification may not be practical unless some means can be found to enhance the stability of the vitamin during manufacture of these products.

12.8 Possible areas for future research

This study has concentrated on Asian noodles and upon selected B group vitamins. It would of value to extend this work to other flour and cereal grain foods and to other nutritional components including other vitamins. One specific example is of steamed breads which represent a staple food in parts of Asia including northern China. Typical formulations contain ingredients which would be expected to result in alkaline conditions in the product. Accordingly studies of these products may also provide further insights on the nutritional value of flour-based foods. It is known that cereals can be a significant source of niacin in the diet and although this is regarded as a relatively stable vitamin, further studies are recommended. Such work may also lead to a reassessment of our understanding of the adequacy of nutrient intakes and the impact of processing.

Another issue highlighted by the studies reported here is the paucity of information on the nutritional status, particularly regarding thiamin, for the populations of both developed and developing nations. The general view that both clinical and sub-clinical deficiencies continue to exist even where fortification has been practiced for many decades is an issue of broad concern.

In the context of nutritional value, milling of the wheat grain is known to be important. Although this issue was outside the scope of the current study, further research may now be warranted. It is well established that milling extraction rates directly affect the content of vitamins and other nutrients in white flours (Ranhotra 1994; Gregory 1996; Fujino and others 1996). The low B group vitamin contents of commercial Asian noodles found in the current study may reflect the low milling extraction rates used for Asian noodle manufacture (Miskelly 1993; Nagao 1995a). There is a lack of recent published data on vitamin contents of milled flours of varying extraction rates. This may be a significant issue for further research because there have been many technological

developments in grain milling since the early data was published. Recent advancements in milling technology and practice may have allowed greater vitamin retention in flours (Stenvert 1995; Forder 1997).

There are further reasons for reinvestigation of the impact of milling on vitamin contents in flour. One of these is the increasing evidence that folate is important nutritionally and the ongoing reassessment of the dietary sources of this nutrient and the desirability of fortification for women considering child-bearing as well as the population as a whole.

A second issue is that the published data on flour milling has relied on analytical methods in wide use some decades ago. Advances in analysis may result in significantly higher results and ultimately in different conclusions. This is reinforced by the findings of the current study that optimisation of extraction and analysis procedures is an important consideration particularly for folate. The procedures validated and adopted here would form a useful basis for further studies in this area.

In some parts of the world, fortification has been used to address the problem of dietary deficiencies of some vitamins and other nutrients in the food supply (Borenstein and others 1991; Iannarone 1991). A number of issues arise here. In the current study yellow alkaline and instant noodles were prepared from a commercial flour that had been fortified with thiamin to meet Australian legislative requirements (minimum of 6.4 mg/kg). The losses of thiamin during production of both yellow alkaline and instant noodles were relatively high. For yellow alkaline noodles the losses were so great as to leave insufficient thiamin for this product to be considered a useful source of dietary thiamin. Thus another area for further research may be to further assess ways to enhance the effectiveness of fortification of these products. The relative stability of different fortifying agents including thiamin hydrochloride and thiamin nitrate could also be pursued.

One of the findings of the current study which cannot be readily explained is the apparent instability of PN during the relatively short time when dough mixing occurs. This is of particular interest because no losses were subsequently found when the noodle dough strands were dried at 40 °C. The same relative losses found for all three

styles of noodles demonstrate that the pH of the dough was not a determining factor. These findings warrant further investigation to elucidate the factors and mechanism of the losses observed.

The findings of this study provide a strong basis of knowledge for further investigations aimed at the development of strategies for enhancing the stability of vitamins in Asian noodles. As different factors are important in the cases of particular vitamins and related to specific styles it is likely that a variety of different strategies will be need to be considered.

One possible way in which vitamin stability might be enhanced is the technology of encapsulation. The general approach has been applied widely to pharmaceutical products. More recently microencapsulation has been used for food ingredients and one of the earlier applications has been in the area of flavouring of food products. Varying techniques as well as encapsulating agents are available to suit different purposes (Versic 1988) and the use of encapsulation continues to expand (Gibbs and others 1999; Augustin and others 2001). Currently there are no specific reports describing the use of encapsulation for enhancement of vitamin stability in food products although the potential of the approach has been mentioned in reviews of the subject (Meyers 1998; Nordmark 1999). It is strongly recommended that further work be carried out to investigate the potential of microencapsulation of B group vitamins to enhance their stability. Based on the factors identified here as influencing stability, the manipulation of the pH environment within microcapsules may be useful for both riboflavin and thiamin. Alternative strategies are likely to be necessary for microencapsulation of vitamin B-6 and folate. Although high temperature processing resulted in losses of all four of the B group vitamins, a combination of microencapsulation reagents might be found which would result in enhanced stability during heating. Thus for example, during the extraction and analysis of thiamin, the use of solutions at pH 1 allows the application of autoclave temperatures for 20 min without loss of the vitamin. Further work is warranted as microencapsulation potentially offers a solution to the losses in the vitamins particularly at higher pH values and even those occurring during high temperature processing.

In conclusion, there have been rapid developments in our knowledge over recent years in the areas of noodle processing and packaging as well as the factors that influence their appearance and consumer appeal. The research reported here is the first systematic investigation of the B group vitamins and their stability in these foods. It is hoped that this work might form the basis of further studies of Asian noodles and other food products, ultimately leading to the enhancement of their nutritional value. It appears that much remains to be done to ensure adequate nutrition for our expanding world population.

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