Folate Content of Asian Vegetables

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Foreword

Folates are one of the most nutritionally significant vitamins. Green leafy vegetables are reported to be an excellent source of folates which occur naturally in many forms or vitamers. This study has provided information on the folate content and vitamer composition in selected Asian vegetables. It has highlighted some of the lesser known vegetables, eg mizuna, tatsoi, kang kong, snake beans, in addition to choy sum are excellent folate sources. This information can be used to promote these vegetables through folate content labelling to increase their market penetration. Consumers can use the information to select from a wider range of vegetables to maintain or increase their folate intakes. With the range of Asian vegetables continually expanding, improved product quality and availability and increasing acceptance by Australian consumers, it would be expected that this industry sector will maintain its growth in production volume, value and market penetration. This research will position Asian vegetables in the market-place as a major fresh produce category due to their beneficial health attributes.

All of the vegetables analysed contained the same folate vitamers in varying proportions. 5-Methyl-tetrahydrofolate, reportedly the most bioavailable folate form, was the major folate vitamer, except for mizuna, tatsoi and flowering choy sum in which 5-formyl-tetrahydrofolate was the predominant folate. Storage trials showed only marginal loss of folate activity occurred in the edible portions of the vegetables during refrigerated storage under similar conditions to those used by consumers. Growers and processors now have preliminary supply chain information indicating that the quality of Asian vegetables relating to folate activity can be maintained from harvest to consumption.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS), a highly specific analytical technique, and a reference microbiological assay were applied in this folate study. The limitations of the microbiological assay are well-recognised and this method appeared to overestimate the folate content. As food composition tables and nutrient databases use data from microbiological assays as the accepted standard reference method, this could have implications for public health agencies. The actual folate intake of the population may be lower than determined from these sources, resulting in an increased risk of compromised folate status.

This report is an addition to RIRDC's diverse range of over 2000 research publications and it forms part of our New Plant Products/Cultural and World Foods sub-program (New Rural Industries Portfolio), which aims to improve crop productivity, sustainability, produce quality and the viability of these new plant industries in Australia.

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Ms Avis Houlihan is a Principal Scientist with Agri-Science Queensland (Department of Employment, Economic Development and Innovation, DEEDI) with extensive biochemistry experience in the analysis of major and minor components in foods, with a specific focus on bioactive and functional components.

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Acknowledgments

Abbreviations

AOAC	Association of Official Analytical Chemists
AQIS	Australian Quarantine and Inspection Service
CRM	Certified reference material
DFE	Dietary folate equivalents
EC-JRC-IRMM	European Commission Joint Research Centre Institute for Reference Materials and Measurement
EU	European Union
FACM	Folic Acid Casei Medium
FSANZ	Food Standards Australia New Zealand
HPLC	High performance liquid chromatography
INMU	Institute of Nutrition Mahidol University
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LSD	Least significant difference
NHMRC	National Health and Medical Research Council
NMI	National Measurement Institute
NRV	Nutrient reference value
PteGlu	Pteroylglutamic acid (folic acid)
RDA	Recommended dietary allowance
RDI	Recommended dietary intake
RM	Reference material
UPH_2O	Ultra-pure water

5-Me-THF, 5-MTHF, 5-methyl-THF	5-methyl-tetrahydrofolate
5-Formyl-THF, 5-FTHF	5-formyl-tetrahydrofolate
10-Formyl-FA, 10-FFA	10-formyl-folic acid
10-Formyl-THF, 10-FTHF	10-formyl-tetrahydrofolate
5,10-CH-THF	5,10-methenyl-tetrahydrofolate
$5,10-CH_2-THF$	5,10-methylene-tetrahydrofolate
DHF	dihydrofolate
THF	tetrahydrofolate

Contents

Tables

Figures

Executive Summary

What the report is about

This report provides information to industry and consumers on the folate content and folate vitamer composition of selected Asian vegetables grown in Australia, using a highly specific analytical technique (liquid chromatography-tandem mass spectrometry, LC-MS/MS). Analysis was also done by the microbiological folate assay, the current 'gold standard' and accepted reference method for folate analysis.

Who the report is targeted at

The findings from this research will provide industry with a marketing message to highlight the health benefits of Asian vegetables through folate content labelling. Consumers will use this information in their food purchase decisions to ensure adequate folate intake for improved health. Health and allied professionals will use the data to provide dietary and nutritional advice. In due course, public health organisations, such as Food Standards Australia and New Zealand (FSANZ), may incorporate the data on individual folate vitamers into food composition tables and nutrient databases.

Background

Folates are one of the most nutritionally significant vitamins. The importance of folate in the diet of pregnant women for the prevention of neural tube defects (spina bifida) in babies is well-known. Folate deficiency has also been implicated in a wide variety of disorders including Alzheimer's disease, cardiovascular diseases, osteoporosis, breast and colon cancers, depression, dementia, cleft lip/palate and hearing loss. The National Health and Medical Research Council (NH&MRC) dietary guidelines recommend greatly increased folate intake to meet daily nutritional requirements and for the reduction of chronic disease risk compared to previous dietary guidelines. Natural sources of folate are reportedly more effective than supplementation or fortification with folic acid. Green leafy vegetables are excellent sources of folates which occur naturally in many forms or vitamers. With an expanding range of Asian vegetables and increasing consumer awareness and acceptance, it is timely to present information on the health attributes of these vegetables.

Aims/objectives

The aim of this project was to obtain information on the folate content and folate vitamer profile of a range of Asian vegetables using an advanced analytical technique (LC-MS/MS) for industry to use as promotional and marketing tool, to increase awareness and knowledge of health and allied professionals and for consumers to become more familiar with the health attributes of Asian vegetables.

Methods used

A liquid chromatography technique incorporating tandem mass spectrometry (LC-MS/MS) was used to determine the folate vitamer composition of selected Asian vegetables. These were buk choy (*Brassica rapa* subsp. *chinensis*), choy sum (*Brassica rapa* subsp. *parachinensis*), gai choy (*Brassica juncea*), gai lan (*Brassica oleracea* var. *alboglabra*), kang kong (*Ipomoea aquatica*), mizuna (*Brassica rapa* var. *nipposinica*), pak choy (*Brassica rapa* subsp. *chinensis*), snake bean (*Vigna unguiculata* subsp. *sesquipedalis*), tatsoi (*Brassica rapa* var. *rosularis)* and wombok (*Brassica rapa* subsp. *pekinensis*). Spinach (*Spinacia oleracea*) was included as a high folate reference vegetable.

A revised and improved microbiological assay for total folate was used as the standard reference method. While the objective of this project was to apply an advanced methodology (LC-MS/MS) to

obtain more definitive information on folates in vegetables, it was also important to have a recognised reference method for quality control purposes.

Results/key findings

Mizuna, tatsoi, choy sum, kang kong and snake beans were found to contain more or similar folate contents to spinach, a recognised high folate source. All of the vegetables analysed contained the same folate vitamers (5-methyl-tetrahydrofolate, 5-formyl-tetrahydrofolate, 5,10-methenyl-tetrahydrofolate, 10-formyl-folic acid and tetrahydrofolate) in varying proportions. 5-Methyl-tetrahydrofolate, reportedly the most bioavailable form, was the major folate vitamer in the vegetables, except mizuna and tatsoi in which 5-formyl-tetrahydrofolate was the predominant folate. Storage trials showed only marginal loss of folate activity occurred in the edible portions of the vegetables during refrigerated storage under similar conditions to those used by consumers. Growers and processors now have preliminary supply chain information indicating that the quality of Asian vegetables relating to folate activity can be maintained from harvest to consumption.

LC-MS/MS is a highly specific analytical technique with a high level of accuracy and reproducibility. In this project, the method has produced detailed information on individual folate vitamers in Asian vegetables. The limitations of the microbiological assay are well-recognised. The assay is time-consuming, labour-intensive, lacks specificity and measures only total folate. This assay consistently gave higher results than the LC-MS/MS method and may overestimate the folate content of foods.

Implications for relevant stakeholders

With the range of Asian vegetables continually expanding, improved product quality and availability and increasing acceptance by Australian consumers, it would be expected that this industry sector will maintain its growth in production volume, value and market penetration. This research will position Asian vegetables in the market-place as a major fresh produce category due to their beneficial health attributes.

If the microbiological assay is overestimating the folate content of foods, there are implications for public health agencies. Food composition tables and nutrient databases use data from microbiological assays as the accepted standard reference method. The actual folate intake of the population may be lower than that determined from these sources, resulting in an increased risk of compromised folate status. Further studies are required to clarify this area.

Recommendations

Further research is recommended to investigate the variation between the two analytical methods used in this project, including spiking of prepared vegetable samples with labelled folate standards to monitor loss of added folate. The application of stable isotope dilution assays would greatly increase the analytical potential of the LC-MS/MS method. Studies of the impact of senescence of whole plants on folate degradation would provide useful information on folate changes during storage. Research on the distribution of folates in plant organelles, inner and outer leaves and flowering stems could assist in consumer selection for high folate intake. Transport and storage across the supply chain could be studied to ensure optimum conditions for the maintenance of folate activity from harvest to consumption. Innovative processing steps which increase folate bioavailability and improve the health benefits of these vegetables could be investigated.

Examination of other bioactive phytochemicals in Asian vegetables would provide industry with additional marketing and promotional information and provide consumers with more information on the health properties of these vegetables leading to increased demand, sales and consumption of this vegetable sector.

1. Introduction

The health benefits of incorporating a wide variety of vegetables in the diet are well-recognised. Green leafy vegetables are reported to be excellent sources of folates which are water-soluble vitamins and occur naturally in foods in many forms or vitamers. It is important to provide consumers with as much information as possible on natural sources of folate, which are more effective than supplementation or fortification with folic acid. The wide range of Asian vegetables now available in Australia provides consumers with many choices. Preliminary evidence indicates that the folate contents of several Asian vegetables are greater than spinach, which is considered a high-folate vegetable (Iwatani et al., 2003).

The importance of folate in the diet of pregnant women for the prevention of neural tube defects (spina bifida) in babies is well-known. Recent research has indicated that an adequate folate intake may be important in minimising the risk of other disease states, including Alzheimer's disease (Seshadri et al., 2002), cardiovascular diseases (Loria et al., 2001), osteoporosis (Gjesdal et al., 2006), breast and colon cancers (Shrubsole et al., 2001) (Kato et al., 1999) (Kim, 1999), depression (Reynolds, 2002), dementia (Ebly et al., 1998), cleft lip/palate (Krapels et al., 2004), and hearing loss (Houston et al., 1999).

Public health organisations are recognising the importance of increased folate in the diet. The US Food and Nutrition Board have nominated a recommended dietary allowance (RDA) for folate of 400 µg for adults, 600 µg during pregnancy and 500 µg during lactation per day. The National Health Federation in the US has recommended higher nutrient reference values (NRV) for folate of 800 µg for adults, 830 µg during pregnancy and 865 µg during lactation per day. In Australia, the National Health and Medical Research Council (NH&MRC, 2006) released revised dietary guidelines "Nutrient Reference Values for Australia and New Zealand, including Recommended Dietary Intakes", which define the recommended dietary intakes (RDI) for a range of nutrients. The RDI for folate has been increased substantially to 400 µg DFE (dietary folate equivalents) per day, a 100 % increase compared to previous guidelines. The NH&MRC has also broadened the concept of dietary intakes, to include recommendations for optimising diets for lowering chronic disease risk, with an additional 100 - 400 µg DFE recommended per day.

The microbiological assay for total folate is the only method accepted by the AOAC International as a standard reference method and is the source of folate data in food composition tables and nutrient databases. The assay is time-consuming, labour-intensive and lacks specificity. Since the micro-organism is unlikely to respond equally to the different folate vitamers, this method may not be a reliable measure of the total folate present in foods (Martin, 1995). More specific chemical techniques, such as HPLC (high performance liquid chromatography), have been developed (Ginting and Arcot, 2004) (Doherty and Beecher, 2003) (Jastrebova et al., 2003) (Pawlosky et al., 2003) and further improved by the addition of mass spectrometry (Freisleben et al., 2003a, Freisleben et al., 2003b) (Thomas et al., 2003) (Nelson et al., 2001) (Stokes and Webb, 1999). These procedures measure individual vitamers and so provide more information on the folate content of foods.

This project was undertaken to provide more information on the folate content and vitamer composition of Asian vegetables available in the Australian market. Individual folate vitamers were measured in selected vegetables using an LC-MS/MS method. The microbiological assay for total folate was also applied as the official standard reference method.

2. Objectives

The objective of this project was to obtain information on the folate content and folate vitamer profile of a range of Asian vegetables using an advanced analytical technique based on liquid chromatography and tandem mass spectrometry (LC-MS/MS). The aim was to provide information for industry to use as a health claim marketing tool (folate content claim), for health and allied professionals to use in dietary and nutritional advice, and for consumers to become more familiar with the health attributes of the many Asian vegetables currently available to enable informed purchase decisions.

3.   Methodology

3.1.   Vegetable samples

With a focus on consumer information needs, common names of selected Asian vegetables were derived from Australian wholesale and retail conventions. The national agreement on names for the more common Asian vegetables (Ekman, 2008) was used where applicable and identification was based on morphology.

3.1.1. Sampling

Fresh vegetables were purchased from a number of retailers, including Asian grocers (Figure 1) and national supermarkets, in the southern area of Brisbane, Queensland, Australia. After a preliminary survey, using a microbiological folate assay, five samples from different retailers of each vegetable found to have good folic acid equivalent activity were obtained over winter and spring in 2009 (see Appendix A).

The Asian vegetables selected for this study were:

- buk choy (*Brassica rapa* subsp. *chinensis*)
- choy sum (*Brassica rapa* subsp. *parachinensis*)
- gai choy (*Brassica juncea*)
- gai lan (*Brassica oleracea* var. *alboglabra*)
- kang kong (*Ipomoea aquatica*)
- mizuna (*Brassica rapa* var. *nipposinica*)
- pak choy (*Brassica rapa* subsp. *chinensis*)
- snake bean (*Vigna unguiculata* subsp. *sesquipedalis*)
- tatsoi (*Brassica rapa* var. *rosularis*)
- wombok (*Brassica rapa* subsp. *pekinensis*)

Spinach (English) (*Spinacia oleracea*), as a known high folate vegetable, was included for comparative purposes.

A sub-sample of approximately 50 g was taken on the day of purchase to represent an edible portion, defined as 'that likely to be consumed in a typical food preparation'. The raw material was washed and dried prior to stabilisation by cryo-homogenisation and storage at -80 °C.

Figure 1.   Retail display of Asian vegetables

3.2.   Microbiological assay

The microbiological assay is currently the 'gold standard' assay for total folate analysis and is the accepted standard reference method for Australian and international food composition tables and nutrient databases. While the objective of this project was to apply an advanced methodology (liquid chromatography-tandem mass spectrometry, LC-MS/MS) to obtain more definitive information on folates in vegetables, it was also important to have a recognised reference method for quality assurance purposes. Although the microbiological assay is widely used, it does have limitations. It is method-specific (small variations in methodology can markedly affect the results obtained) (Shrestha et al., 2000), operator-specific, matrix-specific (requiring optimisation for each food type) and provides information on total folate only (not vitamer composition). Considerable research effort in this project was dedicated to ensuring that the microbiological assay used was rigorously optimised and validated for the analysis of folates in a vegetable matrix to ensure a high level of repeatability and reproducibility.

3.2.1. Principle

A vegetable homogenate is heated in a phosphate buffer with an antioxidant to initiate the protected extraction of folates. A tri-enzyme treatment is used to further digest the food matrix and hydrolyse polyglutamated folates to mono-glutamates and di-glutamates. Extracts are filter sterilised and added to a folate-free basal medium. *Lactobacillus rhamnosus* from a cryo-protected inoculum is cultured with this medium and the growth response is measured turbidimetrically. The response is proportional to the concentration of folates added in the vegetable extract. Folic acid equivalent activity is assigned by comparison to the growth response using folic acid over a defined range. As the micro-organism is reported to have variable responses to the individual folate vitamers, the activity is dependent on the vitamer profile, which varies between food types (eg plants, cereals, meats) and between species within these categories.

3.2.2. Preliminary method development

Establishment of a microbiological assay

A microbiological assay for folate, based on published procedures (AACC, 2000) (AOAC, 2006b, AOAC, 2006a) (Shrestha et al., 2000) (Iwatani et al., 2003) (Sharpless et al., 2008), was established by Associate Professor Prapasri Puwastien and Ms Wasinee Jongjitsin, of the Institute of Nutrition, Mahidol University (INMU, Nakhon Pathom, Thailand), in conjunction with Dr Glenn Graham, at the Queensland Health Forensic and Scientific Services laboratories. Associate Professor Puwastien has extensive experience in folate research and has been involved in several national and international inter-laboratory studies. Queensland Health funded a 4 month sabbatical visit for Associate Professor Puwastien and Ms Jongjitsin to join the project team. A summary of the research undertaken during this time is included in Appendix B.

Preparation of chicken pancreas extract

Chicken (*Gallus gallus domesticus*) pancreas was used as a source of folate γ-glutamyl hydrolase (conjugase) activity. Folate γ-glutamyl hydrolase (EC 3.4.19.9) activity refers to the cleaving of the γ-peptide bonds that couple the glutamate residues together (a different α-peptide bond attaches the first glutamate residue to a folate compound). Folate γ-glutamyl hydrolase from chicken pancreas extract is reported to yield di-glutamates (Leichter et al., 1977). After treatment with chicken pancreas extract, folates may exist as mono-glutamates (those already existing in the food matrix or from endogenous food enzyme activity) and di-glutamates (provided complete deconjugation occurs).

The extract was prepared in-house from a fresh source, based on AOAC method 40.40 (AOAC, 1950), due in part to discontinuation of the commercial source previously available. Chicken pancreas purchased from a commercial operation was homogenised with an equal volume of 0.2 M sodium phosphate buffer, pH 7.2. The homogenate filtrate was slowly mixed for one hour on ice to obtain a crude extract. Aliquots of the extract were lyophilised and stored at -20 °C. Homogeneity was checked by comparison of endogenous folate activity from five separate aliquots and hydrolase activity validated against a certified reference material (CRM), BCR-485 mixed vegetables (European Commission Joint Research Centre Institute for Reference Materials and Measurement (EC-JRC-IRMM), Geel, Belgium). A Horwitz ratio (HorRat) of 0.96 calculated from the relative standard deviation (RSD) for the five samples was deemed within an acceptable range of homogeneity for single laboratory validation (SLV) (Horwitz, 2003).

In-house reference materials

In-house reference materials (RM) were prepared as standards for assay quality control (QC). Fresh broccoli (*Brassica oleracea* var. *italica*) and buk choy (*Brassica rapa* subsp. *chinensis*) were purchased from a local supermarket. 2.4 kg of broccoli was sub-sampled for 1.6 kg of flower head, and 1.8 kg of buk choy was sub-sampled for 1.4 kg of edible portion. Both were chopped, blended and lyophilised, with recovery of 175 g and 30 g of dried material respectively. Each lyophilised sample was divided into small vials containing 1 - 2 g to reduce deterioration from repeated exposure, and stored at -20 °C. Homogeneity of the broccoli RM was checked by comparison of assayed undeconjugated folate activity from seven separate vials. A HorRat of 0.40 calculated from the RSD for the seven samples was deemed within an acceptable range of homogeneity for SLV (Horwitz, 2003).

Subsequent analytical results were charted on a quality control chart (Figure 2) to confirm statistical control of the on-going analytical process. Progress in quality control from the preliminary method and method revision to a working analytical method can be seen.

Figure 2.   Quality control chart
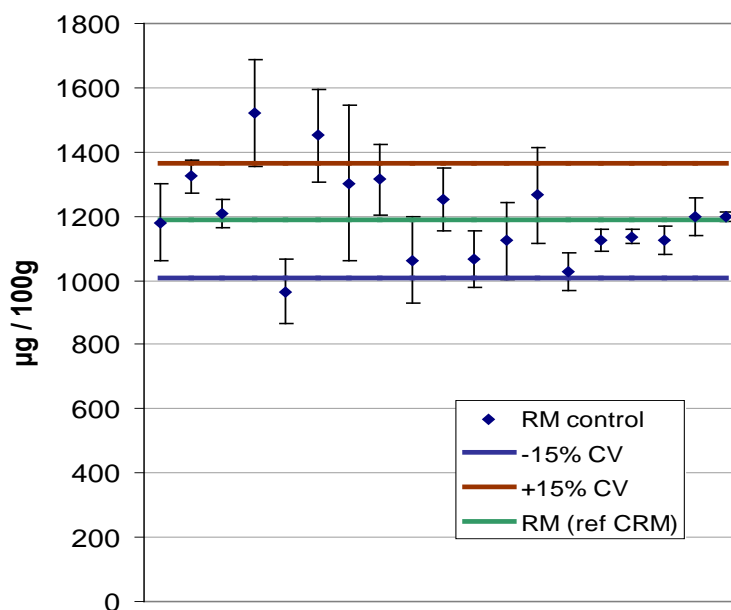
Trial of materials and method

The preliminary microbiological assay was trialled using the in-house chicken pancreas extract and reference materials. Evaluation followed with defatted soybean flour and rice germ, previously analysed at INMU, and fresh vegetable samples. The trial determined an endogenous folate activity for the chicken pancreas extract of $510 \pm 31 \, \mu g \, / \, 100 \, g$ and an undeconjugated folate activity for the lyophilised broccoli of $613 \pm 15 \, \mu g \, / \, 100 \, g$. At this stage, the method was found to require repeated adjustment of inoculum concentration and culture incubation period to achieve an acceptable response. A positive titration drift was overcome by lowering the culturing pH from 6.8 (AOAC, 2006a, AOAC, 2006b) to 6.2 (Arcot and Shrestha, 2005). The assay range for determination was 0 - 1.0 ng folic acid equivalent activity per culture tube.

Evaluation with the defatted soybean flour and rice germ produced folate activities that differed by 40 – 50 % from the determination at INMU. It was considered that the activities of the material may have changed during transit to the Queensland Health laboratories. Evaluation with fresh vegetables found that quantifiable growth was dependent on inoculum concentration and incubation period. Duplicate extracts from fresh Shanghai pak choy (*Brassica rapa* subsp. *chinensis*), mungbean sprouts (*Vigna radiata*) and basil leaves (*Ocimum basilicum*) were assayed for total folate. A comparison with a previous study conducted in Australia (Iwatani et al., 2003) is shown in Table 1. The values obtained in this study were much lower than those reported by Iwatani et al. (2003), except for basil. Overheating of extracts, which could lead to reduced folate activity, was a concern with the autoclaving equipment available and could have contributed to the low results.

Table 1.   Comparison of folic acid equivalent activity (μg/100 g fresh weight)

Vegetable	This study (preliminary)	Iwatani et al. (2003) (mean ± SD)
Shanghai pak choy (*Brassica rapa* subsp. *chinensis*)	92	333 ± 13
Mungbean sprouts (*Vigna radiata*)	20	208 ± 7
Basil leaves (*Ocimum basilicum*)	114	103 ± 10

3.2.3. Revision of method

Based on the knowledge obtained in the preliminary studies, the method was re-assessed after a break in the research program when the Queensland Health scientists on the project team were required for urgent public health activities. It was decided to restructure the method, based on the following key changes.

- Single-use plasticware to eliminate residue and cleaning risk

  The test organism can be sensitive to some residues, and the high temperatures required to neutralise them (AOAC, 2006a, AOAC, 2006b) can be hazardous to staff and detrimental to volumetric glassware calibration (Standards-Australia, 2006). Sterile micro-pipettes were used to dilute extracts and inocula to assay concentrations.

- Filter sterilisation to protect heat labile folates

  Some naturally occurring folates are degraded by oxidation when heated (Chen and Cooper, 1979). Autoclaving can entail additional and variable heat periods during warm-up and cool-down stages. Microfiltration was used instead to sterilise extracts, reducing their exposure to heat and depletion of antioxidant. Sterile extracts were then aseptically added to culture preparations (Herbert, 1961).

- Tri-enzyme incubation time based on response surface methodology

  Incubation times for enzymes in folate extraction remain unresolved. Digestion with pronase for 1.5 hr, α-amylase for 1.5 hr, and γ-glutamyl hydrolase (conjugase) from chicken pancreas for 3 hr was found to be optimal in total folate determination by microbiological assay for the mixed vegetable CRM, BCR-485 (Chen and Eitenmiller, 2007). Their study reported higher measurable folate in a variety of vegetables using these incubation times compared with those prescribed in the AOAC Official Method 2004.05 for folates in cereals (AOAC, 2006b).

- Termination of incubation with chicken pancreas extract by freezing

  Method revision indicated an analyte loss with heat termination of the deconjugation step. Boiling for 5 minutes produced a precipitate from the enzyme and it was concluded that some folate may be a co-precipitant, possibly through protein binding. Termination by freezing was substituted, as used by Tamura (1990) and others, which improved analyte recovery and was implemented as part of the cryo-storage of extracts.

- Cryo-protection of the test organism for reproducible growth response

  This enabled a uniform batch of inocula, prepared directly from the reference strain, to be frozen as single use aliquots. Minimal passage has less possibility of culture divergence than repeated sub-culturing or serial maintenance (Reichgott, 2003). The procedure was based on

Wilson and Horne (1982) where cell washing and folate starvation were replaced by using a low folate medium before cryo-preservation with glycerol.

- Equivalent folic acid activity based on consensus

Equivalency of test organism growth for different folate isomers also remains unresolved. A number of researchers report varying equivalence which implies a dependence on method and materials. Phillips and Wright (1982) observed a difference in growth response with folic acid compared to 5-methyl tetrahydrofolate (5-Me-THF), a major form in vegetables, for an initial assay pH of 6.8 but not when the media buffer capacity was increased and the initial pH lowered to 6.2. Other researches have found equal growth at the higher pH. Early reports (Spronk and Cossins, 1972) suggest that some micro-organisms may preferentially utilise conjugated folates containing more than three glutamic acid residues. Clearly, more work is required as these investigations differ in extent of deconjugation, culturing (media composition, buffer capacity, oxygen tension) and the use of pure folates or natural sources containing other growth factors.

The method revision included using a lower initial culture pH of 6.1, but most extracts showed some difference in equivalency to growth from the folic acid standard. This could be due to:

- a lag or reduced response to natural folates at low concentrations, possibly a metabolic effect

- if equivalency is pH dependent, the buffering capacity of the culture medium is not sufficient to counter the lactic acid produced by the test organism and so pH varies with the amount of growth

- response to pure folic acid may be limited at higher concentrations from exhaustion of another growth factor in the culture medium, but which was supplemented by the sample extracts

- response may be affected by the amount of antioxidant persisting in cultures which could vary in depletion depending on the sample extract; however, the volume of extract involved may be too small for this

- the crude chicken pancreas extract or digestive enzyme preparations may have supplemented the growth from sample extracts

- incomplete deconjugation of polyglutamated folates due to sample extract inhibitors.

Growth drift had been reported during an inter-laboratory study of folates in Brussels sprouts by some participants using a microbiological assay (Finglas et al., 1993). Keagy (1985) advised excluding the less responsive ends of the growth range, cited reported sources of drift, and noted that drift from natural sources is not usually statistically significant with the limited data of single extracts. Koontz et al. (2005) cite reports in which the growth response differs for various folates relative to folic acid.

In the revised method which follows, a zone of equivalency was used where the folic acid standard matched the consensus activity assigned to the certified reference material, BCR-485 (Figure 3).

Figure 3.   Revised microbiological assay zone of equivalency
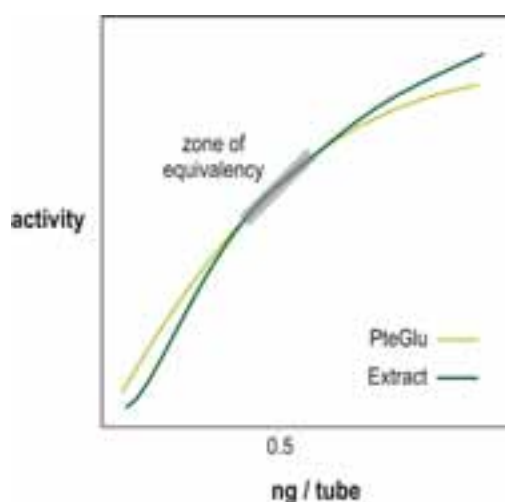
Note: Illustrative comparison for un-purified vegetables extracts using the tri-enzyme treatment with crude γ-glutamyl hydrolase and culturing conditions described below.

Extraction and deconjugation

Sample material and homogenisation

Representative material of the edible portion was taken from each vegetable sample. About 50 - 60 g of this material was chopped and mixed to homogenise, then cryo-homogenised by grinding with liquid nitrogen under subdued lighting. Homogenates were protected from light (Stokstad et al., 1947) and stored in triplicate sub-samples at -80 °C, one of which was prepared first by weighing 2 g of homogenate directly into a 50 mL centrifuge tube ready for extraction.

Extraction buffer

An aqueous buffer of 0.1 M sodium phosphate, 1.0 % L-ascorbic acid (95209, Fluka, Sigma-Aldrich, Buchs, Switzerland) as antioxidant, and pH 6 (Keagy, 1985) was prepared on the day of sample extraction. The literature survey by Arcot and Shrestha (2005) showed that 1.0 % ascorbic acid around this pH is the combination most used to stabilise labile reduced folates during extraction, but optimum extraction pH, and the effect on folate release and isomer interconversion, remains debated among researchers. A pH of 6.0 was selected as being representative of recent vegetable studies (Iwatani et al., 2003) (Devi et al., 2008) and close to the microbiological assay pH of 6.1 used for the activity determination, avoiding the introduction of added variation from pH adjustments.

Heat and enzyme treatments

Heat and enzyme digestion were applied to the thawed vegetable homogenates to increase the release of folate from the sample matrix. Digestion involved the three enzyme (tri-enzyme) scheme (Table 2), where starch and protein are digested to release folate trapped in the sample matrix and polyglutamated folates are deconjugated for an equimolar response by the test organism. Unlike Martin et al. (1990), the enzyme order followed Tamura et al. (1997) with α-amylase first, followed by Pronase E (a commercial non-specific protease) and deconjugation performed after release of folates from the sample matrix.

9

Table 2.   Enzyme preparations (prepared on day of use)

Enzyme	Stock	Concentration [a]	Diluent / Preparation
α-Amylase, Type X-A (EC 3.2.1.1) 31.8 U/mg from *Aspergillus oryzae*	10065, Fluka BioChemika, Sigma-Aldrich, Buchs, Switzerland	20 mg/mL	UPH$_2$O [b]
Protease, Type XIV (EC 232-909-5, Pronase E) 5.4 U/mg from *Streptomyces griseus*	P5147, Sigma-Aldrich Co., St Louis, MO, USA	1.3 mg/mL	UPH$_2$O [b]
Chicken pancreas extract (EC 3.4.19.9, γ-glutamyl hydrolase activity) crude lyophilate	In-house	5 mg/mL	extract buffer / slowly stirred 1 hour, supernatant collected after centrifuging 2000 g for 10 minutes (Keagy, 1985)

[a] Concentrations based on Martin et al. (1990).

[b] All water used for reagent preparation was 18.2 MΩ·cm ultrapure quality (UPH$_2$O), not distilled which may accelerate oxidation of ascorbic acid by heavy metal ions–depending on the source equipment (Herbert 1961).

Extraction and deconjugation were performed with single use sterile plasticware, covered with aluminium foil, following the protocol in Table 3.

Table 3.   Extraction and deconjugation protocol

Sample	weigh homogenate into a centrifuge tube	2 g (1 - 2 µg folate) (exact weight noted)	
pH buffer Antioxidant protect	add extract buffer	20 mL	
Particle break-up Enzyme inactivate Protein denature Vegetative sterilise	heat (water bath) and cool	100 ºC ambient	10 minutes
Starch digest	add α-amylase incubate [a]	2 mL 37 ºC	1.5 hours
Protein digest	add pronase incubate [a] inactivate (water bath) cool	2 mL 37 ºC 100 ºC ambient	1.5 hours 5 minutes
Undeconjugated sub-sample	centrifuge sample supernatant	2000 g	10 minutes
Deconjugate	supernatant to new tube add chicken pancreas extract incubate [a]	6 mL 4 mL 37 ºC	3 hours
Sterilise	centrifuge filter supernatant [b]	2000 g 0.2 µm	10 minutes
Aliquot	cryogenic vials protect from light	1-2 mL	
Digest terminate Cryo-preserve	freeze	-80 ºC	

[a] Incubations were performed in darkness using a shaking water bath.

[b] 0.2 µm polyethersulfone membrane (4652, Acrodisc, Pall Corp., Newquay, Cornwall, UK)

Bacteriological determination

Cryo-protected inoculum

The test organism used to determine folate equivalent activity was a strain of bacteria designated ATCC 7469 (American Type Culture Collection). This is a type strain of the family *Lactobacillaceae* with the current name of *Lactobacillus rhamnosus*, a nutritionally fastidious taxon with strains being isolated from humans (including the intestinal tract) and dairy products (Hammes and Hertel, 2009). Formerly known as *Lactobacillus casei* subsp. *rhamnosus*, it has been elevated to species status by phenotypic, including rhamnose fermentation, and genomic taxonomy as proposed by Collins et al. (1989).

Figure 4. *Lactobacillus rhamnosus* (plate growth / microscope)
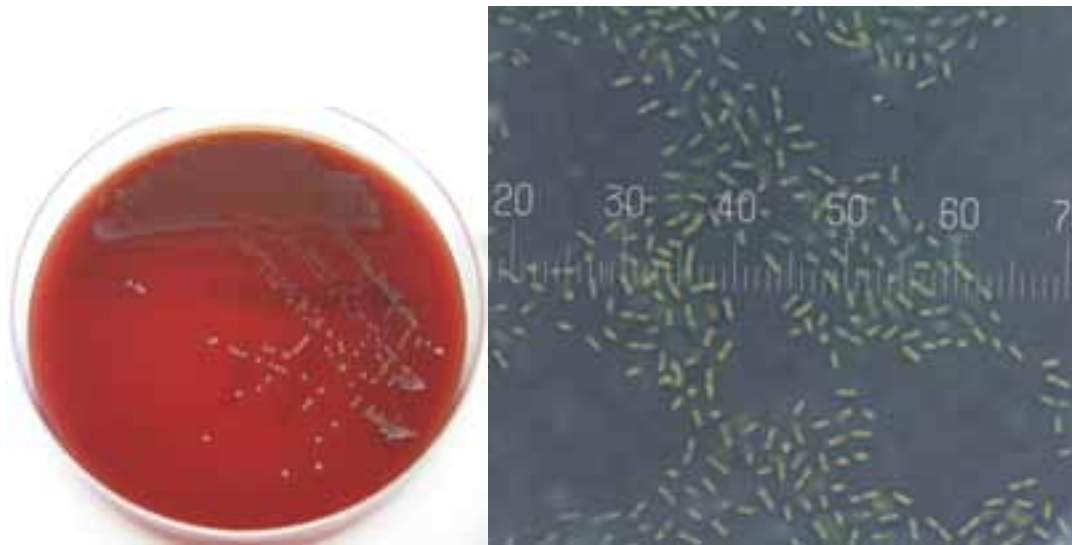
In a review by Quinlivan et al. (2006), the growth *L. rhamnosus* ATCC 7469 is cited as being responsive to all biologically active folate isomers but not to inactive stereoisomers or precursors of folate, and that its response diminishes with folates having more than three glutamate residues. It is the strain used in official methods of AOAC International (formerly, the Association of Official Analytical Chemists) for the microbiological assay of folate. Although growth of the test organism is considered to have an absolute requirement for folate, there are early reports of effects from non-folate substances in the absence of folic acid, but without substantiation or much attention in more recent literature (Wilson et al., 1987) and this remains a question for assay accuracy (Koontz et al., 2005).

The strain was sourced from a lyophilised preparation (0233, MicroBioLogics Inc., North Saint Cloud, MN, USA) that incorporates a desiccated gelatin method (Obara et al., 1981), and obtained from Cryosite Distribution Pty Ltd (Lane Cove, NSW). Figure 4 shows the plate growth and microscopic appearance of *L. rhamnosus*. The certificate of analysis for the strain (Lot 23314) states derivation as four passages from the reference organism. AQIS requirements for quarantine permits and approved facilities were observed.

Inoculum stock and cryo-protection

The freeze-dried strain was revived as per the producer's instructions and cultured (Wilson and Horne, 1982). The lyophilised pellet was resuspended in hydrating fluid, a proprietary sterile phosphate buffered saline supplied with the ampoule, and a swab of suspension used to inoculate 10

mL of sterile FACM (Folic Acid Casei Medium) supplemented with 0.3 µg / L of folic acid. After incubation at 37 °C for 7 hr, 0.5 mL of growth was sub-cultured into 100 mL of the same medium, distributed as 10 mL aliquots, and incubated for a further 22 hr to obtain inoculum growth and deplete the medium and bacteria of folic acid.

The resulting culture was pooled with an optical density of 0.49 measured at A_{600nm}. The test organism was cryo-protected by cooling the culture in an ice bath and then combining with an equal volume of cold sterile glycerol/water (80:20, v/v) to form the inoculum stock. 2 mL aliquots were stored in sterile cryogenic vials at -80 °C. Viable enumeration by plate count using the surface spread method (horse blood agar, HBA), determined a stock concentration of 5.3×10^7 colony forming units mL^{-1}.

Purity and viability of the inoculum was checked by inoculating non-selective media, horse blood agar (HBA, 04059, bioMérieux Australia Pty. Ltd., Murarrie, QLD), and FACM (± folic acid) and incubating aerobically at 37 °C for 24 hr. Materials and apparatus were similarly checked at critical stages throughout the preparation and revived frozen test samples of the cryo-protected stock.

Assay buffer and basal medium

Although the micro-volumetric technique did not require a concentrated stock of culture medium, a double strength medium was prepared to allow for the dilution effect of adding the sample extracts or standards. This allowed the medium to be diluted to culture concentration with an assay buffer to avoid degradation of the antioxidant by the heat required to dissolve the dehydrated medium. A 0.05 M sodium phosphate buffer, with 0.15 % L-ascorbic acid as an antioxidant, pH 6.1 (Keagy, 1985) was prepared at double strength for dilution with the culture medium. It was made on the day of assay as the antioxidant in solution degraded over time even when stored at 4 °C. An additional volume of buffer diluted to single concentration was prepared and filter sterilised (0.2 µm) as a diluent for extracts and the standard.

Folic Acid Casei Medium (FACM, 282210, Difco, Becton Dickinson and Co., Sparks, MD, USA) was prepared at double strength according to the manufacturer's instructions, excepting that ascorbic acid was added to the buffer. This is a commercial dehydrated basal medium with a base of glucose as the carbohydrate, sodium acetate and phosphate buffering, and an enzymatic digest of casein as a non-specific source of metabolites which had been charcoal treated to remove vitamins. An enzymatic digest of casein has been preferred over acid hydrolysis which can result in a precipitate on heating, affecting turbidity measurement (Waters and Mollin, 1961). It was prepared on the day of assay to avoid variation from storage effects, immediately combined with the assay buffer and filter sterilised to prevent contaminating growth. Filter sterilisation removed particulates, improving clarity for turbidity measurement.

Folic acid standard

A concentrated stock solution of folic acid (≥ 97.0 %, 47620, Fluka BioChemika, Sigma-Aldrich, Buchs, Switzerland) was prepared without ethanol, based on the procedure for the commercial culture medium (Zimbro and Power, 2003). 50 mg of folic acid was dissolved in 30 mL of 0.01 M NaOH and made up to 500 mL with UPH$_2$O. The concentration and purity of the intermediate stock was checked by measuring the UV absorbance at pH 13 (a dilution with 0.1 M NaOH), using the molar extinction coefficients of ε_{mol} 24.5×10^3 at 256 nm and ε_{mol} 23.4×10^3 at 283 nm (Keagy, 1985). The concentrated stock was dispensed as 5 mL aliquots in tubes, headed with nitrogen, protected from light, and stored at -20 °C. Working standard stock was made by diluting the concentrated stock with UPH$_2$O to a concentration of 100 ng mL^{-1}. This stock was filter sterilised (0.2 µm), dispensed as 1 - 2 mL aliquots in sterile cryogenic vials, protected from light, and stored at -80 °C.

Culturing

Double strength assay buffer and basal medium were combined in equal volumes, filter sterilised (0.2 µm) and dispensed in 5 mL aliquots to sterile 9 mL polycarbonate culture tubes. Sample extracts, including an extraction blank, and a working standard were thawed and allowed to reach ambient temperature, then diluted with sterile single strength assay buffer to a concentration of 10 ng mL^{-1}, with the dilution being estimated for extracts. 10 - 100 µL of the diluted extracts and standard were aseptically added to separate prepared culture tubes for an assay range of 0.1 to 1 ng equivalent folic acid per tube.

A working inoculum was prepared by diluting a thawed vial of the cryo-protected *L. rhamnosus* (ATCC 7469) stock 1:40 with sterile normal saline. 50 µL of the working inoculum was added to each culture tube, except for medium and sample blanks. Tubes were capped and incubated in a shaking water bath, set to gentle rocking to maintain a uniform temperature, at 37 ºC for 22 hr in darkness. Following incubation, tubes were boiled for 5 mins to terminate growth, cooled and allowed to stabilise at ambient temperature before measuring. Boiling was found not to affect the turbidimetric determination of relative activity.

All procedures were performed aseptically, with sterile apparatus, and under subdued lighting. Non-selective media, HBA, were inoculated from the working and stock inocula and from samples of assay growth, and incubated aerobically at 37 °C for 24 hr to check purity and viability.

Turbidimetric measurement and folic acid equivalent determination

Assay growth was resuspended and immediately aliquoted to a flat bottom micro-titre plate. Turbidity was measured by absorbance at 595 nm using a Multiskan EX photometric micro-plate reader (Thermo Electron, Vantaa, Finland), using the plate shake and measurement lag functions to homogenise the well contents then stabilise any flow birefringence (Keagy, 1985).

A 2nd order polynomial was fitted to the absorbance of growth from the folic acid standard against dilution. Absorbances of growth from the sample extracts and the extraction blank were compared to the standard curve to obtain equivalent folic acid response. Sample extract dilutions with growth corresponding to around 0.5 ng folic acid per tube, the equivalency zone based on CRM consensus, were used to calculate activity. The extraction blank was subtracted from sample activities to correct for endogenous enzymes, folate, or other growth promoters.

3.2.4. Summary of revised microbiological assay

Figure 5 shows a schematic diagram of the revised microbiological assay used for folate analysis in this study.

Figure 5.   Microbiological assay of equivalent folic acid (PteGlu) activity
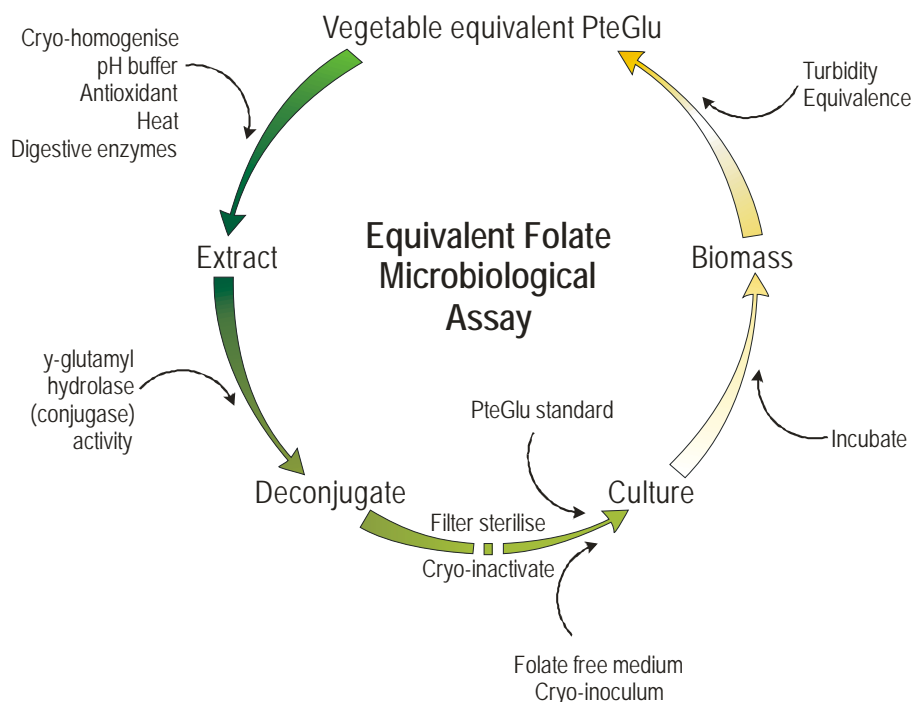
3.2.5. National Measurement Institute Proficiency Study AQA 09-12 Folic Acid in Flour

The revised microbiological assay was employed in a proficiency study (AQA 09-12) conducted by the National Measurement Institute (NMI, Pymble, NSW) during September 2009 to assay folic acid added to a flour and a bread mix. The study was designed to assist Australian testing laboratories evaluate method performance, key to the introduction of mandatory folic acid fortification of wheat flour for bread making in Australia by Food Standards Australia New Zealand (FSANZ). The timing of the proficiency study was particularly opportune in that it provided the present study investigators with an additional and independent check of the accuracy of the determinative step as measured against other competent laboratories using both microbiological and chromatography-based methods for folic acid. 12 laboratories participated in the study which consisted of analysing five 20 g test samples prepared and checked for homogeneity by NMI. Three flour and one grain bread mix samples were fortified with a range of folic acid from 2.3 – 3.6 mg/kg, and one blank sample of flour was unfortified.

3.2.6. Inter-laboratory bench-marking

In the absence of inter-laboratory collaborative studies or proficiency programs on total folates in foods, it was decided to undertake an inter-laboratory bench-marking study to assess the performance of the revised method against those of several other laboratories with known experience in folate analysis. After negotiating a fee-for-service arrangement with each participating laboratory, duplicate

samples of previously prepared vegetable homogenates were dispatched under frozen conditions to two laboratories with instructions to report on total folate levels using their standard analytical procedure. Both laboratories reported their results using the microbiological assay procedure. Detailed procedures for the methodology used to prepare and analyse the samples were not sought or provided by these laboratories.

3.3. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

3.3.1. Analytical parameters

The sample extracts were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in positive multiple reaction monitoring mode (mrm) using an AB/Sciex API4000Q mass spectrometer equipped with an electrospray (TurboV) interface (MDS Sciex, Concord, Ont., Canada). Separation was achieved using a Shimadzu Prominence HPLC system (Shimadzu Corp., Kyoto, Japan) with a 5 µm 150 x 4.6 mm Alltima C18 column (Grace Davison, Baulkam Hills, NSW, Australia) run at 40 ^{o}C, and a flow rate of 0.8 ml min^{-1}. A linear gradient started at 2 % B, ramped to 20 % B in 9 minutes, held for 3 minutes and then to 100 % B in 1.0 minute, held for 4 minutes before returning to 2 % B and equilibrating for 5 minutes (A = 1 % acetonitrile/99 % HPLC grade water; B = 95 % acetonitrile/5 % HPLC-grade water both containing 0.1 % formic acid).

Settings for the mass spectrometer are shown in Table 4. Wherever possible, two transitions were monitored for each analyte, the first transition used for quantification by comparing the response from an appropriate standard and the second as further confirmation for the presence of the particular folate. Ratios of the responses for the two transitions were required to be within 20 % of that of the standard for confirmation.

Analyte concentrations were determined using an external standard method and compared to a four point calibration using standard concentrations from 20 to 200 µg L^{-1} of eight folates, 5-methyl-tetrahydrofolates (5-Me-THF) (mono- and di-glutamates), 5-formyl-tetrahydrofolates (5-formyl-THF) (mono- and di-glutamates), 5,10-methenyl-tetrahydrofolate (5,10-CH-THF), tetrahydrofolate (THF), 10-formyl folic acid (10-formyl-FA) and folic acid (folic acid) (Schircks Laboratories, Jona, Switzerland). 5-Formyl-tetrahydrofolate-tri-glutamate was also included as a control standard to check deconjugation. Using an injection volume of 20 µL, the instrument limit of detection (LOD) for all folates detected was estimated at 0.8 µg L^{-1} at a signal to noise ratio of 3:1 and the limit of quantitation (LOQ also referred to as the limit of reporting) estimated at 2 µg L^{-1}.

Table 4.   Liquid chromatography-tandem mass spectrometry (LC-MS/MS) Parameters

Name	Q1	Q3	Retention time (mins)	Declustering Potential (DP)	Entrance Potential (EP)	Collision Energy (CE)	Collision Cell Exit Potential (CXP)
Folic Acid 1	442.2	295.1	9.59	65	10	20	20
Folic Acid 2	442.2	176.1	9.59	65	10	53	12
5-Me THF 1	460.2	313.2	7.04	65	10	27	20
5-Me THF 2	460.2	180.1	7.04	65	10	55	12
5-Formyl THF 1	474.2	327.2	9.13	65	10	28	20
5-Formyl THF 2	474.2	299.1	9.13	65	10	46	20
10-Me Folic Acid 1	456.2	309.1	nd	65	10	22	20
10-Me Folic Acid 2	456.2	176.1	nd	65	10	55	12
10-Formyl Folic Acid 1	470.2	295.1	8.78	80	10	37	20
10-Formyl Folic Acid 2	470.2	323.2	8.78	80	10	26	20
THF 1	446.2	299.1	nd	65	10	25	20
THF 2	446.2	180	nd	65	10	52	10
5-Me THF diglu 1	589.2	313.2	6.88	65	10	35	20
5-Me THF diglu 2	589.2	180.1	6.88	65	10	60	10
5-Formyl THF diglu 1	603.2	327.2	8.71	65	10	25	20
5-Formyl THF diglu 2	603.2	299.1	8.71	65	10	52	20
THF diglu 1	575.3	299.1	6.48	65	10	29	20
THF diglu 2	575.3	180	6.48	65	10	58	10
5,10-CH+THF	456	412	nd	75	6	40	10
5,10-CH2THF	458	311	nd	70	8	25	8
DHF	444	178	nd	60	4	30	10
10-CHOTHF	474	298	nd	41	10	30	6

nd, not detected

3.4.   Storage trial

There is little information on the effects of low temperature storage (refrigeration) on folate level or potential changes in the folate vitamer profile in vegetables. It is not uncommon for a product to be stored for several days either prior to sale during transport and display, or after sale to a consumer who may not wish to consume the product immediately. The present study investigated the effect of low temperature storage on folate concentration in choy sum, which had been found to have moderately high levels of folate, comparable to spinach (Table 5). A temperature of 4°C was selected, which is a temperature many domestic refrigerators are set to, and the place where the plant material is predicted to be held for the longest period in the supply chain.

3.4.1. Sample preparation and storage

Choy sum plants (*Brassica rapa* subsp. *parachinensis* cv. Karate) grown under hydroponic conditions (Figure 6) were obtained from a commercial grower (Durham Farms) in the Lockyer Valley, Queensland, in mid-October 2009 (mid-spring). The plants were grown under hail-netting to protect against adverse weather conditions. Plants were harvested after the flowering stem had emerged and elongated to approximately 15 cm in length, at which stage most flowers still remained closed (Figure 7). Prior to storage, the plant shoot was severed at its base from the root system, which was discarded. Three to four shoots were packed in macro-perforated polypropylene plastic bags and sealed to

minimise moisture loss during storage. The bags were stored at 4 °C in an atmosphere without modification or control.

Figure 6.   Hydroponic cultivation of choy sum plants grown under hail netting

Figure 7.   Leaves and floral shoot of "Karate" choy sum prior to preparation for folate analysis

3.4.2. Analysis

Leaf colour analysis and moisture loss assessment

Plant samples were weighed and leaf colour measured immediately prior to low temperature storage at 4 °C for 1, 2 and 3 weeks. At each withdrawal, 13 bags were selected randomly for analysis. All bags were analysed for weight loss and from these, 8 bags were measured for change in leaf colour, and the remaining 5 bags reserved for folate activity analysis. After weighing the samples, leaf colours (hue angle) of the largest fully-expanded outer leaf and an inner expanding leaf from 1 - 2 plants within each bag were measured using a Minolta chromameter (D_{65} illumination, CR-200, Minolta, Osaka, Japan), after which the bags were resealed and returned to refrigerated storage. Change in hue angle is considered a quantitative indicator of leafy vegetable senescence, as a decline in hue angle provides an objective and sensitive measure of leaf yellowing, with outer leaves senescing more rapidly than inner leaves (O'Hare et al., 1995).

Folate analysis

Following packaging, five bags were randomly selected and transferred to the Queensland Health laboratory for assay of initial folate activity prior to storage at 4 °C. Bags were held overnight at 4 °C and assayed the following day. Similarly, five bags were withdrawn from storage after 1, 2 and 3 weeks at 4 °C. Only the edible portions were sub-sampled from the plant material in each bag for folate analysis. Outer leaves considered inedible due to either rots, yellowing, or wilting were removed from the sample. Sub-samples were homogenised, extracted and assayed by LC-MS/MS and the revised microbiological assay, as previously described.

3.5. Quality assurance

Implementation and validation of the microbiological assay method was undertaken in the Queensland Health Forensic and Scientific Services laboratory which is technically accredited to ISO 17025 and certified to ISO 9000 quality systems. All analytical operations and project management activities are required to comply with the principles of good laboratory practice as specified under the accreditation and quality systems certification of the relevant ISO standards.

As part of the method development process, several food samples were prepared as in-house reference materials (RM) and used as analytical quality control (QC) samples. Pure folate standards were purchased for use as calibration standards and a mixed vegetable certified reference material, CRM (BCR-485), was employed to establish and monitor method bias for total folates.

The laboratory's participation in the NMI folic acid proficiency study provided an independent evaluation of the validity of the determinative step for free folic acid whilst the inter-laboratory comparison of results for duplicate samples provided a good indication of the likely variation which may be expected between experienced laboratories for analysis of foods for natural levels of bound or complex or conjugated vitamins.

4. Results and Discussion

4.1. Vegetable samples

Retail sampling indicated that the national agreement on names for the more common Asian vegetables is in use, particularly for the better known *Brassicas*, but that a range of names still persists, especially in Asian grocers. This will require consideration when providing information to consumers.

A range of other Asian vegetables (eg daikon, chi qua) were assessed initially, but the folate levels were low so these were not included in the subsequent study. Of the Asian vegetables, only leafy vegetables and snake bean scored well for folate activity.

4.2. Microbiological assay

The microbiological assay was critically assessed and refined to ensure the methodology produced suitable reference results for comparison to those from the LC-MS/MS procedure. This involved scrutiny of all steps in the assay to limit method-specific, operator-specific and food matrix-specific effects on the repeatability and reproducibility of the assay. As a result, an improved microbiological assay for the analysis of total folates in vegetables was developed in this project.

The microbiological assay system still has limitations and, while it is the standard reference method, further research is needed to address these issues (Section 6.2).

Researchers are using a range of techniques, eg radio protein binding assay (Ogle et al., 2001), HPLC (Stea et al., 2006), LC-MS/MS (Rychlik, 2004), to analyse folates in foods. However, comparison to an accepted standard reference method is crucial to assess the performance of these methodologies. These reports have generally used published microbiological assay data from other laboratories. This has many limitations, such as differences between samples, sample treatments, deconjugation steps and extraction techniques. In this project, these variations were eliminated by conducting the analyses by LC-MS/MS and the revised microbiological assay on the same samples using the same folate extraction techniques, permitting a more meaningful comparison of methodologies.

4.2.1. Folate content of Asian vegetables

Table 5 shows the folate content (folic acid equivalent activity) of the selected Asian vegetables obtained using the revised microbiological assay developed in this project. English spinach was analysed as a bench-mark for a high folate vegetable. The folate activities are ranked in Figure 8.

Table 5.   Folic acid equivalent activity for selected Asian vegetables (µg/100g fresh wt, n=5)

Vegetable	Range	Mean (SD)
buk choy (*Brassica rapa* subsp. *chinensis*)	65–113	89 (22)
choy sum (*Brassica rapa* subsp. *parachinensis*)		
no flowering evident	126–212	156 (35)
flowering evident	142–270	215 (54)
gai choy (*Brassica juncea*)	104–162	131 (22)
gai lan (*Brassica oleracea* var. *alboglabra*)	108–161	143 (21)
kang kong (*Ipomoea aquatica*)	157–226	197 (30)
mizuna (*Brassica rapa* var. *nipposinica*)	250–300	268 (24)
pak choy (*Brassica rapa* subsp. *chinensis*)	90–150	125 (30)
snake bean (*Vigna unguiculata* subsp. *sesquipedalis*)	131–178	156 (17)
spinach, English (*Spinacia oleracea*)	161–238	213 (31)
tatsoi (*Brassica rapa* var. *rosularis*)	180–269	231 (35)
wombok (*Brassica rapa* subsp. *pekinensis*)	74–93	83 (9)

Figure 8.   Ranked folic acid equivalent activity for selected Asian vegetables
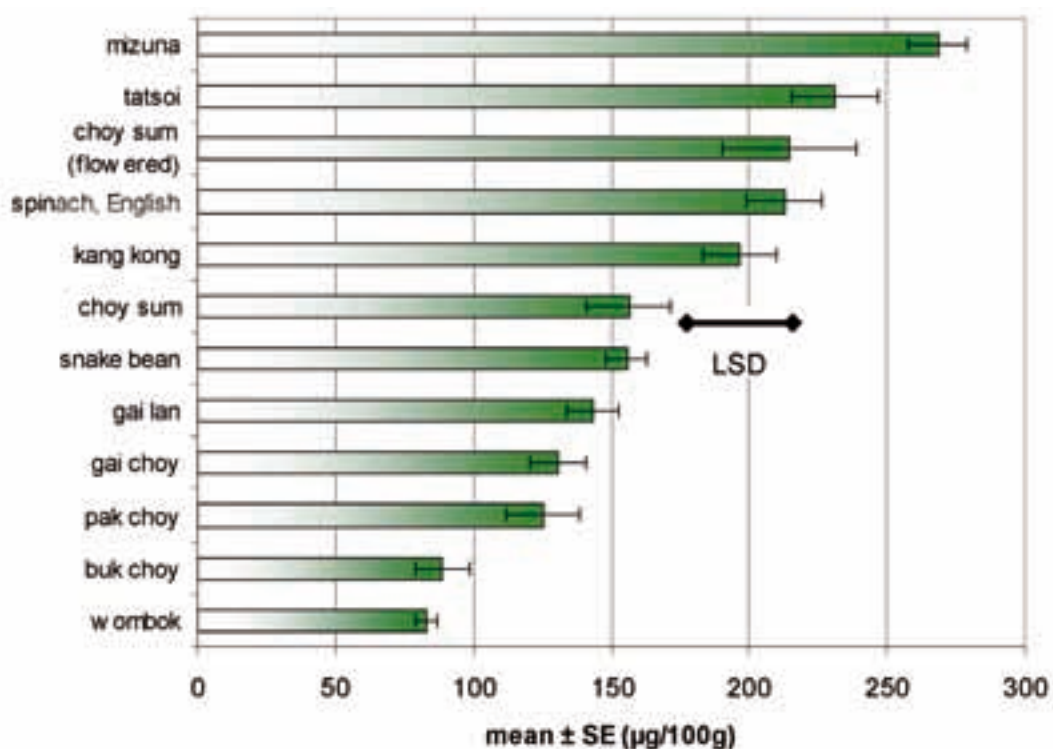
The results show that some Asian vegetables are excellent sources of folate. Although mizuna, tatsoi and choy sum (flowered) had higher mean folic acid equivalent activities than spinach, and kang kong only slightly lower, only the value obtained for mizuna was significantly higher ($p < 0.05$) than spinach (Figure 8). Snake bean, an Asian legume, was also shown to be a good folate source. Snake bean seeds have been reported to contain the highest folate content (658 µg/100 g) of legumes (VegTalk, 2010).

Vegetables at the flowering stage (choy sum) had higher folate contents than when not flowering, although the differences were not statistically different (Figure 8). Similarly, limited studies found folate levels were higher in the more actively growing leaf (lamina) tissues of buk choy and pak choy compared to stems (petiole) (Table 6). More research is needed to further explore these findings as it would provide useful information to consumers wanting a higher folate intake.

Table 6. Folic acid equivalent activity of petioles and laminas

Sample	Folic acid equivalent activity (ug/100g fresh wt)
buk choy - petiole	33
buk choy - lamina	141
pak choy – petiole	42
pak choy - lamina	190

4.2.2. Comparison to literature data for Asian vegetables

Comparison to literature data shows large variations in reported folate contents of some Asian vegetables using a microbiological assay (Table 7). The data may reflect differences between samples and variations in methodologies across the three research groups, such as differences in pH, buffers, type of enzymes, incubation times and temperature and sample clean-up procedures (Shrestha et al., 2000).

Table 7.   Comparison of literature data for folate contents of Asian vegetables (microbiological assay)

Vegetable	Folate content (µg/100 g fresh wt) (SD)		
	This study	Iwatani et al. (2003)	Devi et al. (2008)
buk choy	89 (22)	340 (25)	-
choy sum	156 (35) 215 (54) (flowering)	425 (14)	-
kang kong	197 (30)	225 (19)	-
pak choy	125 (30)	333 (13)	-
snake beans	156 (17)	-	130 (10)
spinach	213 (31)	302 (17)	-
wombok	83 (9)	170 (14)	81 (2)

4.2.3. Proficiency study

The results of the proficiency study confirmed that the revised microbiological assay used for folate analysis in this project provided concordant results with those from other study participants using both chemical and microbiological methods. Satisfactory Z and E_n scores, which include measurement of expanded uncertainty, were returned from NMI for all samples against the assigned values. These results together with the generally good recovery of total folates from the certified reference material used throughout this study instilled a high level of confidence in the revised microbiological assay and further indicated a high level of operator skill and competency. The proficiency study report (AQA 09-12 Folic Acid in Flour) is available on-line at:
http://www.measurement.gov.au/Publications/ProficiencyStudyReports/Pages/default.aspx .

4.2.4. Inter-laboratory bench-marking

The results of the inter-laboratory study are shown in Table 8. The analyses were conducted on duplicates of the same homogenised samples to reduce the impact of sample heterogeneity on data variability. The reported data of our study overall appeared to have a bias towards higher results. Although excellent correlation was observed for the results for the lyophilised broccoli reported by Laboratory 1 and this study, other data reported by Laboratory 1 appeared to be around a third lower than those reported in our study. The lower results reported by Laboratory 2 are more problematic as some of their data on similar vegetable matrices were considerably higher than those observed in this study. This suggests loss of sample integrity between initial preparation at Queensland Health and analysis at Laboratory 2 as a more likely or dominant cause for the bias towards lower values. The Laboratory 2 results for the lyophilised broccoli and kang kong are considered outliers without a clear rationale. Without detailed knowledge of the methodologies employed, or the sample transport and storage conditions prior to testing, it is difficult to rationalise the differences reported. The variability in the results could be attributed to a number of factors, including the analytical method used, operator proficiency, changes within the sample due to storage conditions during transport, degradation by endogenous enzymes, and treatment at the testing laboratory prior to analysis.

Table 8. Comparison of folate contents of Asian vegetables (inter-laboratory bench-marking study)

Vegetable	Folate content (µg/100 g fresh wt) (mean)		
	This study	Laboratory 1	Laboratory 2
broccoli (lyophilised)	1212	1190	129.1/92.1
buk choy	113	nt	43.5
choy sum 1	142	133/171	nt
choy sum 2	152	nt	67.3/64.8
kang kong	226	nt	7.1
mizuna	251	193	137.7
snake bean	178	131	80.4
spinach	238	182	181.6
tatsoi	249	nt	120.6

nt, not tested

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

## 4.2.5. Folate content and vitamer composition of Asian vegetables

The folate vitamer contents of the selected Asian vegetables are shown in Table 9. The major vitamers detected were di-glutamates of 5-methyl-tetrahydrofolate (5-methyl-THF) and 5-formyl-tetrahydrofolate (5-formyl-THF) which are present as a result of deconjugation by the chicken pancreas extract during preparation of the sample extracts (Leichter et al., 1977).

Table 9.   Folate vitamer content in Asian vegetables (µg/100 g fresh wt, mean, SE)

	5-MeTHF mono-glu	5-Formyl THF mono-glu	10-Formyl Folic acid	5-MeTHF di-glu	5-Formyl THF di-glu	THF di-glu	5,10-methenyl THF
buk choy	9.1 (1.4)	6.6 (0.3)	0.6 (0.4)	39.6 (9.1)	37.6 (3.7)	19.3 (8.0)	2.9 (0.9)
choy sum (flowered)	11.4 (2.1)	11.5 (1.7)	1.7 (0.2)	71.1 (9.7)	75.8 (5.2)	18.6 (2.2)	2.8 (1.1)
choy sum	14.5 (3.2)	9.9 (1.8)	0.9 (0.5)	63.3 (6.7)	43.8 (6.3)	22.6 (6.3)	4.9 (1.5)
gai choy	14.8 (3.1)	9.0 (1.2)	1.1 (0.4)	65.6 (17.2)	39.32 (3.6)	21.9 (6.1)	5.3 (1.6)
gai lan	13.3(1.8)	6.9 (1.2)	1.6 (0.6)	54.4 (9.2)	43.6 (5.6)	17.9 (4.2)	4.5 (1.3)
kang kong	14.6 (3.6)	2.2 (0.4)	1.2 (0.3)	106.2 (13.9)	57.0 (4.0)	8.8 (1.3)	1.3 (0.2)
mizuna	14.2 (2.6)	15.9 (2.0)	1.8 (0.2)	80.5 (5.8)	118.1 (7.3)	22.2 (1.6)	3.0 (0.5)
pak choy	9.7 (2.8)	7.9 (0.4)	0.5 (0.3)	46.5 (5.6)	43.3 (8.6)	15.7 (2.2)	3.7 (1.4)
snake bean	27.7 (3.1)	16.0 (2.1)	3.5 (0.5)	33.3 (2.4)	39.5 (2.9)	10.8 (1.0)	4.9 (1.4)
spinach	45.8 (7.3)	26.1 (7.8)	1.8 (0.5)	35.4 (5.8)	51.5 (6.4)	5.6 (1.2)	5.8 (3.1)
tatsoi	13.4 (2.3)	13.4 (0.7)	2.1 (0.3)	57.2 (6.1)	86.9(7.1)	16.5 (1.5)	3.0 (0.3)
wombok	11.8 (2.1)	3.1 (0.4)	0.2 (0.2)	34.0 (1.1)	19.5 (2.3)	12.0 (1.3)	1.3 (0.5)

The data were converted to folic acid equivalents (µg/100 g fresh wt) to examine the forms of folate present. Folic acid equivalents of the mono- and di-glutamate forms of 5-methyl-THF and 5-formyl-THF were summed to give the content of each vitamer form in the vegetables (Figure 9). 5-Methyl-tetrahydrofolates were the major vitamers in the vegetables, except mizuna, tatsoi and flowering choy sum in which 5-formyl-tetrahydrofolates predominated. Kang kong contained the highest level of 5-methyl-THF, reportedly the most bioavailable folate form. Lower levels of tetrahydrofolates, 10-formyl-folic acid and 5,10-methenyl-tetrahydrofolate were present in all samples. It should be noted that significant and rapid changes in the concentration of 5-formyl-THF can occur within the same vegetable during storage, as shown in the storage study with choy sum (Section 4.3). In the present trial, it is possible that different vegetables were of different ages at the time of purchase from individual retailers, which may have affected the levels of 5-formyl-THF present.

Figure 9. Folate vitamer content in Asian vegetables (µg folic acid equivalents/100 g fresh wt) (mean, SE)
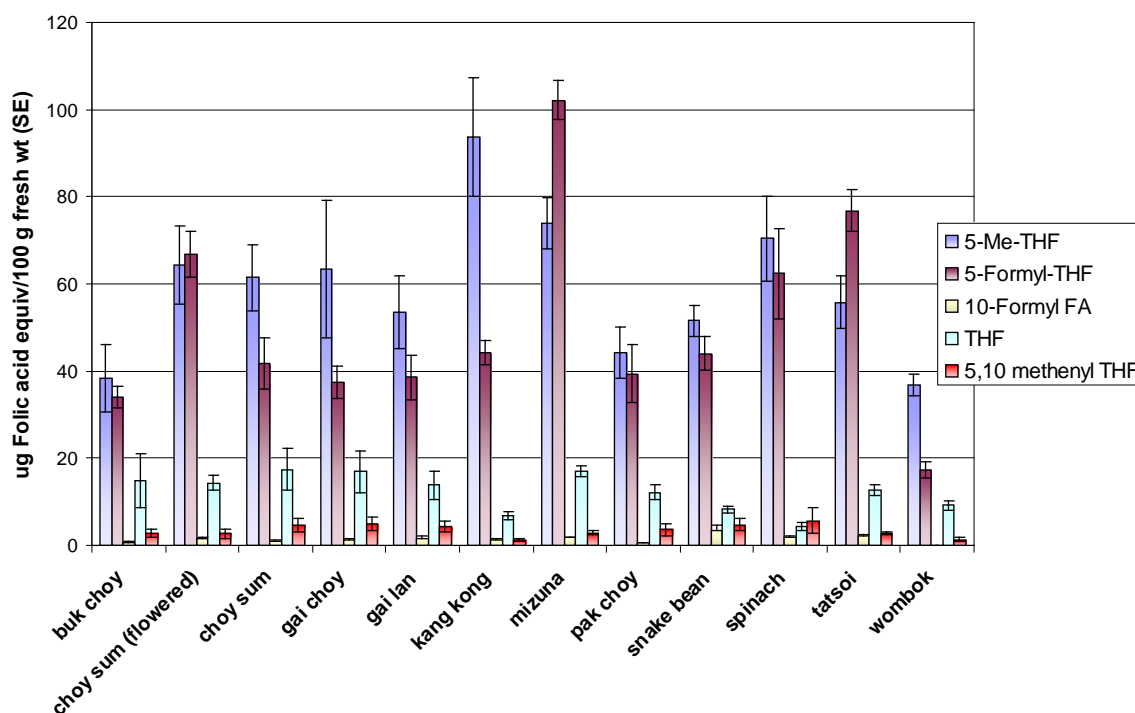
The vitamer profiles of petiole and lamina samples were quite different (Table 10). Petioles contained lower levels of 5-formyl-THF-diglutamates than laminas. Examination of the folate forms as folic acid equivalents showed 5-methyl-THF was the major vitamer in petioles while 5-formyl-THF was the predominant folate in laminas (Figure 10). Petioles contained less than a third of the folic acid equivalent activity of laminas (Table 12). These data are from a very limited study and require further investigation.

Table 10. Folate vitamer content in petiole and lamina samples (µg/100 g fresh wt)

	5-MeTHF -mono-glu	5-Formyl THF- mono-glu	10-Formyl -folic acid	5-MeTHF -di-glu	5-Formyl THF-di-glu	THF-di-glu	5,10-methenyl - THF
buk choy – petiole	5.3	nd	nd	18.6	8.7	13.9	nd
buk choy – lamina	9.8	13.5	nd	32.2	57.3	25.6	6.9
pak choy – petiole	6.3	1.0	nd	25.5	6.6	14.9	0.8
pak choy - lamina	11.5	24.3	nd	42.8	87.9	24.1	9.1

nd, not detected

Figure 10. Folate vitamer content in petioles and laminas (µg folic acid equivalents/100 g fresh wt)
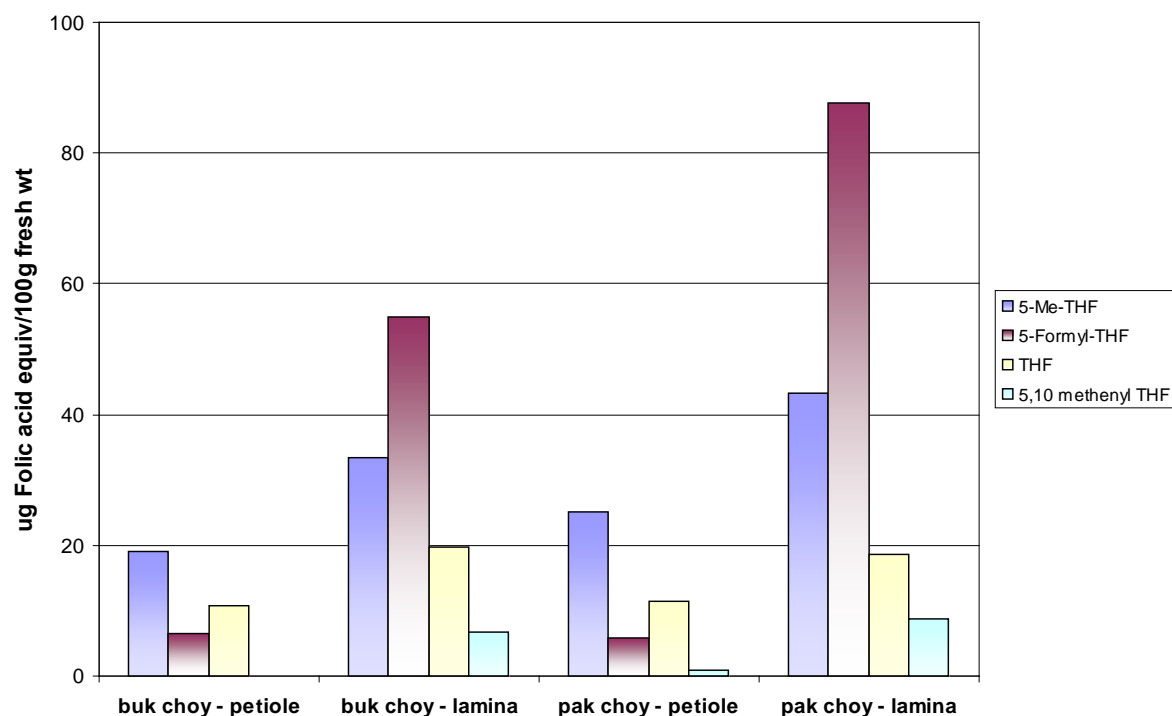
4.2.6. Comparison to microbiological assay

The levels of individual folate vitamers (expressed as folic acid equivalents) were added to enable comparison to the results from the revised microbiological assay (Table 11).

Table 11. Comparison of liquid chromatography-tandem mass spectrometry (LC-MS/MS) and microbiological assay (mean, SE)

Vegetable	LC-MS/MS (µg folic acid equiv/100 g fresh wt)	Microbiological assay (µg folic acid equiv/100 g fresh wt)
buk choy	91 (6)	89 (10)
choy sum (no flower)	126 (10)	156 (15)
choy sum (flowered)	150 (16)	215 (24)
gai choy	124 (18)	131 (10)
gai lan	112 (11)	143 (9)
kang kong	147 (14)	197 (13)
mizuna	198 (5)	268 (11)
pak choy	100 (10)	125 (13)
snake bean	112 (9)	156 (8)
spinach	145 (19)	213 (14)
tatsoi	150 (6)	231 (16)
wombok	65 (5)	83 (4)

LC-MS/MS analysis produced consistently lower results than the microbiological assay, although there was a strong relationship (R^2 0.85) (Figure 11). Vegetable extracts may contain components which activate the response of the micro-organism, resulting in higher apparent folate contents. Interestingly, the limited studies on petioles and laminas showed similar results for petioles analysed by both methods (Table 12), while the lamina results fitted the correlation graph below. This may reflect differences in the response of the micro-organism to the folate vitamer profiles of these plant parts.

Figure 11. Comparison of liquid chromatography-tandem mass spectrometry (LC-MS/MS) and microbiological assay

$y = 0.7535x$
$R^2 = 0.8526$
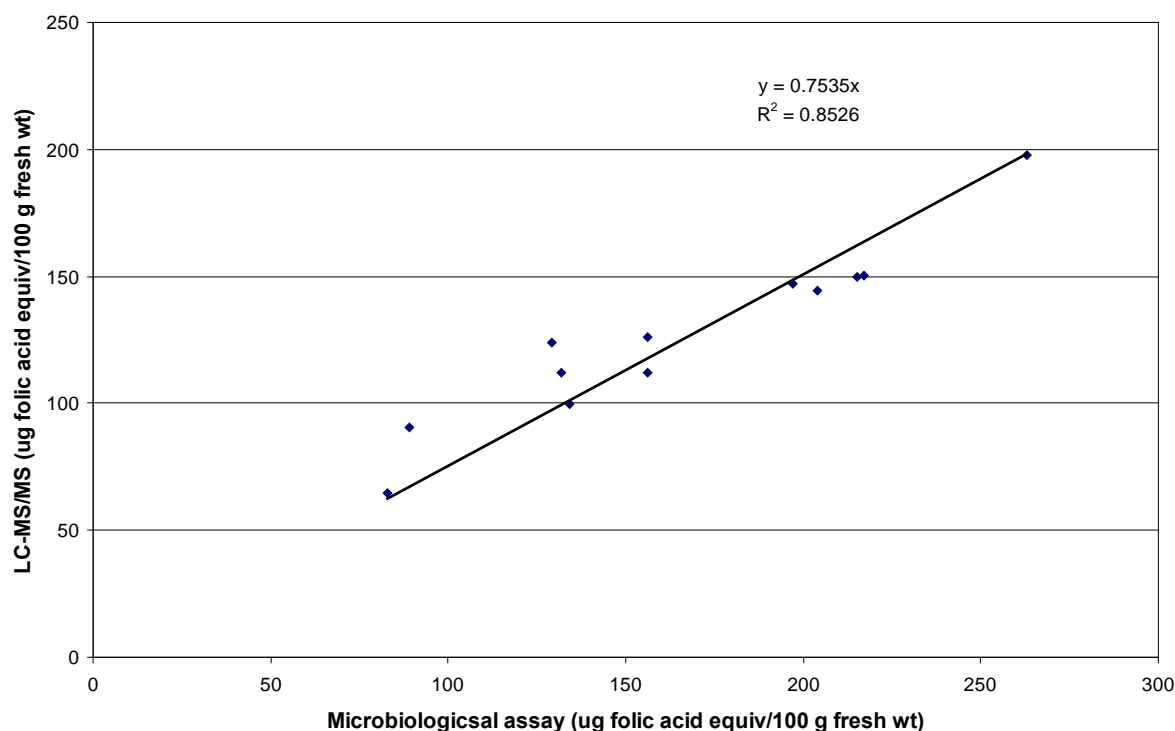
Table 12. Comparison of liquid chromatography-tandem mass spectrometry (LC-MS/MS) and microbiological assay of petioles and laminas

Vegetable	LC-MS/MS (µg folic acid equiv/100 g fresh wt)	Microbiological assay (µg folic acid equiv/100 g fresh wt)
buk choy – petiole	36	33
buk choy – lamina	115	141
pak choy – petiole	43	42
pak choy - lamina	158	190

LC-MS/MS is a highly specific analytical technique and would be expected to have a higher level of specificity than a microbiological assay which is less specific in regard to the deconjugated folates. If the microbiological assay overestimates the folate content of foods, there are implications for public health agencies. Food composition tables and nutrient databases use data from microbiological assays as the accepted reference method. The actual folate intake of the population may be lower than that determined from these sources, resulting in an increased risk of compromised folate status. Further studies are required to clarify this area.

4.2.7. Comparison to literature data

There is limited data in the literature on folate vitamers in Asian vegetables (Table 13). Vahteristo et al. (1997) reported 5-methyl-THF and THF present in *Brassica pekinensis* (Lour.) Rupr. or wombok. Formyl vitamer forms were not reported, in contrast to this study's data (Table 9, Figure 9). In the present study, data for 5-methyl-THF (mono- & di-glutamates) in spinach are within in the range of literature data, while higher levels of 5-formyl-THF (mono- & di-glutamates) were found (Table 9, Figure 9).

The differences between data from this study and literature data could be attributed to:

- variations in methods eg sample preparation, extraction, enzyme hydrolysis conditions, pH

- different plant varieties/cultivars (generally not specified in the literature)

- agronomic factors eg geographical location, hydroponic vs in-ground cultivation, time of year, hours of sunlight

- storage time of vegetable prior to analysis.

Table 13. Folate vitamers (μg/100 g fresh wt) (literature data)

Vegetable	5-MTHF	5-FTHF	10-FTHF	5,10-CH-THF	THF	DHF	10-FFA	Ref
Chinese cabbage*	50	nd	nd	nd	4	nd	nd	1
spinach	43.2±2.1	21.2±2.0	3.0±1.2	3.3±0.6	9.2±4.4	20.0±4.5	-	2
	72.8-140.0	4.8-54.7	nd	nd	nd-18.7	nd	nd	3
	137.0	12.0	nd	nd	15.5	nd	nd	4
	84.2	32.1	nd	nd	44.4	nd	3.1	5
	79	nd	24	nd	31	nd	nd	6

nd, not detected

* *Brassica pekinensis* (Lour.) Rupr. (wombok)

1   Vahteristo et al. (1997)
2   McKillop et al. (2003)
3   Freisleben et al. (2003b)
4   Freisleben et al. (2003a)
5   Zhang et al. (2005)
6   Hefni et al. (2010)

4.2.8. Summary of folate activity of selected Asian vegetables

Based on the results from LC-MS/MS analysis and the microbiological assay, the top five (5) of the Asian vegetables analysed in this study are shown below in Table 14. Both methodologies identified the same Asian vegetables as high folate vegetables, with only slight differences in the order of activity.

Table 14.   Top five (5) Asian vegetables for folate activity

LC-MS/MS	Microbiological assay
mizuna	mizuna
choy sum (flowering)	tatsoi
tatsoi	choy sum (flowering)
kang kong	kang kong
choy sum (non-flowering)	choy sum (non-flowering)

4.3.   Storage trial

The microbiological assay and LC-MS/MS method used to quantify folate activity in the present trial indicated a slight, but not statistically significant ($p > 0.05$) decline in folate levels in the edible portion of choy sum shoots stored for up to 3 weeks at 4 °C (Figure 12). While the microbiological assay averaged 141 μg folic acid equivalent activity / 100 g fresh wt across the storage period, the LC-MS/MS analysis yielded an average of 80 μg folic acid equivalent activity / 100 g fresh wt, approximately 43 % lower than the microbiological assay.

Figure 12.   Folate activity of the edible portion of choy sum shoots over a three week storage period as derived from microbiological assay (●) and LC-MS/MS (○)
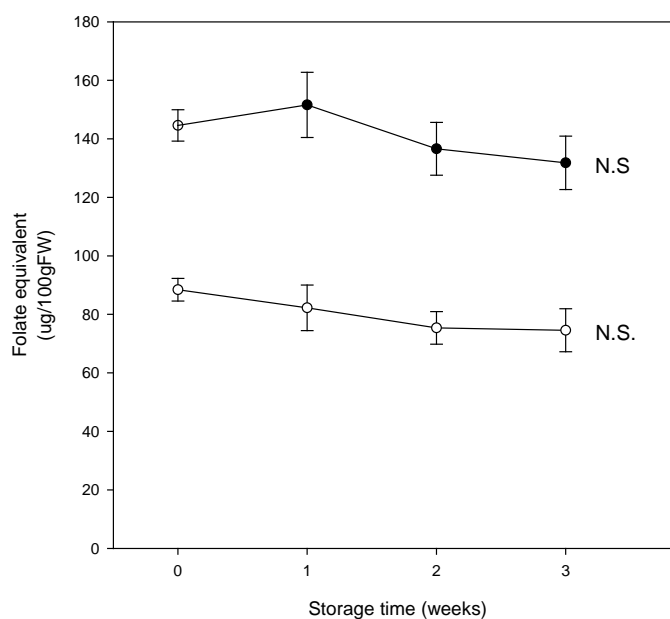
Data are the mean of 5 replicates (±SE). 'N.S.' indicates no significant change ($p > 0.05$) in folate activity occurred during storage.

The principal folate vitamers present in choy sum following deconjugation treatment were identified as the di-glutamate and mono-glutamate forms of 5-methyl-THF and 5-formyl-THF, and to a lesser extent, THF. Minor vitamers included 5,10-methylene-THF and 10-formyl-FA.

During storage, the concentration of 5-methyl-THF (di-glutamate) did not significantly change, contrasting with 5-formyl-THF (di-glutamate) which rapidly declined (Figure 13). Slight decline was also observed for the mono-glutamate form of 5-formyl- THF, while 5-methyl-THF (mono-glutamate) slightly increased. Combining the mono-glutamate with the di-glutamate forms of 5-methyl-THF or 5-formyl-THF (as folic acid equivalents) (Figure 14) gave a similar result to the di-glutamate fraction of these vitamers.

THF was observed to slightly increase at 1 week storage, but then decrease slightly again over the storage period (Figure 13). The folate vitamers, 5,10-methylene THF and 10-formyl FA, exhibited no significant change over the storage period, averaging 1.0 and 1.2 ug folic acid equivalent activity / 100g fresh wt, respectively.

Figure 13. Change in concentration (±SE) of the principal folate vitamers (●, 5-methyl-THF di-glutamate; ○, 5-formyl-THF di-glutamate, ▼, 5-methyl-THF mono-glutamate; ▽, 5-formyl-THF mono-glutamate, ■,THF) present in deconjugated choy sum extract during storage at 4°C for 3 weeks.
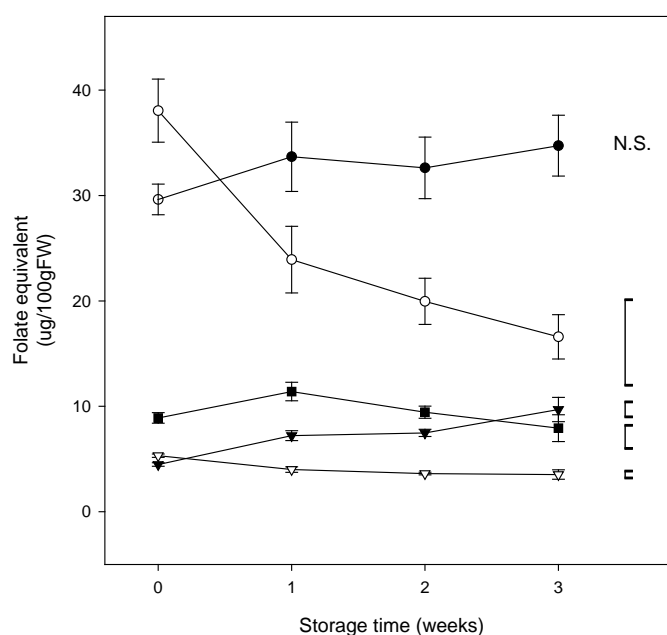
Vertical bars on the right of the plots indicate LSD (p<0.05) where applicable for that plot.

Figure 14. Change in concentration (±SE) of the combined mono and di-glutamate vitamers of 5-methyl-THF (●) and 5-formyl-THF (○) present in deconjugated choy sum extract during storage at 4°C for 3 weeks.
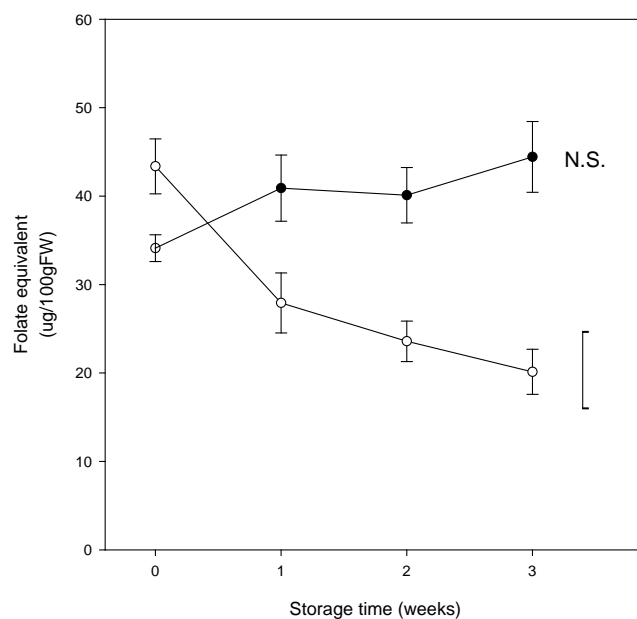
A vertical bar on the right of the plot indicates the LSD (p<0.05) for 5-methyl-THF.

During storage, a gradual increase in moisture loss was recorded from 2 % at 1 week to 4 % at 3 weeks for choy sum shoots (Figure 15A). In regard to changes in leaf colour, both inner and outer leaves were observed to significantly decline in hue angle over the storage period (Figure 15B). Although the decline in hue angle was statistically significant over time, it accrued less than 4 ° in difference for both inner and outer leaves, such that the leaves remained green. A figurative indication of this colour change is shown in Figure 16.

Decline in hue angle occurred at approximately the same rate for inner and outer leaves (Figure 15B). The inner leaves generally maintained a higher hue angle than the outer leaves, with the only exception to this being at 1 week of storage, at which time the inner and outer leaves presented a similar hue angle reading.

31

Figure 15. Moisture loss (A) and decline in hue angle (B) of choy sum shoots stored over a three week period at 4°C (hue angle: ●, outer leaves; ○, inner leaves).
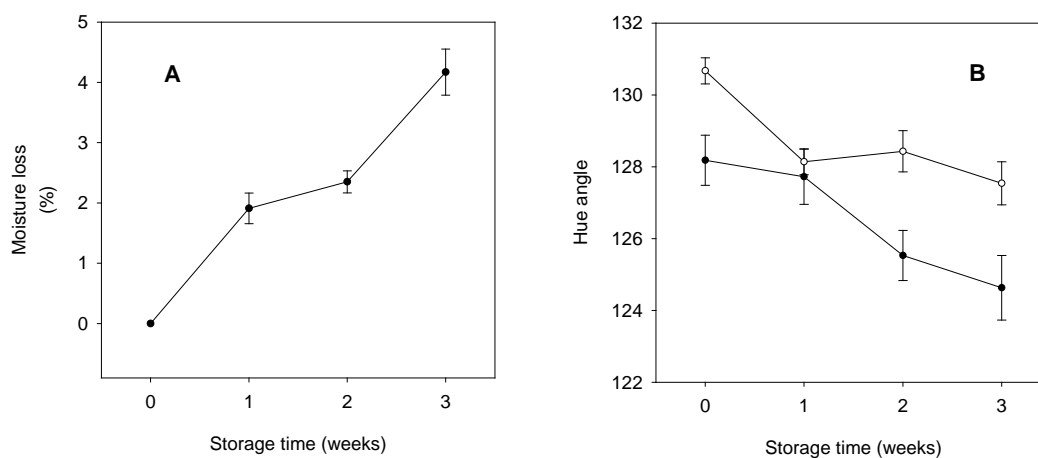
Figure 16. Figurative representation of the change in leaf colour in choy sum leaves over a three-week storage period at 4°C.
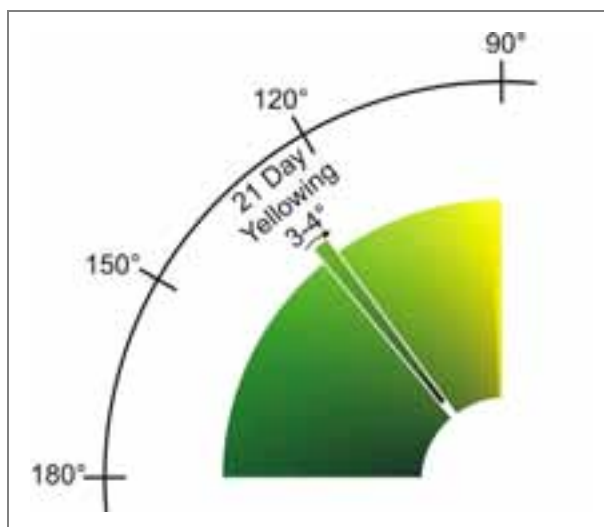
The present trial indicated that folate activity does not significantly decline in choy sum shoots when stored for up to 3 weeks at 4 °C. From a consumer perspective, this is a beneficial outcome as choy sum does not necessarily have to be eaten immediately after harvest to maintain optimum folate-related health benefits. Although in the present trial it may have been possible that folate concentration could have declined in the non-edible fraction removed prior to analysis, this portion by its very nature would not be consumed.

The absolute difference in folic acid equivalent activity between the microbiological assay and LC-MS/MS (Figure 12) can only be speculated at this stage. Studies indicate that the concentrations of folate vitamers can decline following the deconjugation process prior to LC-MS/MS analysis, with degradation increasing as the time-lapse between these two processes increases, particularly at ambient temperatures.

A study by Pandrangi and LaBorde (2004) found folate levels in spinach significantly declined by 47 % after 8 days of storage at 4 °C. In the study however, the authors reported greater than 35 % weight loss from the sample, as opposed to 2 % over the same period in the current trial (Figure 15A). Furthermore, the non-edible portion was included with wilted, damaged and discoloured leaves also analysed. In a similar trial with spinach, where damaged or discoloured leaves were removed at the start of the trial, a decline in folate of only 26 % after 7 days was reported (Chen et al., 1983). It is uncertain if this decline was again associated with a large concurrent loss in moisture, or whether non-edible leaves were included in the analysis during storage, or is simply an inherent difference between spinach and choy sum folate metabolism. In the present trial, apart from moisture loss, leaf yellowing was another indicator of senescence during storage (Figure 15B). Loss in hue angle was minimal however (but significant), but this didn't correspond with any decline in folate concentration (Figure 12).

LC-MS/MS analysis of the folate vitamers present in choy sum indicated a significant and rapid decline in 5-formyl-THF, while 5-methyl-THF made no significant change (Figure 14), although a slight increase was observed for the mono-glutamate form (Figure 13). While 5-methyl-THF is considered to have an active role in the 'methyl cycle' by providing the methyl group required for transformation of homocysteine into methionine (Rebeille et al., 2006), one of the roles of 5-formyl-THF is that of a stable storage form of folate (Stover and Schirch, 1992). It is thus possible that the observed decline in 5-formyl-THF during storage may be a means of maintaining 5-methyl-THF concentrations at a functional level by transforming one to the other. Similarly, 5-formyl-THF may have been used as a substrate to maintain the minor folate vitamers, 5,10-methylene-THF and 10-formyl-FA, at a functional level over the storage period.

The actual folate activity of the choy sum material used in the present trial was approximately 20 % lower than that previously recorded for an unnamed cultivar of choy sum at the same flowering stage (Table 5). It is possible that this difference may have been due to cultivar, or possibly growing conditions, which were not known at time of purchase for the latter. Clearly, variation in folate activity is possible within choy sum, which is likely to be the case with most vegetables. Despite this, choy sum appears to be a good source of folate, and displayed a folate activity similar to spinach tested in the present project (Table 5).

Although not examined in the current trial, it would have been useful to monitor folate activity in the non-edible fraction generated during storage at 4 °C, as well as the folate concentration and vitamer profile in different parts of the vegetable (outer leaves, inner leaves, flowering stalk). It is possible that both the concentration and profile of folate vitamers may vary between different physiological tissues, especially those undergoing rapid cell division.

4.4.   Conclusions

Folates are one of the most nutritionally significant vitamins. This study has provided information on the folate content and folate vitamer composition in selected Asian vegetables. It has highlighted that some of the lesser known vegetables, eg mizuna, tatsoi, kang kong, snake beans, in addition to choy sum, are excellent folate sources. This information can be used to promote these vegetables through folate content labelling to increase their market penetration. Consumers can use the information to select from a wider range of vegetables to maintain or increase their folate intakes. Health and allied professionals can use the data to provide dietary advice. This research will position Asian vegetables in the market-place as a major fresh produce category due to their beneficial health attributes.

The comparative folate data reported for a number of vegetables in this study against those reported by the other laboratories on duplicate samples as well as the variation in data obtained from two different techniques has raised a number of technical and quality issues which can best be resolved by closer inter-laboratory collaboration to harmonise sample preparation and analytical methodology.

The deficiencies of the microbiological assay are well-recognised. If this assay overestimates the folate content of foods, there are implications for public health agencies as the actual folate intake of the population may be lower than believed, resulting in an increased risk of compromised folate status. Further studies are required to clarify this area.

In this project, we have applied a more accurate and reproducible methodology, LC-MS/MS, to obtain the 'next generation' of folate data and detailed information on the folate vitamer profiles of Asian vegetables, providing industry with robust analytical tools for future research in this area.

5. Implications

5.1. Outcomes for industry in Australia

- Information for industry regarding folate content of Asian vegetables for use in marketing, promotion and on packaging as content claims.

- Information for industry on effects of storage of Asian vegetables to ensure folate activity is maintained across the supply chain.

- Increased profile for Asian vegetables in the market due to positive health messages.

- Increased consumer awareness and understanding of nutritional and health benefits of Asian vegetables, resulting in a positive influence on consumer food choices and increased sales.

- Increased knowledge of folate levels in Asian vegetables by health and allied professionals for dietary and nutritional advice to consumers.

- Expanded information on folate vitamers in vegetables which is useful information for public health agencies, regulatory bodies, eg FSANZ, and for incorporation into food composition tables and nutrient databases.

6. Recommendations

6.1. Dissemination

- Conference poster and oral presentation: First International Vitamin Conference, Copenhagen, Denmark, 19 – 21 May 2010 (Appendix C).

- Peer-reviewed publications: Research papers will be prepared for submission to the Journal of Food Composition & Analysis and Postharvest Biology & Technology.

- Media releases: These will be developed through the media units in the Queensland Government Department of Employment, Economic Development and Innovation and Queensland Health for distribution to industry newsletters, popular print media etc in consultation with RIRDC.

- Retail sampling indicated that the national agreement on names for the more common Asian vegetables is in use, particularly for the better known *Brassicas*, but that a range of names stills persists, especially in Asian grocers, and this will be considered when providing information to consumers.

6.2. Future research

- Microbiological assay – standardisation of procedure:

    - purification or cloning of deconjugase enzymes to eliminate unknown effects of other components (eg enzymes, inhibitors, activators) present in in-house or commercial preparations

    - selection of deconjugase enzyme to ensure complete deconjugation to mono-glutamate forms to minimise variations in response by the micro-organism

    - optimisation of sample treatment procedures for each vegetable matrix to ensure complete deconjugation and folate extraction

    - more definitive data on response of micro-organism to different folate vitamers

    - conversion of folates to one form (eg 5-methyl-tetrahydrofolate) prior to analysis to eliminate variations in response between folate vitamers

    - availability of a wider range of certified reference materials (CRM) (eg mixed green leafy vegetables, mixed *Brassica* species or individual vegetables) to minimise sample matrix effects.

- LC-MS/MS analysis:

    - increase in the analytical potential of LC-MS/MS through the application of stable isotope dilution assays in this area and for future research (eg suitable for the identification and quantitation of folate vitamers and metabolites in food as well as biological samples such as blood and urine for bioavailability studies (Monch et al., 2010)).

- Vegetable studies:

  - investigate impact of senescence (eg yellowing leaves) during storage on folate degradation

  - investigate folate vitamer profile and concentration in different physiological organs (eg inner leaves, outer leaves, flowering stalks) to identify which organs may be selected for highest folate activity

  - investigate impact of photosynthetic light during storage on folate synthesis and maintenance

  - examine other bioactive phytochemicals (eg anti-oxidants, anti-inflammatory components) in Asian vegetables to provide industry with additional marketing and promotional information and to provide consumers with more information on the health properties of these vegetables

  - study transport and storage processes for Asian vegetables across the supply chain to ensure optimum conditions for the maintenance of folate and phytochemical bioactivity from harvest to consumption.

- Processing trials:

  - investigate the application of processing techniques, such as high pressure processing, to increase the bioavailability of dietary folates (eg through deconjugation of polyglutamates to form mono- and di-glutamates) and other bioactive phytochemicals in Asian vegetables.

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8. Appendices

Appendix A. Asian vegetables selected for this study

Buk choy (*Brassica rapa* subsp. c*hinensis*)

Buk choy laminas

Buk choy petioles

Choy sum (*Brassica rapa* subsp. *parachinensis*)

Flowering choy sum

Gai choy (*Brassica juncea*)

Gai lan (*Brassica oleracea* var. *alboglsbra*)

Kang kong (*Ipomoea aquatica*)

Mizuna (*Brassica rapa* var. *nipposinica*)

Pak choy (*Brassica rapa* subsp.*chinensis*)

Pak choy laminas

Pak choy petioles

Snake bean (*Vigna unguiculata* subsp. *sesquipedalis*)

Tatsoi (*Brassica rapa* subsp. *rosularis*)

Wombok (*Brassica rapa* subsp. *pekinensis*)

Spinach (*Spinacia oleracea*)

Appendix B. Summary of preliminary method development (microbiological folate assay) at Queensland Health Forensic and Scientific Services

Preliminary development of a microbiological assay for folate in vegetables was undertaken at the laboratories of Queensland Health Forensic and Scientific Services (QHFSS) by Associate Professor Prapasri Puwastien and Ms Wasinee Jongjitsin from Mahidol University, Thailand, in collaboration with Dr Pieter Scheelings and Dr Glenn Graham from QHFSS, over a 4 month period from May to August 2008.
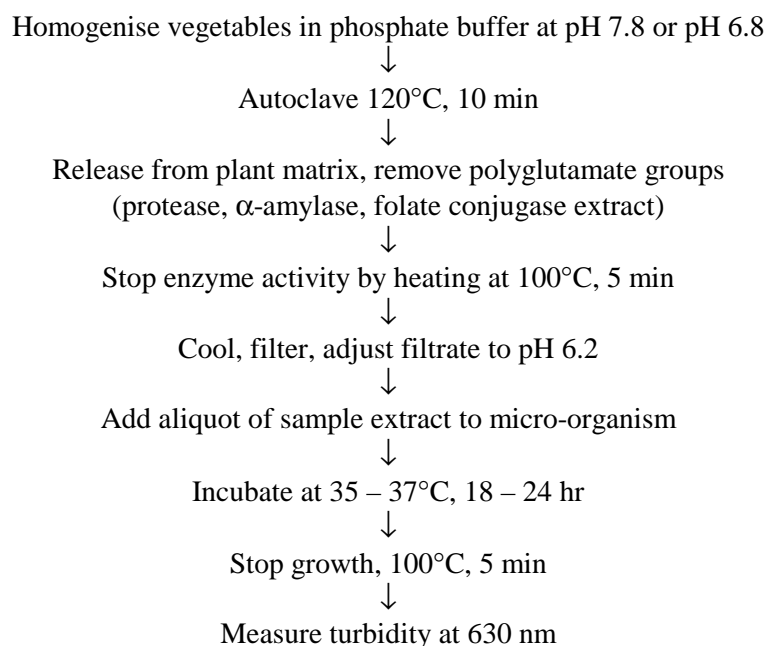
The microbiological assay for folate in foods is based on the growth of a micro-organism in a folate-free medium after the addition of a sample extract containing deconjugated folates (AACC, 2000, AOAC, 2006a,b, Iwatani et al, 2003, Sharpless et al, 2008, Shrestha et al, 2000). Growth is proportional to the folate content of the sample extract. Sample extracts are prepared by enzymatic hydrolysis to release folates from the sample matrix in a form which can be utilised by the micro-organism. Single or tri-enzyme treatments can be used, depending on the nature of the matrix. Protease and α-amylase are used to hydrolyse protein and starch components and to release folates. Polyglutamated folates are hydrolysed to mono- or di-glutamates using a folate conjugase preparation, usually a crude form, from chicken pancreas, hog kidney, rat serum or combinations of these enzymes. For these studies, crude folate conjugase extract was prepared from fresh chicken pancreas (AOAC, 1950, AOAC, 2006b).

The micro-organism used in this preliminary study was *Lactobacillus rhamnosus* (ATCC 7469), previously know as *Lactobacillus casei*, which was grown either as stab or colony cultures. The working inoculum was prepared from these cultures on the day before the assay, by subculturing the stock culture into micro-inoculum broth (MIB) and incubating at 35 - 37°C for 18 – 24 hr.

Reference samples were used for quality control purposes. In-house reference materials were prepared from fresh broccoli and Chinese chard, sourced from local supermarkets. Broccoli stalks were discarded and the florets were chopped, blended in a food processor and lyophilised. Lyophilised material was prepared from the edible portion of Chinese chard following a similar procedure. BCR-485, a certified reference material prepared from mixed vegetables (tomato, carrot, sweetcorn), was obtained from the European Commission Joint Research Centre Institute for Reference Materials and Measurement (EU-JRC-IRMM, Geel, Belgium).

A range of experiments was conducted to establish the most effective conditions for sample preparation and inoculum preparation, the concentration range of micro-organism to use in the assay, the amount of sample extract to add, and the incubation conditions (time, temperature, pH). The results of these experiments were used to fine-tune the assay and the developed procedure (see flow chart below) was applied to the analysis of the reference materials, BCR-485 and in-house preparations. Good agreement was obtained with the folate content of the certified reference material (BCR-485), however, there appeared to be a vegetable matrix effect with the in-house reference materials, with a greater coefficient of variation for broccoli (a fibrous vegetable) compared to bok choy (a leafy vegetable) (see results below).

The flow chart for the microbiological assay developed in this period (May – August 2008) is shown below.

Homogenise vegetables in phosphate buffer at pH 7.8 or pH 6.8
↓
Autoclave 120°C, 10 min
↓
Release from plant matrix, remove polyglutamate groups
(protease, α-amylase, folate conjugase extract)
↓
Stop enzyme activity by heating at 100°C, 5 min
↓
Cool, filter, adjust filtrate to pH 6.2
↓
Add aliquot of sample extract to micro-organism
↓
Incubate at 35 – 37°C, 18 – 24 hr
↓
Stop growth, 100°C, 5 min
↓
Measure turbidity at 630 nm

Folate contents (µg folic acid equivalent activity/100 g) of reference materials determined by the preliminary microbiological assay				
Reference material[†]	Range	Mean (n)	SD	CV (%)
BCR-485	223 – 363	306 (11)	39	13
Broccoli	709 – 1929	1050 (18)	292	28
Bok choy	1472 - 2253	1717 (13)	266	15

[†] Certified value: BCR-485, 315 ± 28 µg folic acid equivalent activity/100 g,
[†] Expected values: Broccoli, 1188 µg folic acid equivalent activity/100 g; Bok choy, unknown.

These preliminary studies provided the foundation for further method development as described in detail in the body of this report.

Appendix C. Poster presented at the First International Vitamin Conference, Copenhagen, Denmark, 19 – 21 May 2010

Department of **Employment, Economic Development and Innovation**

Folate Analysis of Asian Leafy Vegetables:
Chromatographic or Microbiological Assay?

Avis Houlihan[1], Matthew Pyke[1], Pieter Scheelings[2], Glenn Graham[2], Geoffrey Eaglesham[2], Timothy O'Hare[1], Lung Wong[1], Prapasri Puwastien[3] & Wasinee Jongjitsin[3]

1. Queensland Primary Industries and Fisheries, Australia; 2. Queensland Health Forensic and Scientific Services, Australia; 3. Institute of Nutrition, Mahidol University, Thailand

Aims

• To compare total folate results obtained by LC-MS/MS and a microbiological assay

• To study folate vitamer composition of Asian leafy vegetables using liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Methods

Vegetables

• 10 (x5) Asian leafy vegetables grown in Australia

• Homogenised in phosphate buffer, pH 6.0

• Digested using a tri-enzyme treatment (amylase, protease, chicken pancreas extract)

LC-MS/MS

• Positive multiple reaction monitoring mode using a mass spectrometer with an electrospray interface.

• Separation using a C18 column

Microbiological assay

• Vegetable extracts added to *Lactobacillus rhamnosus;* growth monitored by turbidity measurement

• Folic acid equivalent activity determined by comparison to growth of micro-organism in presence of standard amounts of folic acid.

Results

• LC-MS/MS results were consistently lower than those obtained from a microbiological assay (Figure 1)

• 5-Methyl-tetrahydrofolate (5-Me-THF) and 5-formyl-tetrahydrofolate (5-formyl-THF), major folate vitamers in all vegetables (Figure 2)

• 5-formyl-THF, major folate in those vegetables with the higher total folate contents (mizuna, choy sum (flowering), tatsoi) (Figure 2).

Discussion

• Does LC-MS/MS under-estimate folate content or does the microbiological assay overestimate folate levels?

• LC-MS/MS is a highly specific and reproducible analytical technique, however some losses of labile vitamers could occur.

• The microbiological assay may be dependent on folate vitamer composition

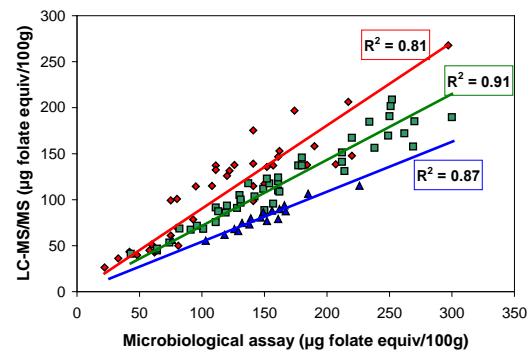• Activators in the vegetable matrix may influence micro-organism's response

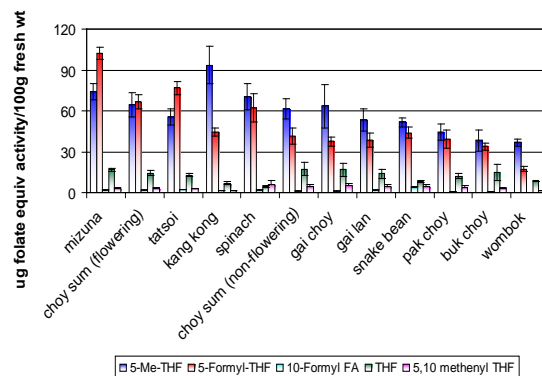Figure 1: Correlations between LC-MS/MS and microbiological assay

Figure 2: Folate vitamer composition of Asian leafy vegetables

Folate Content of Asian Vegetables

by Avis Houlihan, Matthew Pyke, Pieter Scheelings, Glenn Graham,
Geoffrey Eaglesham, Tim O'Hare, Lung Wong, Prapasri Puwastien and Wasinee Jongjitsin

Folates are one of the most nutritionally significant vitamins. Natural sources of folate are reportedly more effective than supplementation or fortification with folic acid. Green leafy vegetables are excellent sources of folates which occur naturally in many forms or vitamers. With an expanding range of Asian vegetables and increasing consumer awareness and acceptance, it is timely to present information on the health attributes of these vegetables.

This report provides information to industry and consumers on the folate content and folate vitamer composition of selected Asian vegetables grown in Australia, using a highly specific analytical technique - liquid chromatography-tandem mass spectrometry. Analysis was also done by the microbiological folate assay, the current 'gold standard' and accepted reference method for folate analysis.

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