

# **Medical importance of dietary betaines**

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## Abstract

The medicinal effects of betaines have only recently been studied, and this thesis furthers this field. Chapter 2 describes the process of developing a betaine assay in order to assay the betaine content of foods. The final methods are described in section 2.5. Using those methods, foods commonly found in the New Zealand diet were assayed and a database of betaine contents in foods can be found in chapter 3. Foods that were high in particular betaines have been studied further, to get an indication of the variation between food samples that is present. This study found that there are only three betaines present in the diet at levels high enough to have potential metabolic effects: glycine betaine, trigonelline and proline betaine. The database has been used to estimate the average daily intake of glycine betaine, trigonelline and proline betaine at 150-200, 20 and 15 mg/day respectively. A diet was also designed to maximize glycine betaine intake, resulting in an intake of 620 mg/day. Chapters 4 and 5 describe experiments on the effect of dietary betaines on different medical problems. Chapter 4 shows that there is an increase in plasma homocysteine concentrations associated with the consumption of coffee, which is consistent with the hypothesis that trigonelline in coffee interacts with homocysteine homeostasis. Trigonelline has some antimicrobial properties in certain conditions, and the question of trigonelline in the diet being a potential to be utilized in the treatment or prevention of urinary tract infections (UTI) is addressed in Chapter 5. Due to a number of factors (discussed in section 5.5), the antimicrobial properties of trigonelline have no effect in human urine, and trigonelline is unable to be used to treat or prevent UTI.

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Mum, Dad, Derek, Ico, Eyk and Lis. I know I was slack, and hardly ever told you what I was doing. I wish it were not so, and I am sorry. If you ever read this, just know that I thought about you, and often longed to hear your voices.

And finally, I would like to dedicate this to Andy, who is no longer with us playing the game of life. You were a great inspiration to me, and you will not be forgotten.

## Abbreviations used in this thesis

AC	acetyl carnitine	MTHFR	5,10-
ACC	Accident Compensation Corporation		methylenetetrahydrofolate reductase
ACN	acetonitrile	OD	optical density
Ars	arsenobetaine	osmol	osmole: one mole of osmotically active particles
Bet	betonidine		
BHMT	betaine-homocysteine methyl transferase	PB	proline betaine
But	butyrobetaine	pH	potenz hydrogen
Carn	carnitine	Pro	propionobetaine
CBS	cystathionine $\beta$ -synthase	R <sub>f</sub>	retention factor
CCl <sub>4</sub>	carbon tetrachloride	RMM	relative molecular mass
cfu	colony forming units	rpm	revolutions per minute
CL	cystathionine $\gamma$ -lyase	SCX	strong cation exchange
Cre	creatinine	sec	seconds
CVD	cardiovascular disease	SEM	standard error of the mean
diglyme	2-methoxyethyl ether	TEA	triethyl ammonium
DMG	<i>N,N</i> -dimethylglycine	THF	tetrahydrofolate
DMSP	dimethylsulfonopropionate	TLA	three letter acronym
DNA	deoxyribonucleic acid	TLC	thin layer chromatography
Erg	ergothionine	TMAH	tetramethyl ammonium hydroxide
GB	glycine betaine	TMAO	trimethylamine oxide
HPLC	high performance liquid chromatography	Trig	trigonelline
K <sub>m</sub>	Michaelis-Menten constant	tsp	teaspoon
M	mol <sup>-1</sup>	UTI	urinary tract infections
MDCK	Madin Darby Canine Kidney	UV	ultraviolet light
min	minutes	v	volume
mol	mole	V	volt
monoglyme	1,2-dimethoxyethane	Vit B <sub>6</sub>	vitamin B <sub>6</sub> (pyridoxal phosphate)
MS	methionine synthase	V <sub>max</sub>	maximum velocity (of an enzyme)
MTHF	5-methyltetrahydrofolate		

# Chapter 1

## Background

*"Anyone who isn't confused really doesn't understand the situation."*

Edward Murrow

## 1.1. Betaines

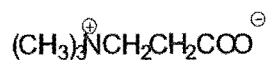
Betaine was the name given to a substance that was isolated and crystalized from beet (*Beta vulgaris*) by Scheibler (1869). This compound proved to be *N,N,N*-trimethylglycine (figure 1.1), now commonly known as glycine betaine. The term betaine is often still used to describe glycine betaine, although it is common for betaine to describe glycine betaine or its analogues. For the purposes of this thesis, betaines will be defined as analogues of *N,N,N*-trimethylglycine that have a quaternary amino group, bearing a positive charge at all pH, and a carboxylate moiety that has a negative charge at physiological pH, giving the compound a net charge of zero. Also covered are compounds that have a positively charged moiety that is not a quaternary amine. For example, arsenobetaine, found in seafood has a quaternary arsonium centre, and dimethylsulfoniopropionate (DMSP), found in shellfish, has a tertiary sulfonium centre.

### 1.1.1. Distribution and function in nature

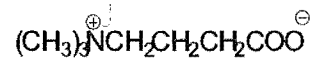
Betaines can be found in all kingdoms of life. The biological roles of betaines are not completely understood, but betaines are involved in cell volume regulation, as methyl donors, in cell to cell signalling and as carrier molecules. Some betaines, such as glycine betaine, are accumulated by cells, and play important roles in cell volume regulation (sections 1.1.1.1, 1.2.1). Glycine betaine can be a source of methyl groups, and is involved in methyl group metabolism (section 1.2.2). Carnitine is a betaine that is essential in all eucaryotic cells for the transport of long-chain fatty acids across the mitochondrial membrane. The fatty acids are trans-esterified to carnitine (figure 1.1), and the resultant long-chain acylcarnitine esters are transported into the mitochondria via a specific translocase, allowing the metabolism of long-chain fatty acids (reviewed in Rebouche 1992). The betaines trigonelline and proline betaine are used by legumes as signals to rhizobia, initiating the formation of root nodules (Phillips et al. 1994; Phillips et al. 1995). Proline betaine can also be found in citrus fruits (reviewed in Nolte et al. 1997), and trigonelline in coffee (Stennert and Maier 1994), although these betaines have no known function in these plants. Arsenic is found in sea water as arsenate, and arsenobetaine is believed to be the non-toxic metabolic end product of arsenate in marine fauna (Shibata et al. 1992; Hanaoka et al. 1995). Some of these topics are elaborated in the following sections.



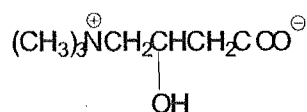
Glycine betaine



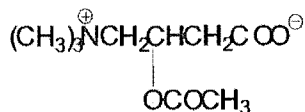
Propionobetaine



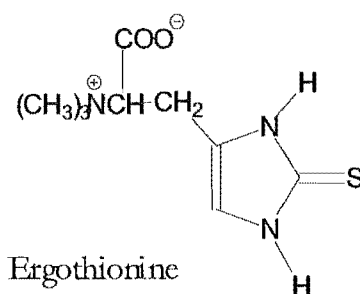
Butyrobetaine



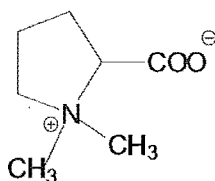
Carnitine



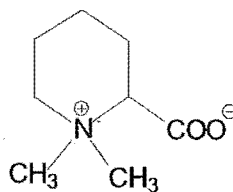
Acetyl carnitine



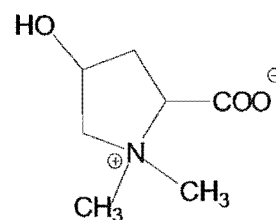
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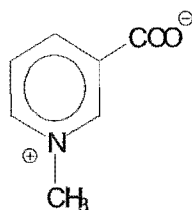
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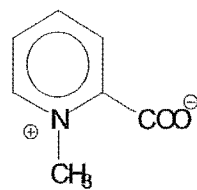
Pipecolobetaine



Betonidine



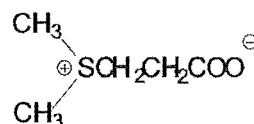
Trigonelline



Homarine



Arsenobetaine



Dimethylsulfoniopropionate

**Figure 1.1:** Chemical structures of known betaines that were expected to be found in the normal human diet.



### 1.1.1.1. Compatible solutes and cell volume regulation

A compatible solute is a compound that can be present in high concentrations ( $>1 \text{ mol l}^{-1}$ ) within cells without having an adverse effect on cellular components (enzymes, DNA, membranes, etc.) or on normal cellular functions (Le Rudulier et al. 1984). Classes of compatible solutes are polyols (eg. sorbitol), amino acids (eg. proline) and methyl amines (eg. glycine betaine) (reviewed in Somero and Yancey 1997). The concentration of compatible solutes in vitro can be adjusted without altering the function of cellular components (Somero and Yancey 1997) and in vivo experiments have also shown that cells do better (eg. grow more rapidly) in hyperosmotic media when supplied with some compatible solutes (Randall et al. 1996; Somero and Yancey 1997). Changes in cell volume have drastic effects on the concentrations of cell constituents, which would have massive metabolic effects. To counter this, some cells have the ability to actively accumulate compatible solutes, the effect being that the volume and constituents of the cell (eg. water and inorganic salts) remain constant except for the concentration of the compatible solute(s) (reviewed in Somero and Yancey 1997). The compatible solute(s) used by cells depends on the environment and type of cell. For example, *Escherichia coli* K12 synthesizes trehalose after a transfer to hyperosmotic medium, unless glycine betaine or proline are present, in which case these compatible solutes are accumulated instead (Dinnibier et al. 1988). Glycine betaine is one of the most common compatible solutes in nature, and many organisms are able to synthesize or selectively accumulate glycine betaine as a means of surviving osmotic changes (Somero and Yancey 1997).

### 1.1.1.2. Counteracting solutes

A special class of compatible solutes are counteracting solutes. These solutes have the ability not only to be present at high concentrations, but also to reverse the effect of some physical and chemical stresses such as heat, urea and alcohol (reviewed in Somero and Yancey 1997). Thermodynamic calculations suggest that glycine betaine should be one of the most effective counteracting solutes (Timasheff 1992). Counteracting solutes are able to maintain native enzyme structures (and other biological macromolecules) in the presence of stresses that would normally denature them. A generally accepted mechanism for the action of these solutes is that they are preferentially excluded from the surface of macromolecules, which makes the compact, folded form (native) of the macromolecule thermodynamically favourable (Timasheff 1993).

An example of counteracting solutes can be found in the mammalian urinary tract.

where invading bacterial cells are frequently subjected to high osmolality and urea concentrations. In minimal media, the urea and salt concentrations are often inhibitory to bacterial growth, but bacteria are able to colonize the urinary tract. Glycine betaine is present in mammalian urine (Lever et al. 1994) and is an important factor in making the urine a suitable growth medium (Chambers and Kunin 1985), thus allowing bacteria to colonize the urinary tract.

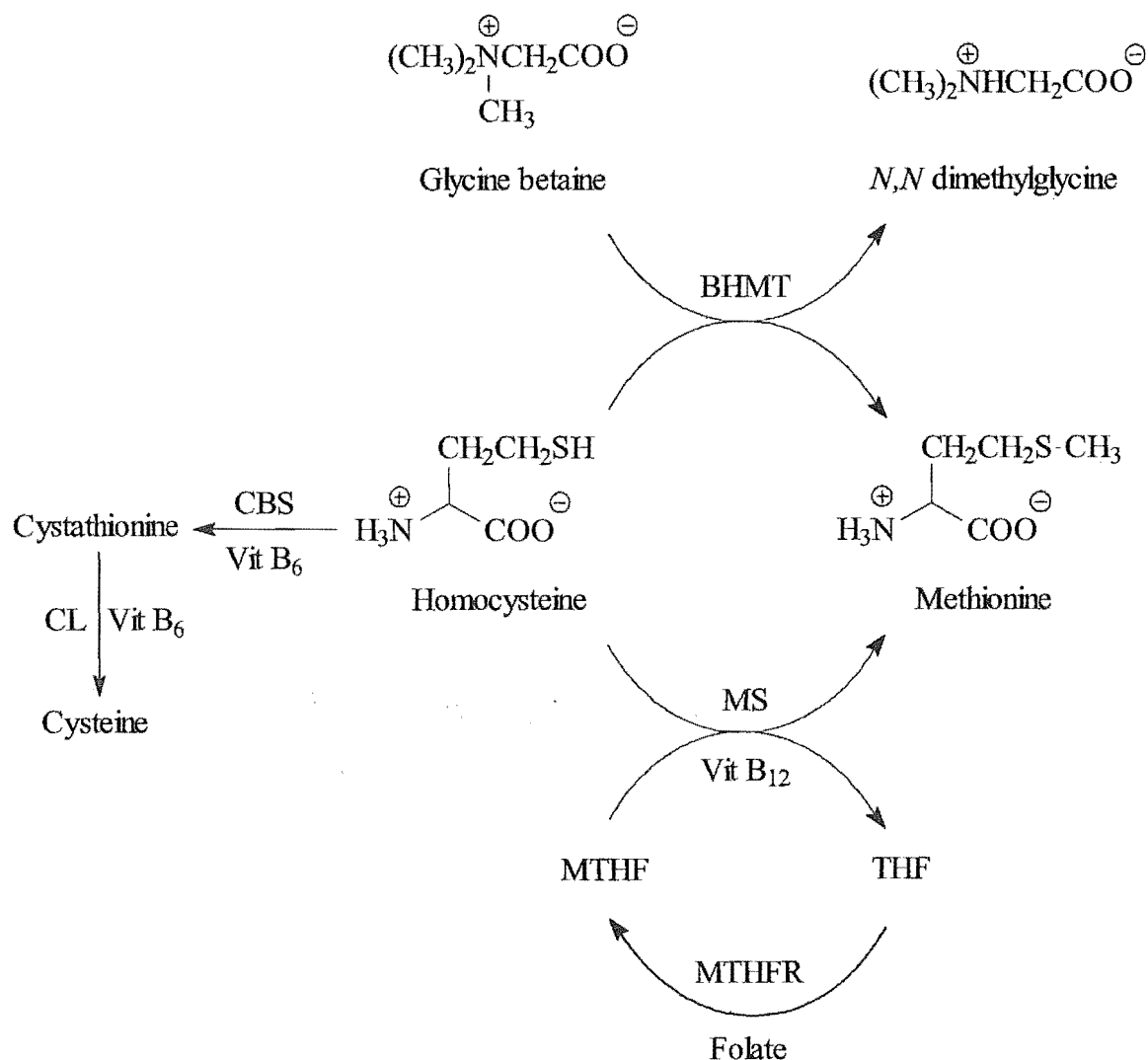
## **1.2. Betaines in humans**

### **1.2.1. Osmolytes and cell volume regulation**

Glycine betaine is used as an osmolyte in kidney cells (Bagnasco et al. 1986; Sizeland et al. 1993), and it is important for cell volume regulation of kidney cells (Kwon 1998). Active accumulation of glycine betaine normally only occurs in the kidney, but osmo-sensitive glycine betaine transporter pumps can be found in many different human cell types including liver, kidney (Huassinger et al. 1998) and endothelial (Petronini et al. 2000) cells. Most human cells other than kidney cells are surrounded by interstitial fluid which in healthy humans has a constant osmolality. There are a number of other osmolytes that are utilized by the kidneys, including inositol and sorbitol (Schmolke et al. 1991). Glycine betaine is used as an osmolyte in other tissues when required; for example, hypernatremia (high blood  $\text{Na}^+$  levels) causes more than a 50% increase in the glycine betaine content of brain cells (Lien et al. 1990).

### **1.2.2. Role as a methyl donor**

In animals, as well as some unicellular organisms, glycine betaine donates a methyl group to homocysteine in a reaction catalysed by betaine homocysteine methyl transferase (BHMT; E.C. 2.1.1.5) forming the products N,N-dimethylglycine and methionine (figure 1.2). Regulation is complex in mammals, with both transcriptional and post translational regulation present as well as feedback inhibition by N,N-dimethylglycine (Garrow 1996). BHMT is present in the liver and kidney of mammals (Sunden et al. 1998), and has been reported in other tissues such the eye (Rao et al. 1998). There is no apparent correlation between BHMT activity and the level of plasma glycine betaine in rats, and the activity also appeared to be insensitive to tissue glycine betaine



**Figure 1.2:** Metabolic pathway showing the metabolism of homocysteine.

**CBS:** cystathionine • -synthase. **CL:** cystathionine • -lyase. **MS:** methionine synthase.

**MTHF:** 5-methyltetrahydrofolate. **MTHFR:** 5,10-methylenetetrahydrofolate reductase.

**THF:** tetrahydrofolate. **Vit B<sub>6</sub>:** vitamin B<sub>6</sub> (pyridoxal phosphate).

concentration (Finkelstein et al. 1983). Proline betaine can act as a substrate for purified porcine BHMT (Mulligan et al. 1998), but whether this occurs in human physiology is unknown.

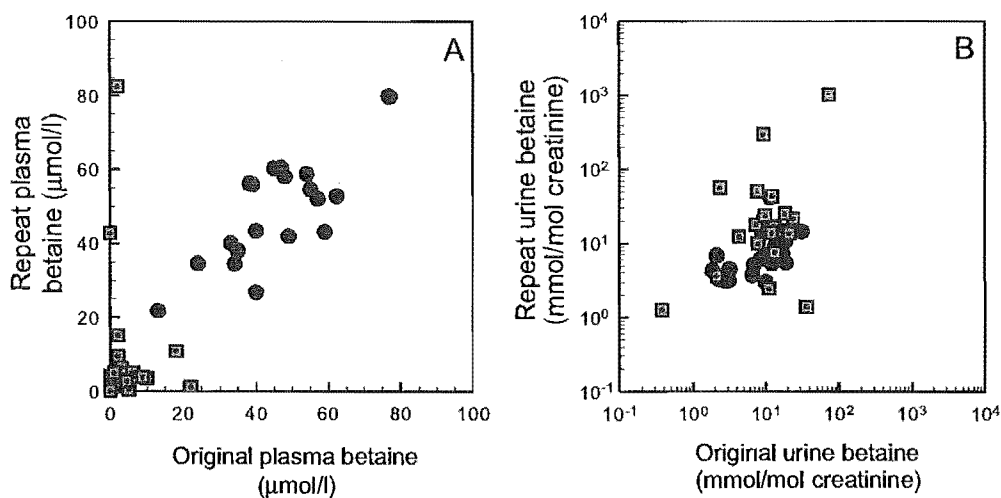
### 1.2.3. Glycine betaine homeostasis

#### 1.2.3.1. Regulation in blood plasma

The level of glycine betaine in blood plasma is highly regulated in mammals. Plasma levels are regulated with variable individual set points generally between 20-70  $\mu\text{mol}^{-1}$  in healthy humans, and concentrations generally remain within 20% of the individual's set point. Within an individual the set point appears to be constant, at least over three years (figure 1.3A), and possibly longer (Chambers and Lever 1996). Plasma concentrations are 23% (female) and 32% (male) lower ( $P < 0.1$  and  $P < 0.001$ ) in patients with renal disease, but there is no significant difference in the plasma glycine betaine concentration in diabetics and healthy people.

#### 1.2.3.2. Source of glycine betaine

Glycine betaine can be obtained either through the diet, or it can be endogenously synthesized from choline via the enzymes choline dehydrogenase (E.C. 1.1.99.1, an alcohol dehydrogenase) and betaine aldehyde dehydrogenase (E.C. 1.2.1.8) (Flower et al. 1972). Synthesis occurs in the



**Figure 1.3:** Plots showing homeostasis of glycine betaine and proline betaine over three years. Plot A shows the change in plasma glycine betaine (red circles) and proline betaine (blue squares) concentrations over three years. Plot B shows the change in urinary glycine betaine (red circles) and proline betaine (blue squares) concentrations over three years. Graph taken from Lever et al. (unpublished data).

liver and kidney (reviewed in Zeisel 1981). Conversion of choline to glycine betaine also occurs in the stomach (Flower et al. 1972), due to either bacteria, or the cells in the epithelium of the stomach. Approximately half of the choline consumed in the diet is converted to glycine betaine in the gut of hamsters (Flower et al. 1972). Some choline that is ingested is instead converted to trimethylamine by bacteria in the gut (de la Huerga and Popper 1952; Asato and Simenhoff 1965). Prior to this project, there was little data on the glycine betaine or choline content of the diet, other than for a small number of foods that have high levels. There is strong evidence that glycine betaine consumed is absorbed into the blood stream, as patients with chronic renal failure (McGregor 2001) and homocystinuria (reviewed in Sakura et al. 1998) given oral glycine betaine supplements show a marked increase in plasma glycine betaine.

### **1.2.3.3. Loss of glycine betaine**

Glycine betaine can be converted to N,N-dimethylglycine by the transfer of a methyl group to homocysteine as described earlier (section 1.2.2), and some glycine betaine is excreted in the urine (Lever et al. 1994a). Healthy kidneys are able to efficiently reabsorb glycine betaine, with a fractional clearance of only 3-5% (Lever et al. 1994a). Most of the resorbance occurs in the proximal tubules, but it can also occur in Henle's loops in rats (Pummer et al. 2000). The urinary excretion of glycine betaine has been shown to be increased in diabetes mellitus (both type 1 and type 2) and renal failure (Lever et al. 1994b; Dellow et al. 1999).

### **1.2.4. Dietary betaines**

At least three betaines that are not known to be synthesised or required by humans have been detected in human specimens, indicating that betaines obtained in the diet are absorbed into the circulatory system in humans. Trigonelline and proline have been found both in plasma and urine (Lever et al. 1994a), and arsenobetaine has been found in urine (Fowler 1977). There is a correlation between the plasma concentrations of proline betaine and trigonelline and the presence of these compounds in the urine. These compounds are not accumulated in the kidney, unlike glycine betaine (Lever et al. 1994a). The concentrations of these betaines in the blood is very variable (figure 1.3A), and the physiological effects of these compounds are unknown. A correlation between proline betaine excretion and glycine betaine excretion was noted by Lever et al. (1994a), but no explanation for this was given and the reason(s) is still unknown. Trigonelline has been

proposed as an antidiabetic substance, but no studies into this have been carried out (reviewed in Al-Habori and Raman 1998). Arsenobetaine is a non-toxic arsenic compound that can sometimes be found in urine after ingestion of seafood. Arsenobetaine has caused problems in the measurement of arsenic in humans, because traditional tests are unable to distinguish between inorganic (toxic) and organic (non-toxic) forms of arsenic (reviewed in Fowler 1977). Recently techniques for distinguishing the forms have been developed (Mester and Fodor 1997), allowing the concentrations of toxic arsenic to be accurately determined.

### 1.3. Homocysteine

Homocysteine is a non-protein amino acid (figure 1.2) which is an intermediate in methyl transfer reactions and a byproduct of protein metabolism. Plasma homocysteine concentrations are homeostatically controlled in mammals, primarily by the liver and, to a lesser extent, the kidneys (Finkelstein 1998). Normal levels are between 5 and 17  $\mu\text{mol l}^{-1}$  in plasma (Schreiner et al. 1997). Homocysteine can be metabolized through three different pathways in mammals (figure 1.2). Two of these pathways are dependent on B vitamins; the folate dependent THF pathway, and the vitamin B<sub>6</sub> dependent pathway (Finkelstein 1998). The other pathway, described earlier, utilizes glycine betaine as a methyl donor, producing methionine and N,N-dimethylglycine. It should be noted that homocysteine converted to methionine is ultimately recycled via the SAM/SAH (S-adenosylmethionine/S-adenosylhomocysteine), whereas the vitamin B<sub>6</sub> dependent pathway, which forms cysteine, alters the carbon backbone, and removes homocysteine from this metabolic cycle.

In 1962, the first disease involving homocysteine metabolism (homocystinuria) was described (Gerritsen et al. 1962), (Carson and Neill 1962), and homocysteine was soon linked to atherosclerosis (McCully 1969). Classical homocystinuria is a genetic metabolic disorder caused by a deficiency in one of the enzymes cystathionine  $\beta$ -synthase (CBS), methylenetetrahydrofolate reductase (MTHFR), or methionine synthase (MS) (figure 1.2). The symptoms of classical homocystinuria include mental retardation, skeletal abnormalities, and premature atherothrombotic disease (reviewed in Welch and Loscalzo 1998). The levels of homocysteine in the blood plasma of patients presenting with classical homocystinuria range from 50-500  $\mu\text{mol l}^{-1}$  (Jacobsen 1998). The incidence of homocystinuria is low, with the homozygous trait occurring only once in about 200 000 births (reviewed in Welch and Loscalzo 1998).

In recent years there has been a lot of interest in homocysteine as an independent risk factor for cardiovascular disease. Elevated homocysteine levels have also been associated with other disorders such as fetal abnormalities (Ronsenquist et al. 1996) and cognitive impairments (reviewed in Nygård et al. 1998). Mildly elevated homocysteine concentrations occur in 5-7% of the population (reviewed in Welch and Loscalzo 1998), and are affected by lifestyle factors including smoking, and inactivity. Males tend to have higher homocysteine concentrations, and homocysteine concentrations increase with age. There is no apparent threshold for the risk of CVD, and it has been estimated that an increase of  $5 \text{ mol}^{-1}$  confers a risk increase of 50% (Boushey et al. 1995).

### **1.3.1. Folate studies**

A significant decrease in circulating homocysteine can be achieved with B-vitamin supplementation (Boushey et al. 1995; Rosenquist et al. 1996; Surtees et al. 1997; Healy et al. 1998; den Heijer et al. 1998; Malinow et al. 1998 and Riddell et al. 2000: reviewed in Jacobsen 1998 and Deutsch et al. 1998) and, at present, patients presenting with high homocysteine levels are generally put on a vitamin therapy, specifically receiving folate, vitamin B6 and vitamin B12. Although reductions in circulating levels of homocysteine are often observed, a return to normal levels is not common.

### **1.3.2. Other factors effecting homocysteine**

There have been a number of recent studies which have indicated that B-group vitamins are not the only factors in altering plasma homocysteine levels. Nygård et al. (1995) in the Hordaland study found that circulating homocysteine levels were increased in smokers and heavy coffee drinkers, whereas they were lower in physically active people. Broekmans et al. (2000) gave subjects a high fruit and vegetable diet for four weeks and showed no significant change in either folate or vitamin B<sub>12</sub> levels but a 10% decrease ( $P < 0.05$ ) in circulating homocysteine. DeRose et al. (2000) showed a large reduction in circulating homocysteine levels after transferring subjects to a vegan based diet. Stickel et al. (2000) noted a large (60%) ( $P < 0.01$ ) increase in circulating homocysteine after giving rats high levels of alcohol, despite the provision of more than 20 times the basal requirement of folate and the achievement of high blood and hepatic folate levels. The

effect that alcohol has on homocysteine concentrations may be due to the negative effect it has on folate concentrations (Hillman and Steinberg 1982). All of these studies strongly suggest that B vitamins are not the only important factor in homocysteine metabolism, and other factors need to be considered.

### **1.3.3. Glycine betaine and homocysteine**

Glycine betaine metabolism has not enjoyed the same level of interest that B-vitamin dependent pathways have. This is likely to be due to the difficulty of measuring physiological concentrations of glycine betaine (chapter 2). There have been a number of studies that have looked at the effects of glycine betaine administration on homocysteine metabolism. Wilcken et al. (1983) gave 10 patients with cystathionine  $\beta$ -synthase deficiency who were unresponsive to pyridoxine therapy (the standard treatment) 6 g/day of glycine betaine for between three and 13 months. All of the subjects experienced a marked (between 50% and 97%) decrease in circulating homocysteine, but only two subjects' homocysteine levels were lowered to within the normal range. Other changes noted in the patients included two subjects who showed a darkening of previously fair hair, and there were improvements in behaviour reported by school teachers or parents in six patients. These were not formally tested. Storch et al. (1991) administered 3 g/day glycine betaine to healthy young men, and showed that there was no significant increase in glycine betaine excretion, although in one subject, increased excretion accounted for ~17% of administered glycine betaine. They also observed an increase in the transmethylation rate of methionine, suggesting increased cycling of homocysteine and methionine. Brouwer et al. (2000) gave healthy volunteers 6 g/day glycine betaine, and showed a significant decrease in plasma homocysteine after 2 weeks ( $10.9 \mu\text{mol l}^{-1}$  to  $10.0 \mu\text{mol l}^{-1}$ ,  $P = 0.01$ ), suggesting that some of the increased methionine transmethylation observed by Storch et al. was due to increased flux through BHMT.

### **1.3.4. Other effects of glycine betaine**

Betaine has also been given in studies unrelated to homocysteine metabolism or cell volume regulation. Söderling et al. (1998) reported that betaine added to toothpaste relieves symptoms of dry mouth, an oral condition that causes discomfort, increased susceptibility to oral candidosis and difficulty in speech and eating. It is likely that this is due to the counteracting nature of glycine



betaine. Glycine betaine also protects the liver (of rats) against carbon tetrachloride (CCl<sub>4</sub>) induced lipidosis (fatty infiltration of the liver) (Junnila et al. 1998) and glycine betaine has been hypothesized to protect the liver from the damage that can be caused during niacin therapy (used to treat hyperlipidaemia) (McCarty 2000). The ability of glycine betaine to protect the liver is likely to be due to the methionine sparing effect of betaine, and its role in methyl transfer. The toxic effects of aluminium in rats is reversed by administration of betaine for 30 days, starting the day after administration of aluminium (Ramakrishna et al. 1998) and it was suggested that betaine may prevent formation/stop progression of amyloid plaques and also augment choline levels, both of which would lessen the symptoms of Alzheimer's disease.

There are no reports of any adverse effects associated with the supplementation of glycine betaine.

## **1.4. Aims**

The aim of this thesis was to investigate some of the aspects of dietary betaines. Initially, a database of the betaine content in foods was required. This knowledge allows estimation of the intake of glycine betaine, which is important to compare to the dosages that have been given in some clinical trials. This knowledge also enables comparison of different diets and lifestyles, in particular, comparing the “normal” diet to vegetarian/vegan diets, coeliacs’ (gluten free) diets, and the diets recommended to diabetics and heart and renal patients. Also gained is the ability to design a diet that is high in glycine betaine. The formation of a database also determines which betaines in the diet are present at levels that may be of physiological importance. Before this work could commence, modification of the assay to fulfil the requirements for the formation of the database was needed.

The effect that glycine betaine has on human metabolism has been examined in a number of studies, but few studies have been carried out on the effect of other betaines on human metabolism. This thesis aims to elucidate some of the effects of dietary betaines on human health. In particular, the effect of trigonelline, a betaine present in coffee, is investigated in chapter 4, the potential of trigonelline to modulate homocysteine levels is investigated, and in chapter 5, the antibacterial properties of trigonelline in urine are tested.

## Chapter 2

### Assay Development

*"Result! Why, man, I have gotten a lot of results.  
I know several thousand things that won't work."*

Thomas Edison

## 2.1. Introduction

This chapter outlines the steps taken to optimize and test the methods used to measure the betaine content of a range of foods. These methods include the removal of hydrophobic compounds from the sample, derivatization procedure, and a range of HPLC separation systems. The resulting methods are summarized in section 2.5.

The betaine assay at Canterbury Health Laboratory has evolved since 1988. During that time it has been optimized for the quantification of different betaines that are commonly found in plasma and urine samples, as well as some synthetic betaines that were candidate antimicrobial agents. The original assay (Lever et al. 1992) was unable to fulfil a number of requirements of measuring the betaine content in the constituents of the diet:

- 1) Earlier experience in this laboratory has shown that hydrophobic compounds found in some foods, especially animal homogenates, cause a decrease in the performance of HPLC columns, so a means of removing these compounds was required (section 2.2).
- 2) The chromatograms have always had some reagent-derived artefact peaks which had not caused major problems and had been tolerated. However, some of the naturally occurring betaines run in the same area as these interfering compounds. For detection and quantification of these compounds, the interference must be reduced (section 2.3).
- 3) Unique to this project was the possibility that different compounds co-elute with known betaines. This cannot be avoided, but it can be detected by using separation systems with different selectivities (section 2.4).

## 2.2. Sample preparation

Measurement of betaines requires the derivatization in aqueous solution (section 2.3), and then separation of the derivatives using HPLC (section 2.4). As most foods are solid, a means of forming an aqueous extract was required.

## **2.2.1. Preparing an aqueous extract**

Analysis can only be carried out on liquid samples, so a method of obtaining an aqueous extract from foods was required. A general method (described in section 2.5.1.1) of obtaining an aqueous extract was designed in association with Crop and Food Research Ltd (Athar 2000). Alterations to the method were made as required, and these are described in sections 2.5.1.2 - 2.5.1.4.

### **2.2.1.1. Dichloromethane extraction**

To make the final solution suitable for analysis by HPLC, hydrophobic compounds needed to be removed. A simple method was envisaged, involving a liquid-liquid extraction. The solvent to be used for this extraction needed to be hydrophobic, immiscible with water, preferably denser than water, and relatively non-toxic. A solvent with these properties could be added to the sample, and then centrifugation would result in a distinct and easily accessible upper aqueous layer. Dichloromethane has these properties, so was used as the extraction solvent. The extraction procedure is described in section 2.5.1.5, and testing is described in sections 2.2.2.1 - 2.2.2.3.

## **2.2.2. Methods**

### **2.2.2.1. Recovery of dichloromethane extraction**

To ascertain if any betaines of interest are transferred into the dichloromethane layer, a standard was extracted with dichloromethane as described in section 2.5.1.5. The two layers were separated, and the dichloromethane layer was evaporated and any residue resuspended in H<sub>2</sub>O. The resuspended solution, the original aqueous standard and the extracted aqueous standard were derivatized (2.5.2) and assayed using the Phenosphere (2.5.3.3) chromatography system. This was carried out in duplicate, and results are shown in section 2.2.3.1. A full description of the methodology can be seen in section 7.2.1.

### **2.2.2.2. Meat**

To test whether the extraction procedure removes hydrophobic compounds from the aqueous solution, a number of meat extracts (2.5.1.1) were combined. This solution was extracted with dichloromethane as described in section 2.5.1.5. The two liquid layers were separated, and

compared to the original mixture using thin layer chromatography (TLC) with dichloromethane used as the mobile phase. The TLC plate was viewed under normal, blue (366 nm) and UV (254 nm) light.

### **2.2.2.3. Butter**

Butter was suspended in dichloromethane (0.5 g in 3 ml), and the resultant suspension was extracted as described in section 2.5.1.2. The two liquid layers were separated, and compared to the original mixture using thin layer chromatography (TLC) with dichloromethane used as the mobile phase. The TLC plate was viewed under normal, blue (366 nm) and UV (254 nm) light.

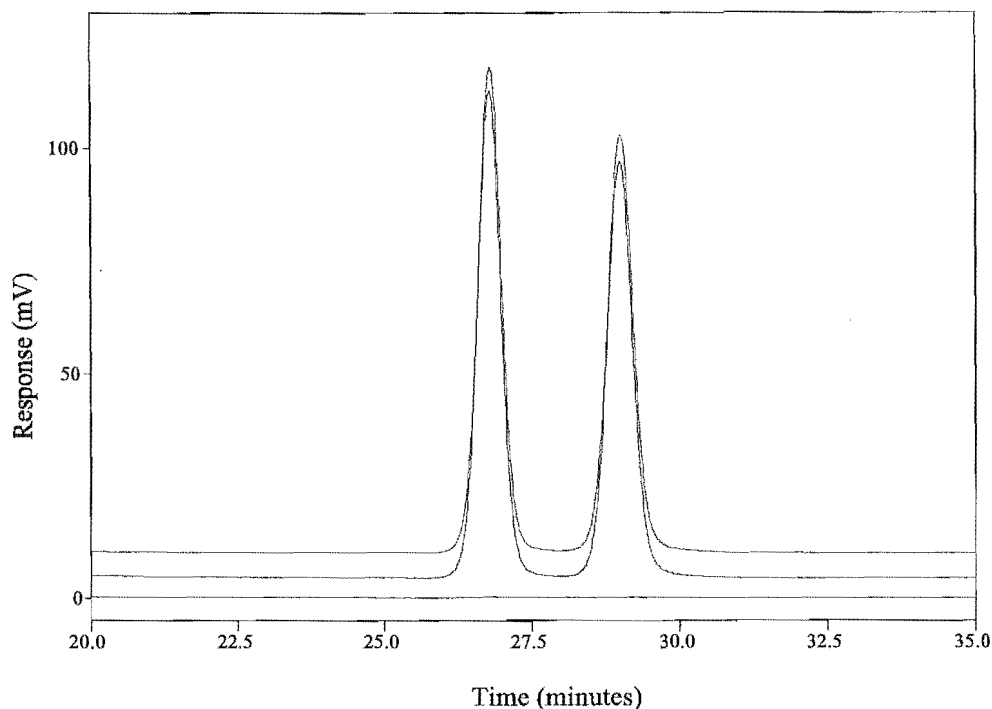
## **2.2.3. Results**

### **2.2.3.1. Recovery**

Figure 2.1 shows the resultant chromatograms from the betaine transfer experiment. The peak areas can be seen in table 2.1. More than 99% of the betaines were recovered in the extracted sample.

### **2.2.3.2. Meat**

After centrifugation, three distinct layers were observed. The lowest and highest layers were liquid, and the middle layer was solid. This corresponds to the dichloromethane (lower) layer, a layer of denatured proteins and other solids, and an aqueous layer. At least two compounds could be seen in the dichloromethane layer ( $R_f = 1$ ,  $R_f = 0.8$ ) when the TLC plate was viewed under UV light, but no hydrophobic compounds were visible in either the post-extraction or the pre-extraction aqueous samples.



**Figure 2.1:** Chromatogram showing the betaine (glycine betaine and trigonelline) recovered after an extraction. The blue trace is a derivatization of the dichloromethane layer. The green trace is prior to dichloromethane extraction and the red trace is the aqueous layer after dichloromethane extraction.

Trace		Glycine betaine area (mV.sec)	Glycine betaine (%)	Trigonelline area (mV.sec)	Trigonelline (%)
Before Extraction		3100	100	2930	100
After Extraction	H <sub>2</sub> O layer	3070	99.1	2910	99.4
	Dichloromethane layer	5	0.2	4.4	0.2

**Table 2.1:** Efficiency of betaine extraction from a standard betaine solution. Betaine peak areas and percentages of unextracted sample standard areas.

### 2.2.3.3. Butter

The TLC of the butter extract showed the presence of some hydrophobic compounds in dichloromethane (pre- and post- extraction) ( $R_f = 0.6$ ) whereas the only compounds seen in the water layer did not migrate from the initial point ( $R_f = 0$ ).

### 2.2.4. Discussion

These results indicate that the extraction procedure is suitable for the removal of hydrophobic compounds from a wide range of foods without substantially altering the concentration of betaines that are of interest. No hydrophobic compounds were seen on the TLC plates, and virtually all of the betaines were recovered after extraction.

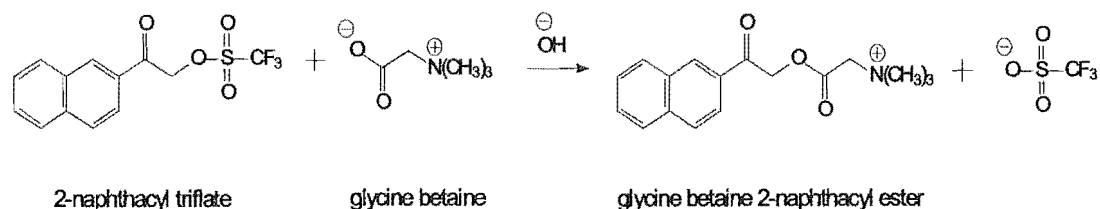
## 2.3. Derivatization

### 2.3.1. Background

Betaines are intrinsically difficult to detect at physiological concentrations, and to measure them by HPLC a chromophore must be added to the molecule. A description of the original derivatization reaction can be seen in section 7.2.2. Two different chromophores have been used in this project. The naphthacyl functional group was favoured because of its purity and availability, but the 2-fluorenyl group was used when 2-naphthacyl trifluoromethanesulfonate was in short supply. The chromatography of betaine derivatives with the two chromophores was essentially the same (data not shown).

The chromophore was synthesized as the trifluoromethanesulfonate by the Chemistry Department, University of Canterbury. The chromophore is transferred from the trifluoromethanesulfonate to the carboxyl group of the betaine as shown in figure 2.2 (Ingalls et al. 1984). This reaction only occurs in the presence of base, and the resulting betaine ester is unstable in the presence of base (Wittman and Ziegler 1988).

The first part of this procedure was the drying stage. The aqueous sample was diluted in derivatization solvent and anhydrous  $\text{Na}_2\text{SO}_4$  was added in excess. The  $\text{Na}_2\text{SO}_4$  absorbed any water, and the  $\text{Na}_2\text{SO}_4$  was then removed by centrifugation. Because the base used



**Figure 2.2:** The derivatization reaction. 2-Naphthacyl trifluoromethanesulfonate reacts with glycine betaine in the presence of [OH<sup>-</sup>] to form glycine betaine 2-naphthacyl ester, which can then be measured by HPLC.

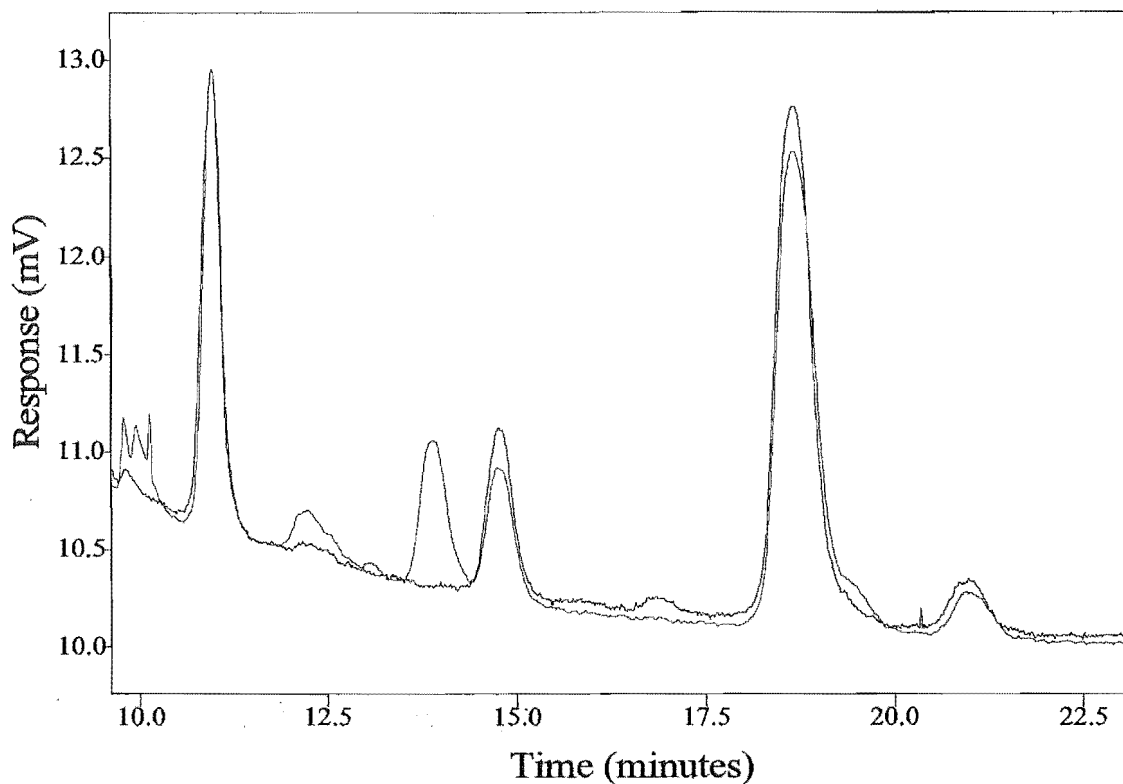
in the reaction is only slightly soluble, the water concentration had a major effect on the concentration of base in solution. As the rate of reaction is dependent on the amount of base present, it was thought to be essential to keep the amount of base constant. The water content was approximately 1.6% (v/v), and this comes solely from the addition of base suspension. The product of the reaction breaks down to the betaine and phenacyl alcohol in the presence of base (Wittman and Ziegler 1988), so basic conditions are required for formation but also cause degradation of the derivative. Five minutes after reactants are combined, the reaction was effectively stopped by centrifugation. All of the solid base was removed, making the solution no longer basic. The betaine derivatives were no longer formed nor degraded.

### 2.3.1.1. Derivatization solvent

The traditional solvent system used for this derivatization reaction is acetonitrile (Ingalls et al. 1984). This has fulfilled all expectations so far, but for this project this derivatization solvent was not suitable. The problem with acetonitrile based systems is that acetonitrile itself is derivatized, and this appears on the chromatograms as an interfering peak (figure 2.3). This has been suspected for some time, and was finally shown by Paton et al. (unpublished data) using mass spectroscopy. Due to the inadequacies of acetonitrile, another solvent was required. The following requirements need to be met:

- 1) The solvent must allow derivatization to occur. Both betaines and derivatizing reagent need to be soluble in the solvent.
- 2) It is important that the solvent itself is not derivatized.
- 3) If the solvent absorbs strongly in the UV, and the betaine derivatives are being





**Figure 2.3:** Chromatogram showing the peak (c.13 minutes) that is created by the presence of acetonitrile. The solution that gave the blue trace was the same derivatized in acetone, and then diluted 1:1 with acetone. The solution that gave the red trace was the same derivatization, but was diluted 1:1 with acetonitrile. This is peak also occurs if the derivatization reaction is carried out in acetonitrile.

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detected using UV absorbance, then the baseline UV absorbance would increase the baseline noise, and reduce the sensitivity of the assay. If the betaine derivatives are to be detected using fluorescence (a more sensitive detection method), the solvent must have low fluorescence.

- 4) The solvent must be pure enough so that there are no observable artefacts on the HPLC traces. Purity is also important for reproducibility.
- 5) The solvent must be readily available, so that the availability of the solvent is never an issue.
- 6) As a number of different workers will be exposed to the solvent, it is advantageous if the solvent is non-toxic.

- 7) A non-volatile solvent is desirable, because solvents with low boiling points may evaporate leading to high and inaccurate results.

### **2.3.1.2. Order of reactants**

Initially, the derivatizing reagent was added prior to the base, but the reverse has been the case in the past. The reaction would not be predicted to occur until both base and derivatizing reagent are present, and there is no known reason why the order should make a difference. This was important to standardize the methods used by different people, and also to see if any reduction in steps and/or time could be achieved.

## **2.3.2. Methods**

### **2.3.2.1. Derivatization solvent**

Properties 1, 2, 3 and 5 (section 2.3.1.1) are absolute requirements of the derivatization. A number of solvents that fulfilled requirements 3, 4 and 5 were tested as described in the section 7.2.3. The HPLC traces were compared to show the relative efficiencies of derivatization (section 2.3.3.1).

### **2.3.2.2. Effect of water on derivatization**

A standard solution was derivatized in acetone with varying amounts of water and the resultant HPLC traces were compared. The results can be seen in section 2.3.3.2. Details can be seen in the section 7.2.4.

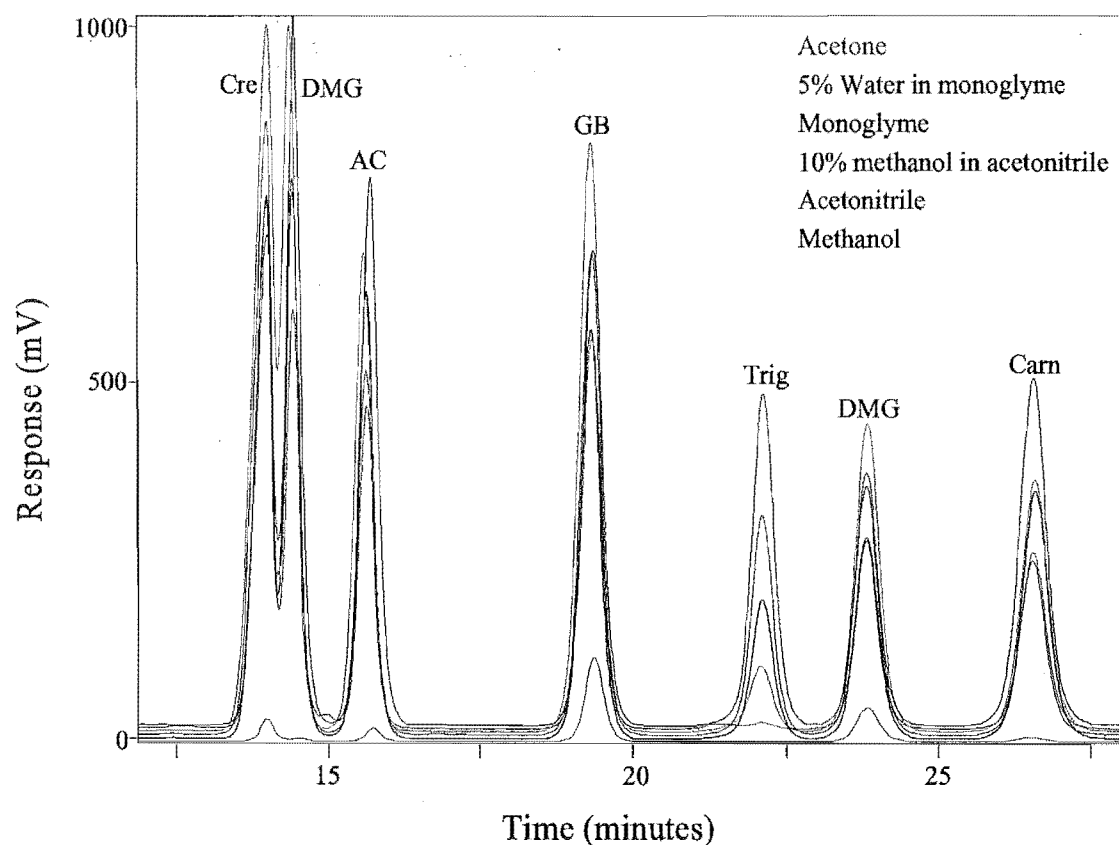
### **2.3.2.3. Order of reactants**

A series of derivatizations were carried out on a standard betaine solution and a water blank. The order of addition of base and derivatizing reagent was altered, and were analyzed using the Zorbax system. The chromatograms were compared (2.3.3.3). Full experimental details can be seen in the section 7.2.5.

### 2.3.3. Results

#### 2.3.3.1. Derivatization solvent

After centrifugation, the pellets in the samples derivatized in methanol and propan-2-ol showed some brown discolouration. The pellets of the other derivatizations were white. The peaks on the sample derivatized in acetone were consistently larger than the other peaks (figure 2.4). A derivatization solvent consisting of 5% H<sub>2</sub>O in monoglyme generally gave the next largest peaks. The peaks corresponding to trimethylamine and creatinine were smaller in the acetone derivatization, relative to some of the other solvents tested.

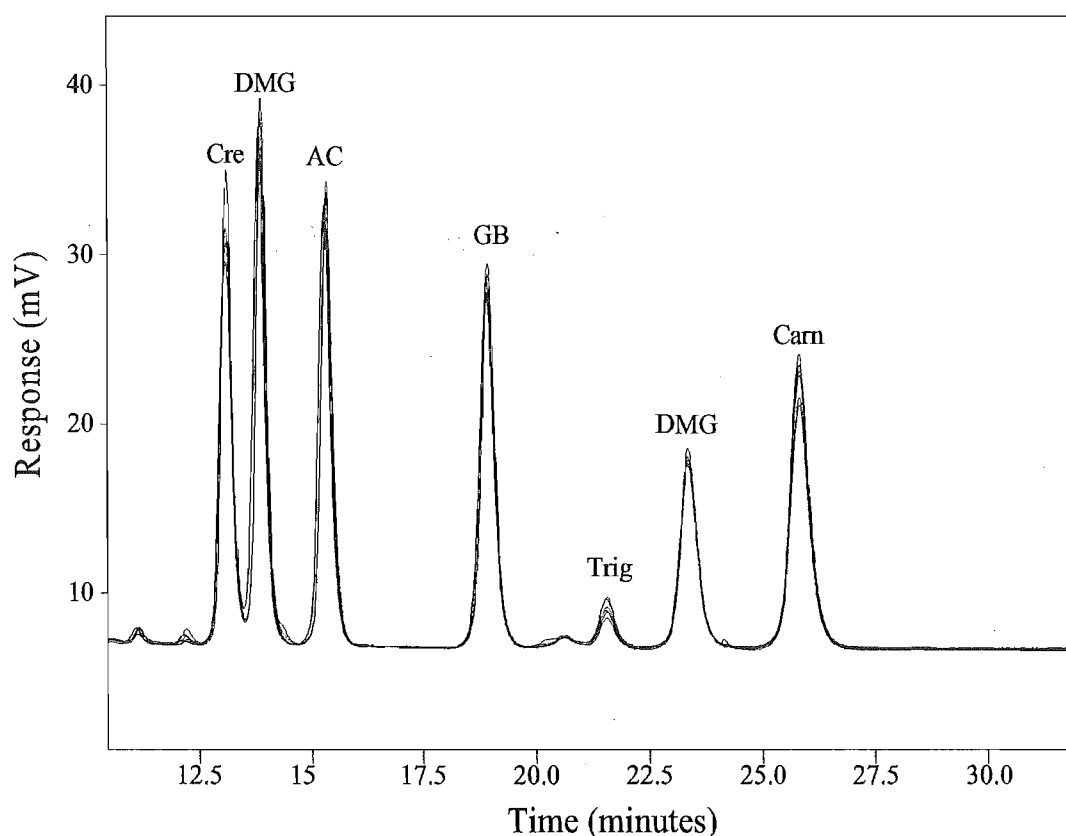


**Figure 2.4:** Overlay of chromatograms showing the effect of different derivatization solvents on betaine recovery. The chromatographic conditions used were the Zorbax conditions (section 2.5.3.1).

**AC:** acetyl carnitine. **Carn:** carnitine. **Cre:** creatinine. **DMG:** *N,N*-dimethylglycine. **GB:** glycine betaine. **Trig:** trigonelline.

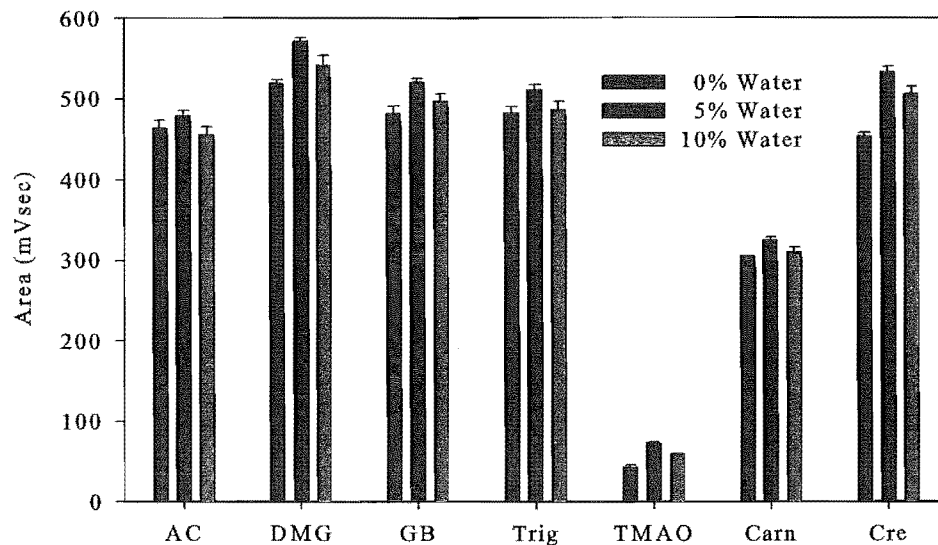
### 2.3.3.2. Effect of water

Figure 2.5 shows six HPLC traces of a standard derivatized in acetone with 0%, 5%, and 10% water (each in duplicate). The variation seen between the chromatograms is shown in figure 2.6. The variation in the betaine derivative peak areas was less than 5% between the different treatments. The peak area variation of trimethylamine oxide and creatinine was greater than 5%, but these two compounds are not betaines, and are not of interest in this study.



**Figure 2.5:** Overlay of 6 chromatograms. These were derivatized in acetone with 0, 5, and 10% H<sub>2</sub>O present (two of each). The chromatographic conditions used were the Zorbax conditions (section 2.5.3.1).

**AC:** acetyl carnitine. **Carn:** carnitine. **Cre:** creatinine. **DMG:** *N,N*-dimethylglycine. **GB:** glycine betaine. **Trig:** trigonelline.



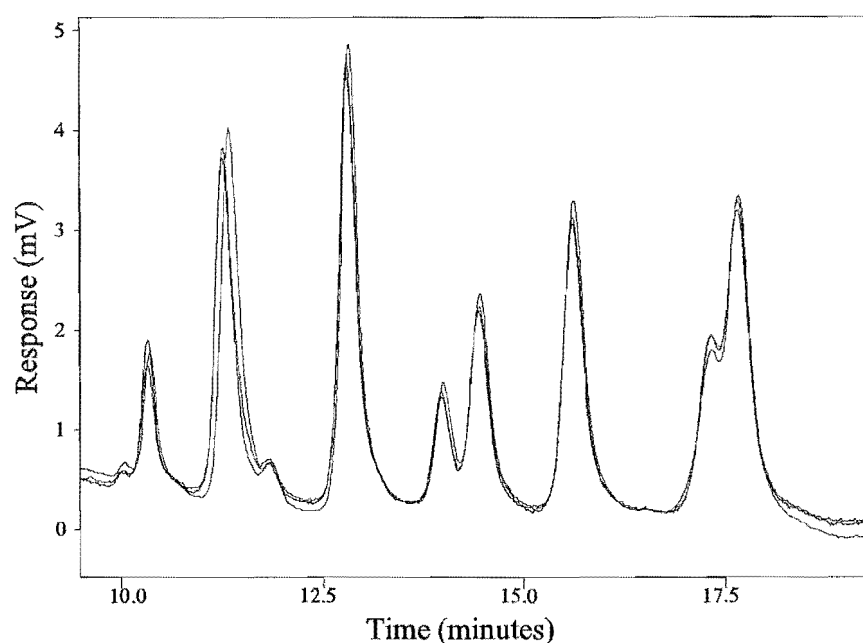
**Figure 2.6:** Graph showing the change in peak area due to water in the derivatization solution. The values graphed are the mean of two repeats. Error bars show the maximum value.

**AC:** acetyl carnitine. **Carn:** carnitine. **Cre:** creatinine. **DMG:** *N,N*-dimethylglycine. **GB:** glycine betaine. **TMAO:** trimethylamine oxide. **Trig:** trigonelline.

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### 2.3.3.3. Order of reactants

No major difference between the three treatments on the betaine standard solution was seen (figure 2.7). The chromatograms of the water blank showed only minor differences. The main difference, as shown in figure 2.8, was the size of the larger contamination peak seen when the base was added first.



**Figure 2.7:** Overlay of chromatograms showing no significant difference when the order of addition of derivatization reactants is altered. The chromatographic conditions used were the Zorbax conditions (section 2.5.3.1).

**AC:** acetyl carnitine. **Ars:** arsenobetaine. **But:** butyrobetaine.  
**DMG:** *N,N*-dimethylglycine. **GB:** glycine betaine.

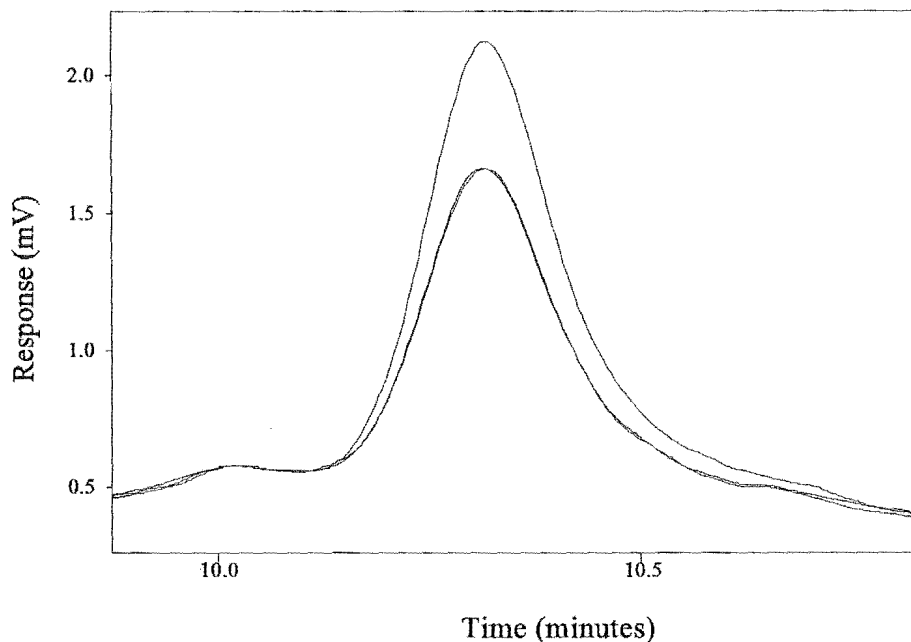
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## 2.3.4. Discussion

### 2.3.4.1. Derivatization solvent

The results shown in section 2.3.2. indicate that of the solvents tested, derivatization was most efficient in acetone. Acetone does not fulfill all of the criteria defined earlier (2.3.1.1), because the boiling point of acetone is 57°C. Ensuring that the microfuge tubes have lids on at all times except when required, means that acetone's volatility does not cause any problems. Monoglyme showed promise as a possible derivatization solvent, but its acute toxicity made acetone the more favourable choice.

The peaks corresponding to trimethylamine and creatinine were substantially smaller in the acetone derivatization than with the other derivatization solvents. These two compounds



**Figure 2.8:** Enlargement showing the largest contamination peak. The red trace was derivatized with the base added before the derivatizing reagent.

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aren't betaines, but are frequently seen in some biological samples and are derivatized.

The discolouration observed in the pellets of the methanol and isopropanol samples can be explained by a number of different possibilities. The triflate, or an impurity present in the triflate, may be insoluble in these solvents, or a side reaction forms insoluble products in these solvents. The later is consistent with Wittman and Ziegler (1988), who reported that the betaine ester is broken down in the presence of alcohols, forming an insoluble product. No further investigations into the nature of the discolouration of the pellet were carried out.

#### **2.3.4.2. Effect of water**

This experiment showed that up to 10% water had a small (< 5%) affect on peak area. The amount of water present in the derivatization mixture is approximately 3.2%, and any variation in the water content in the derivatization mixture would have an insignificant effect. For this reason, the drying step (shaking with anhydrous  $\text{Na}_2\text{SO}_4$  for an hour) in the derivatization procedure was discontinued, removing a liquid transfer and making the procedure simpler and quicker.

### **2.3.4.3. Order of reactants**

The only difference noted was a larger contamination peak observed in the blank when the base was added prior to the trifluoromethanesulfonate. It should be noted that the contamination peaks observed in these chromatograms were all insignificant, and all of the methods gave suitable results. Because no significant differences were observed, the simplest method and quickest method was chosen.

### **2.3.5. Discussion**

The resulting derivatization reaction is simpler than the original method, and the baseline is cleaner. The main disadvantage of the final method is that evaporation may occur, and needs to be minimized. The full method can be seen in section 2.5.2.

## **2.4. HPLC**

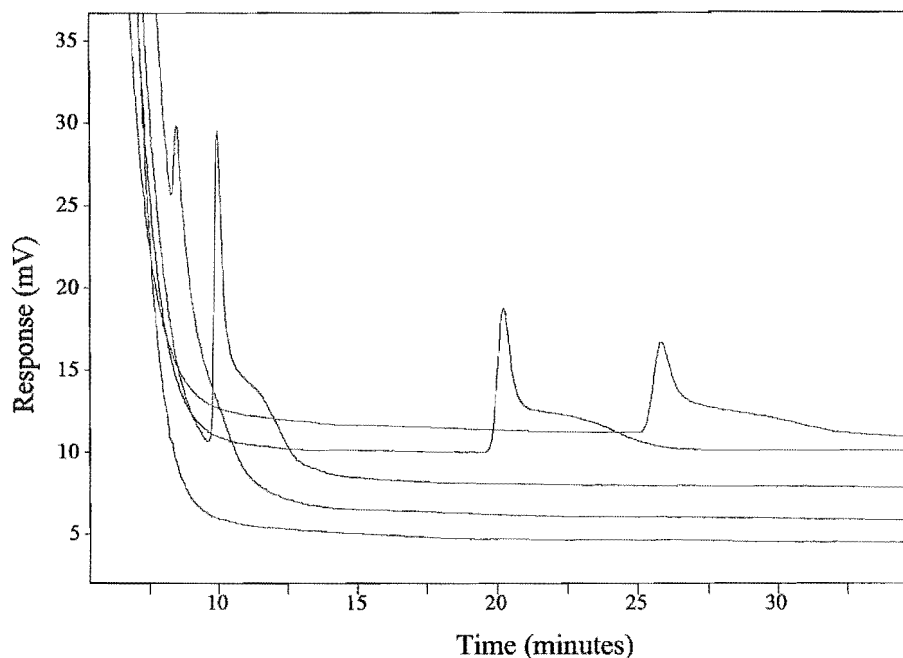
### **2.4.1. Columns**

#### **2.4.1.1. Zorbax**

A previous worker used this column (Zorbax 300 SCX, 250x4.6 mm) in an attempt to separate acetyl carnitine, carnitine, N,N-dimethylglycine, glycine betaine, proline betaine and trigonelline (ie, the betaines commonly seen in human samples). The existing HPLC system (Allwood, unpublished data, section 2.5.3.1) was tried with betaines of interest, and separation was excellent for them, so the system was used.

The pressure slowly increased, and this system required constant cleaning of the column and hardware. To counter this, the system was washed regularly (after every 40 hours of use) with propan-2-ol and dichloromethane. This complemented the rinsing (with 10% water in acetonitrile) that the column received after each run, and kept the column pressure within acceptable limits. Eventually the decrease in column efficiency (peak sharpness) and asymmetrical peaks (figure 2.9) became too great to achieve sufficient selectivity.





**Figure 2.9:** Overlay showing typical asymmetric peaks on the Zorbax column. The column was retired after this recording. Each trace is of a single betaine standard.

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#### 2.4.1.2. Alumina

The alumina system, which was optimized by other investigators in the laboratory, was not altered. This system takes 60 minutes per sample, so occupies the HPLC unit for long periods of time, and uses excessive volumes of solvent. This system is resilient, and has excellent resolution (system described in 2.5.3.3).

#### 2.4.1.3. Nucleosil

A Phenomenex Nucleosil 5  $\mu$ m SA 100A 250x4.0 mm column was obtained. A buffer consisting of tetramethyl ammonium hydroxide and glycolic acid in a ratio of 1:2 in a mixture of acetonitrile and water was suggested as a starting point.

#### 2.4.1.4. Phenosphere

A Phenomenex Phenosphere 5  $\mu\text{m}$  SCX 80A 250x4.8mm column was obtained. This column has some similarities to the Zorbax column, and given the success of the Zorbax system, it was used as base for development of the Phenosphere system.

### 2.4.2. Methods

#### 2.4.2.1. Nucleosil

The mobile phase initially tested was the same as the Zorbax system (table 2.2). No peaks were seen, so the buffer content was lowered (to 1/10th and then 1/20th of the initial concentration by diluting the mobile phase) in an effort to increase retention times.

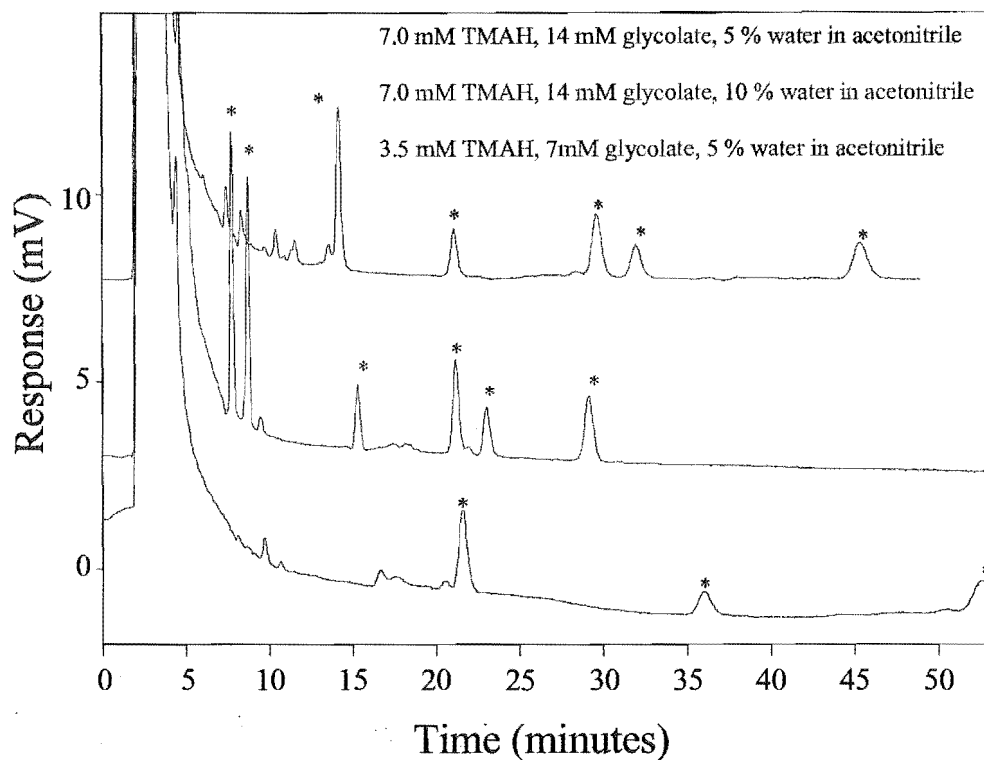
#### 2.4.2.2. Phenosphere

A standard was derivatized and separated using the same mobile phase as the Zorbax system (table 2.2) but with half the buffer concentration. The retention times were too long (figure 2.10). Increasing the buffer concentrations increases the competition for the stationary phase (ie, the column), which shortens the run time (Johnson and Stevenson 1978). The buffer

System	Column	Guard column	Solvent	Buffer	Run Time
Phenosphere	Phenosphere 5 $\mu\text{m}$ SCX 80A 250x4.8 mm serial #351541	SecurityGuard™ SCX 4x3.0 mm cartridge	10% H <sub>2</sub> O in ACN	7 mmol <sup>-1</sup> TMAH and 14 mmol <sup>-1</sup> glycolic acid	35 min
Zorbax	Zorbax 300 SCX, 250x4.6 mm, P.N no. 880962.704	SecurityGuard™ SCX 4x3.0 mm cartridge	5% H <sub>2</sub> O in ACN	7 mmol <sup>-1</sup> TMAH and 14 mmol <sup>-1</sup> glycolic acid	35 min
Alumina	Merck 5 $\mu\text{m}$ alumina 250x4 mm cartridge	Merck 5 $\mu\text{m}$ LiChroCART 4-4 aluspher AI 100	9% H <sub>2</sub> O in ACN	10 mmol <sup>-1</sup> succinic acid, and 3.7 mmol <sup>-1</sup> TEA	60 min

**Table 2.1:** Conditions of the different HPLC systems used. All chromatograms were recorded and analyzed using Delta Chromatographic Software (Version 5.0, Dataworx Pty Ltd).

**ACN:** acetonitrile. **TMAH:** tetramethyl ammonium hydroxide. **TEA:** triethyl ammonium



**Figure 2.10:** Overlay of chromatograms of standard betaine mix ( $100 \mu\text{mol l}^{-1}$  each of acetyl carnitine, carnitine, glycine betaine, *N,N*-dimethylglycine and trigonelline) using different mobile phases. The \* represent peaks corresponding to betaines.

concentration was increased, using the same mobile phase as the Zorbax system. The run time of this was longer than ideal, and the separation, especially of earlier betaines, was not good. Increasing the buffer further would make the early peaks even earlier and also increases the likelihood of salt precipitation, so instead the water content of the mobile phase was increased.

### 2.4.3. Results

HPLC columns are designed to work at an optimal flow rate. The columns used in this project have all been designed to work best at approximately 1 ml/min. Resolution is sometimes enhanced by lowering the flow rate. Chromatograms recorded at 0.5 ml/min and 1 ml/min were compared, and no difference in resolution was seen (data not shown).

#### **2.4.3.1. Nucleosil**

No peaks were seen after 60 minutes when the buffer used was the same as the Zorbax buffer. When the buffer was decreased to 1/10th and then 1/20th of the original concentration, peaks were still not seen. No more attempts to separate betaine derivatives with this column were made.

#### **2.4.3.2. Phenosphere**

Figure 2.10 shows a series of chromatograms of the same sample separated using different mobile phases. The first important peak (N,N-dimethylglycine) on the blue trace had a retention time of about 22 minutes, and the third peak (glycine betaine) to come out had a retention time of about 53 minutes. The remaining two betaines eluted at a later time, but the run time required for this mobile phase was deemed to be excessive and recording was stopped after 55 minutes. The buffer concentration was increased for the next chromatogram (red trace), giving retention times for the five betaine derivatives ranging between 15 and 45 minutes. A chromatogram of a different range of derivatized betaines showed that separation was achieved, but could be improved, especially for betaine derivatives with early retention times (data not shown). A run time of 45 minutes would be usable, but a shorter run time would be preferred. The water content of the mobile phase was increased for the green trace. This altered the selectivity, so that the betaines of interest had retention times between 15 and 30 minutes.

### **2.5. Conclusion**

The following sections describe extraction, derivatization and HPLC methods that are suitable for analyzing a range of food samples for betaine content. These methods were used for all analyses described in chapter 3.

#### **2.5.1. Extraction procedure**

##### **2.5.1.1. Solid foods**

Foods were uncooked, and all edible parts were put into a standard kitchen food processor (Moulinex Multimoulinette, Model T71). The food was processed so that no pieces were greater than 5 mm across. From this, a portion (0.5 - 1 g) was transferred to a pre-weighed test tube. This

was homogenized for at least one minute using an Ultra Turrax homogenizer. If a homogeneous paste could not be achieved, water (recorded volume) was added. This mixture was then shaken for a minimum of five minutes, and centrifuged (5 min at 4000 rpm). This yielded a liquid layer, which was removed and extracted with dichloromethane as described in section 2.5.1.5.

### **2.5.1.2. Fatty foods**

Some foods were unsuitable for the method described in section (2.5.1.1), due to their hydrophobicity (ie, butter and oils). Instead of the food being homogenized in aqueous solvent, the food was suspended in dichloromethane. This suspension was then extracted with an equal volume of water. After shaking for five minutes, the two solvents were separated by centrifugation, and the top (aqueous) layer was transferred and stored for assay.

### **2.5.1.3. Liquid foods**

Liquid foods were shaken before analysis with equal volumes of dichloromethane as described in section 2.5.1.5. After centrifugation, the top aqueous layer was removed and stored until analysis.

### **2.5.1.4. Other foods**

All foods were able to be processed using the above methods except for cheese. Knowing that cheese has a relatively low melting point, I decided to homogenize cheese in hot water. One part cheese and two parts water were combined in a test tube, and heated to c.70°C in a water bath, forming a homogeneous solution. This was allowed to cool, and then a dichloromethane extraction was carried out as described below. No thermal degradation of betaines occurs below temperatures of 200°C (Payne and de Zwart, unpublished data).

### **2.5.1.5. Dichloromethane extraction**

To remove any hydrophobic compounds present in a sample, a dichloromethane extraction was carried out. An aliquot of the aqueous extract was transferred to a microfuge tube, and an equal amount (v/v) of dichloromethane was added. This was shaken for five minutes before centrifugation. After centrifugation, the top (aqueous) layer was transferred to a fresh microfuge tube and stored for analysis.

## 2.5.2. Derivatization procedure

A 5  $\mu\text{l}$  aliquot of sample was added to 250  $\mu\text{l}$  of acetone. This was vortexed immediately to break up any denatured proteins. Derivatizing reagent (50  $\mu\text{l}$ , 100  $\text{mmol l}^{-1}$  naphthacyl trifluoromethanesulfonate in acetone) was added, followed by addition of 5  $\mu\text{l}$  of  $\text{Mg}(\text{OH})_2$  solution (0.1 g/ml MgO suspended in distilled water) and the solution was shaken for five minutes. This mixture was then centrifuged (5 min, 11000 rpm) and the supernatant was transferred to an HPLC vial for analysis. Vials were capped immediately to stop concentration by evaporation.

## 2.5.3. HPLC

Three different chromatographic conditions were used in this thesis, as described below.

### 2.5.3.1. Zorbax

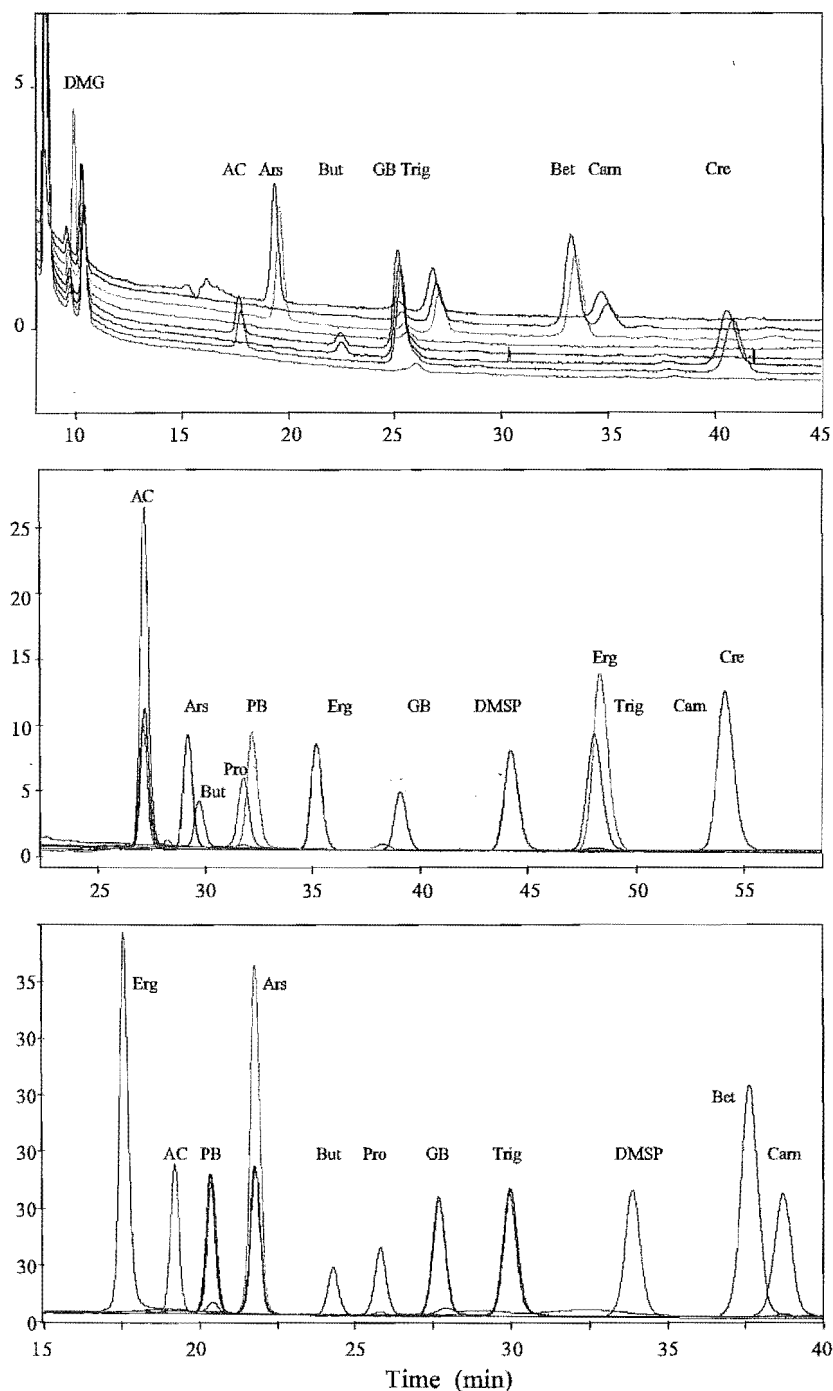
The conditions of this system are described in table 2.2. A chromatogram showing the separation of a range of naturally occurring betaines can be seen in figure 2.11 (A).

### 2.5.3.2. Alumina

The conditions of this system are described in table 2.2. The alumina system that was optimized by Dellow (Dellow et al. 1999) to separate glycine betaine from acetyl carnitine, carnitine, trigonelline and proline betaine. A chromatogram showing the separation of a range of naturally occurring betaines can be seen in figure 2.11 (B).

### 2.5.3.3. Phenosphere

The conditions of this system are described in table 2.2. A chromatogram showing the separation of a range of naturally occurring betaines can be seen in figure 2.11 (C).



**Figure 2.11:** Overlays showing the separation achieved by the different chromatographic systems. Each colour represents a different standard. **A:** Zorbax system. **B:** Alumina system. **C:** Phenosphere system.

**AC:** acetyl carnitine. **Ars:** arsenobetaine. **Bet:** betonicine. **But:** butyrobetaine. **Carn:** carnitine. **Cre:** creatinine. **DMSP:** dimethylsulfonopropionate. **DMG:** *N,N*-dimethylglycine. **Erg:** ergothionine. **GB:** glycine betaine. **PB:** proline betaine. **Pro:** propionobetaine. **Trig:** trigonelline.

## Chapter 3

### Dietary Betaines

*"Tell me what you eat and I will tell you what you are."*

Anthelme Brillat-Savarin

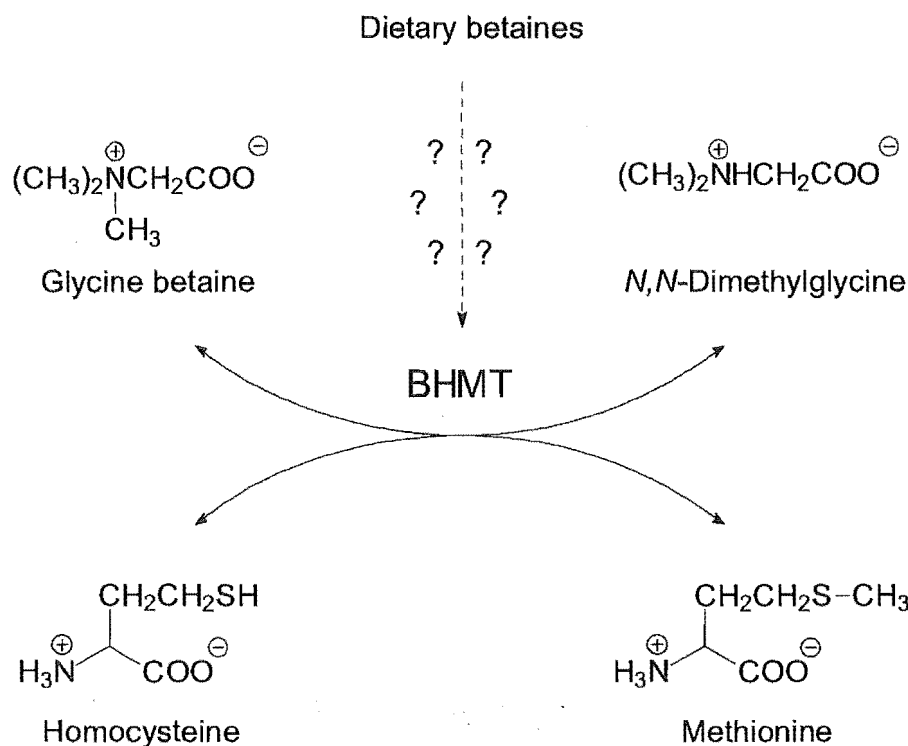


### 3.1. Introduction

A number of betaines are known to be present in the diet, and investigations suggest that they may have important physiological effects. Glycine betaine has been used as a therapeutic agent to treat homocystinuria and regulate mild hyperhomocysteinemia, and has been used to treat other disorders (see sections 1.3, 1.4). Some studies have documented no effect, whereas others have shown positive effects. No studies documenting negative effects on the health of patients have been published. For more information on this topic, see section 1. The enzyme betaine-homocysteine methyl transferase (BHMT) metabolizes glycine betaine in mammals, and studies on purified porcine BHMT have revealed that proline betaine, which is present in citrus and legumes, is a substrate (albeit a poor one) (Mulligan et al. 1998), but proline betaine may act as a competitive inhibitor under *in vivo* conditions. DMSP (dimethylsulfonopropionate) is a betaine that is present in the sea and is accumulated by filter-feeders (Ishiba 1995). Purified BHMT studies show that DMSP is approximately seven times more effective as a substrate than the natural substrate, glycine betaine (reviewed in Garrow 1996). These two examples show that dietary betaines may interact with BHMT, and thus have an effect on plasma homocysteine concentrations (figure 3.1), which may, in turn, affect the risk of cardiovascular disease (see section 1.3). There is an association between high coffee consumption (>9 cups/day) and plasma homocysteine concentrations (Nygård 1997), but no mechanism for this has been published. One possibility is that a betaine in coffee interacts with BHMT, resulting in higher homocysteine concentrations. Trigonelline is a betaine that is found in coffee (Wu 1997; Stennert 1994; Martin 1997) and it may be responsible for the association between coffee and homocysteine concentrations. This topic is discussed and tested in chapter 5. Trigonelline reduces the growth rate of *E. coli* (*in vivo*) in some conditions, which may make it antibacterial in urine. The testing of this forms the basis of section 5.

The betaines trigonelline, arsenobetaine and proline betaine can be detected in human urine (Chambers and Kunin 1985; Lever et al. 1994a), and their presence is dependent on the diet of the individual. This shows that dietary betaines are absorbed in to the blood stream, so may interact with BHMT *in vitro*.

Since dietary betaines may be of medical importance, it is important to assess the betaine content consumed in the typical diet. At the start of this project, no data was available to estimate the levels of betaines in the diet, and this chapter outlines the steps taken in the formation



**Figure 3.1:** The potential interaction of dietary betaines with the reaction catalysed by BHMT.

of a database of betaine content of foods commonly found in the average New Zealand diet. These are summarized in tables 3.3 - 3.5. Foods that have high betaine contents were also analysed for variation (table 3.6, figure 3.2). The data were used to approximate the average daily intake, and a diet high in various betaines has been designed.

### 3.1.1. Strategy

The aim of any food survey is to discover the content in a range of different foods. Ideally, this would yield the average content and variation between samples, but to do this with absolute certainty, the content of each sample needs to be measured. As this was not possible, a sampling strategy was required.

The initial strategy was to survey a wide range of common foods and food groups to establish which foods contain significant amounts of known betaines. This survey would also

establish if any unknown betaines present in the diet. A detailed analysis followed, to establish the variation of betaines in foods with high betaine levels.

## 3.2. Methods

### 3.2.1. Assay

Aqueous extracts were prepared as described in section 2.5.1. The prepared samples were then derivatized as described in section 2.5.2. The derivatized samples were separated and betaines quantified using the Zorbax, alumina and Phenosphere chromatographic systems as described in section 2.5.3.

Fruit/vegetables	Dairy	Meat/fish	Miscellaneous
Alfalfa sprouts	Butter	Beef	Canola oil
Bean	Cottage Cheese	Chicken	Chocolate
Beetroot	Cream	Clams	Coffee
Cabbage	Cream Cheese	Cod	Egg
Carrot	Milk	Lamb	Flour
Celery	Sour Cream	Monkfish	Honey
Chick peas	Yoghurt	Mussel	Milo
Corn		Pork	Olive oil
Courgette			Olivio
Cranberry			Pasta
Cucumber			Peanuts
Feijoa			Rice
Garlic			Rolled oats
Grape			Sugar
Grapefruit			Tea
Lemon			
Lentils			
Mango			
Nectarine			
Orange			
Passionfruit			
Pea			
Pear			
Pumpkin			
Silverbeet			
Snow pea shoots			
Spinach			
Swede			
Tamarillo			
Tomato			

**Table 3.1:** Foods that chromatograms from two systems gave consistent results...

Fruit/vegetables	Dairy	Meat/fish	Miscellaneous
Apple Avocado Banana Broccoli Brussel Sprouts Cauliflower Kiwifruit Kumara Leak Lettuce Mushroom Onion Parsnip Pepper Plum Potato Yam	Cheese	Groper Gurnard Perch Salmon Terakihi Tuna	Marmite Wine (red) Soy sauce Vegemite

**Table 3.2:** Foods which chromatograms from two systems gave differing results.

### 3.2.2. Foods to be assayed

A list of foods was assembled in conjunction with Crop and Food Research Ltd., where the New Zealand Food Composition Database is housed. The list of foods surveyed can be seen in tables 3.1 and 3.2.

### 3.2.3. Food Sampling

The initial aim was to quantify the betaine levels in all food types, and then select foods that have significant levels and study these further to obtain data on the natural variation. A sampling procedure (below) was drawn up in conjunction with Crop and Food in Palmerston North.

Wherever possible, at least two different sources of each type of food were sampled. In the cases where only one brand was available, two items from the same source were sampled. In the case of dried foods (eg, flour, sugar, pasta, etc.), approximately a cup from different sources was sampled. In the case of fruits, at least two individual pieces from different sources were sampled. Sampling of only two individuals was considered appropriate for large items (eg, potato, pumpkin), but inappropriate for small fruits (eg, grapes) because of the representation a small volume gives. For these, two cups (or equivalent) from different sources were sampled. For meats, it can be assumed that meat from one source may come from the same animal, so meats were obtained from different sources, eg, different butchers or supermarkets.

### 3.2.4. Analysis of chromatograms

Each sample was analyzed using at least two chromatographic systems (section 2.5.3). A series of standards containing glycine betaine, trigonelline, proline betaine and arsenobetaine were included in each HPLC run. If the results from the two chromatographic systems were consistent with each other (within 15 %) then values obtained from the different systems were accepted and the mean concentration was reported. If the two chromatographic systems gave inconsistent results, the analysis was repeated using the initial two systems and a third set of conditions.

After repeated measurements, the values from the chromatographic system giving the lowest concentration was used. Two different compounds may co-elute on some chromatographic systems, giving the impression of a higher concentration than is present. If this occurs, it is common to see a distorted peak, but this is not always the case. Using three different chromatographic systems allowed this to be accurately detected. The foods listed in table 3.2 had differing values of betaines on different chromatographic systems. Each of these are discussed in the section 7.3.1.

The UV detector required some maintenance during the food analyses. During the month that it was unavailable, a lower sensitivity UV detector was used. Using this detector, the assay had a detection limit of approximately  $25 \mu\text{mol l}^{-1}$ , whereas the usual detector gave the assay a detection limit of approximately  $1 \mu\text{mol l}^{-1}$ . This detector was able to detect samples with high levels of betaines, but was unable to detect the lower levels found in many foods. The concentration of betaine in food that the limit of sensitivity corresponds to is dependent on the dilutions made, but generally if less than  $1 \mu\text{mol l}^{-1}$  was detected, then less than  $1 \mu\text{g/g}$  was present in the food. If no betaines were detected, the concentration of betaines in that food has been reported as less than  $1 \mu\text{g/g}$ . Any sample which has less than  $10 \mu\text{g/g}$  betaine present has been reported as either less than  $10 \mu\text{g/g}$  or less than  $5 \mu\text{g/g}$ .

### 3.2.5. Calculations

The following equation was used to calculate the betaine content of food:

$$X = \frac{C \times P}{A} \times \frac{V \times RMM}{M}$$

**Equation 3.1:** Equation used to calculate betaine concentration that has been reported. **X:** Reported betaine content in food ( $\mu\text{g/g}$ ). **C:** HPLC standard concentration ( $\mu\text{mol l}^{-1}$ ). **P:** Sample peak area (mV.sec). **A:** Standard peak area (mV.sec). **V:** Volume of water added (l). **RMM:** Relative Molecular Mass ( $\text{g mol}^{-1}$ ). **M:** Mass of food sampled (g).

To obtain a value for the concentration of betaine in food, a number of assumptions have been made. The effect of these assumptions is the value reported will be lower than the actual betaine content. It has been assumed that all of the water present in the homogenated sample originates from the addition of water. This assumption can be justified by the assumption that any betaine present will be distributed evenly throughout the sample; ie, it will be present in the pellet as well as in the supernatant.

For liquid foods, 1 ml was sampled, and the following equation was used:

$$X = \frac{C \times P}{A} \times RMM$$

**Equation 3.2:** Equation used to calculate betaine concentration that has been reported. **X:** Reported betaine content in food ( $\mu\text{g/ml}$ ). **C:** HPLC standard concentration ( $\mu\text{mol l}^{-1}$ ). **P:** Sample peak area (mV.sec). **A:** Standard peak area (mV.sec). **RMM:** Relative Molecular Mass ( $\text{g mol}^{-1}$ ).

### 3.2.6. Variation Studies

Foods that were identified as being high (greater than 200  $\mu\text{g/g}$ ) in betaines (listed in table 3.2) were further analysed. The same procedures as described above (3.2.1 - 3.2.6) were used. A

number of samples ( $2 < n < 15$ ) from different sources were assayed using the chromatographic system that gave the cleanest chromatography during the initial food survey.

### 3.3. Results

#### 3.3.1. Betaine levels in food

Table 3.2. shows the list of foods that gave consistent results between different chromatographic systems. Table 3.3 shows the remainder of the foods that gave inconsistent results between the different systems. The values reported for the foods in table 3.3 are discussed in the section 7.3.1. The betaine content in foods is summarized in tables 3.4 - 3.6.

	Proline betaine	Glycine betaine	Trigonelline
Beef	<1	58	<1
Chicken	<1	200	<5
<b>Clams</b>	15	<b>2500</b>	<10
Cod	<5	25	<1
Groper	<1	12	<1
Gumard	<1	<10	<1
Lamb	<1	72	<1
<b>Monkfish</b>	40	<b>500</b>	10
<b>Mussel</b>	26	<b>1630</b>	83
Perch	<1	26	<1
Pork	<1	41	<1
Salmon	<1	19	<1
Terakihi	<1	<5	<1
Tuna	<1	<10	<1

**Table 3.3:** Betaine concentrations in meats and seafood. All values are in  $\mu\text{g/g}$ . The foods that are in **bold** are those which have a high content of a specific betaine, and variation studies on these foods were carried out. Values that are displayed in blue are discussed in the section 7.3.1.

---

	Proline betaine	Glycine betaine	Trigonelline
<b>Alfalfa sprouts</b>	<b>240</b>	35	181
Apple	<1	<5	<5
Avocado	<1	<10	<1
Banana	<1	<5	23
Bean	<1	30	58
<b>Beetroot</b>	<5	<b>750</b>	<5
Broccoli	<1	<10	<1
Brussel Sprouts	34	<5	31
Cabbage	<1	<1	<1
Carrot	<1	<1	<1
Cauliflower	<1	<10	<1
Celery	<1	<1	<1
<b>Chick peas</b>	<1	<1	<b>350</b>
Corn	<1	<5	<5
Cougette	<1	<1	15
Cranberry	<1	<1	<1
Cucumber	<1	<1	<5
Feijoa	<1	<1	<1
Garlic	<1	<10	<10
Grape	<1	<1	<1
<b>Grapefruit</b>	190	<1	<1
Kiwifruit	<1	<5	<1
Kumara	<5	110	<1
Leak	<1	<1	<1
Lemon	120	<1	<1
<b>Lentils</b>	<1	<10	<b>250</b>
Lettuce	<1	<10	<5
Mango	<1	<1	<1
Mushroom	15	<10	<5
Nectarine	<1	<1	<1
Onion	<5	<5	<10
<b>Orange</b>	<b>510</b>	<1	18
Parsnip	<5	<5	<1
Passionfruit	<1	<1	<1
<b>Pea</b>	<1	<5	<b>280</b>
Pear	<5	<10	<1
Pepper	<1	<10	<1
Plum	<1	<5	<1
Potato	<1	<10	<1
Pumpkin	<1	<1	30
<b>Silverbeet</b>	50	<b>910</b>	<5
<b>Snow pea shoots</b>	<1	<10	160
<b>Spinach</b>	<1	<b>740</b>	100
Swede	<5	<5	<1
Tamarillo	<1	<1	<1
Tomato	<1	<1	<1
Yam	<1	<5	<10

**Table 3.4:** Betaine concentrations in fruits and vegetables. All values are in  $\mu\text{g/g}$ . The foods that are in **bold** are those which have a high content of a specific betaine, and variation studies on these foods were carried out. Values that are displayed in blue are discussed in the section 7.3.1.



	Proline betaine	Glycine betaine	Trigonelline
Butter	<1	<1	<1
Canola oil	<1	<1	<1
Cheese	<1	<1	<10
Chocolate	<1	<5	<5
<b>Coffee</b>	<1	<1	<b>2200</b>
Cottage Cheese	<1	<5	<1
Cream	<1	<5	<1
Cream Cheese	<1	<10	<1
Egg	<1	<10	<1
<b>Flour</b>	<1	<b>730</b>	<1
Honey	<1	<1	<1
Marmite	<1	<10	<1
Milk	<1	<10	<1
Milo	<1	34	<1
Olive oil	<1	<1	<1
Olivio	<1	<5	<1
<b>Pasta</b>	<1	<b>820</b>	<1
Peanuts	<1	<5	<1
Red Wine	<1	<5	14
Rice	<1	<1	<1
<b>Rolled oats</b>	<1	130	<b>230</b>
Sour Cream	<1	<5	<1
Soy sauce	<5	<1	<1
Sugar	<1	<1	<1
Tea	<10	24	16
Vegetemite	<1	<5	<1
Yoghurt	<1	<5	<1

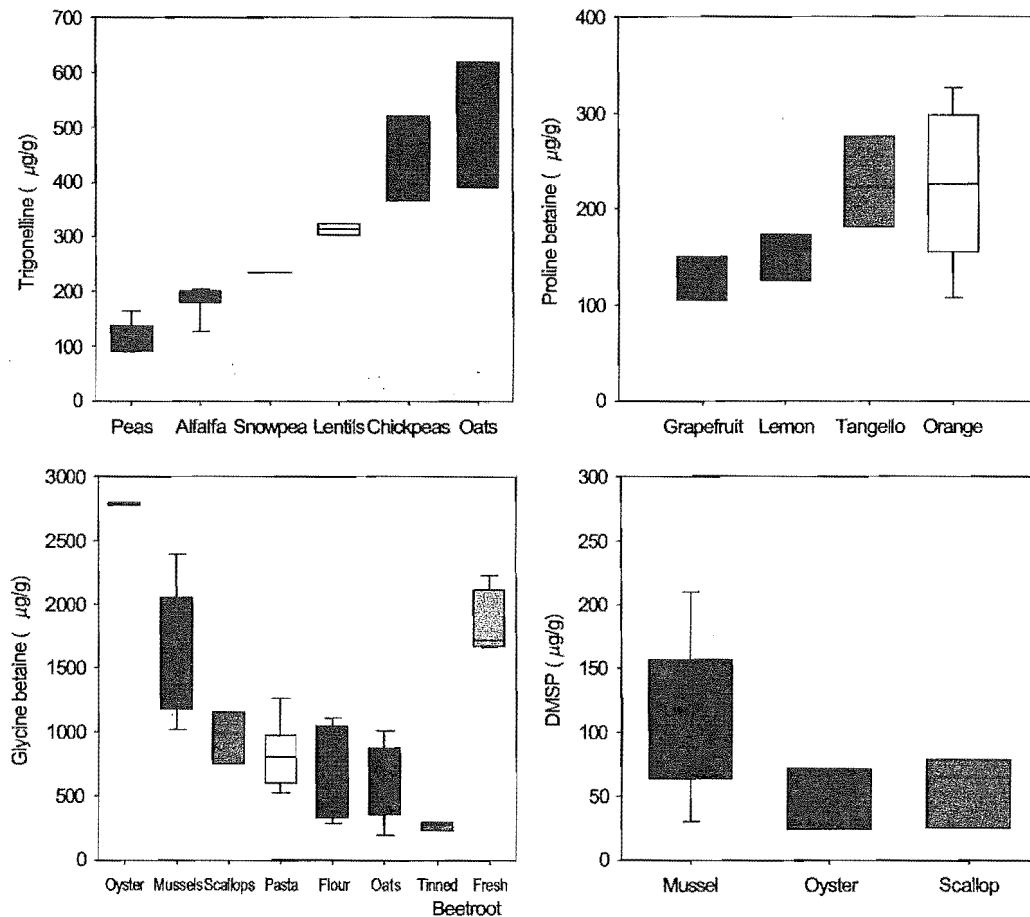
**Table 3.5:** Betaine concentrations in miscellaneous foods. All values are in  $\mu\text{g/g}$  for solid foods and  $\mu\text{g/ml}$  for liquids. The foods that are in **bold** are those which have a high content of a specific betaine, and variation studies on these foods were carried out. Values that are displayed in blue are discussed in the section 7.3.1.

Glycine betaine	Trigonelline	Proline Betaine
Shellfish	Sprouts	Citrus fruit
Pasta	Pea	Sprouts
Flour	Oats	
Oats	Lentils	
Sprouts		
Beetroot		

**Table 3.6:** Foods that have been analysed for variation.

### 3.3.2. Variation studies

The foods that were analysed for variation of betaine content are listed in table 3.7. The variation can found can be seen in figure 3.2.



**Figure 3.2:** Graphs showing the variation of different betaines (glycine betaine, trigonelline, proline betaine and dimethylsulfonopropionate (DMSP) in food. Between 2 and 15 samples of each food type were analyzed. Boxes represent the interquartile range, error bars represent the 95<sup>th</sup> percentile.

### 3.4. Summary

Glycine betaine can be found in animal products, especially shellfish, and in some plants, specifically members of the beet family (eg. beetroot, spinach) and grains. Trigonelline is found in legumes, and in high levels in coffee. Proline betaine can be found in legumes and citrus fruit. DMSP was only detected in shellfish with levels ranged from 20 to 200  $\mu\text{g/g}$  (figure 3.2). It should be noted that foods in which DMSP was detected also had high (c. 10 fold higher) levels of glycine betaine.

A small amount of arsenobetaine was detected in the gurnard extract in all chromatographic systems, corresponding to between 2 and 10  $\mu\text{g/g}$ . This has been reported as less than 10  $\mu\text{g/g}$ . A similarly small level was detected in perch (5.6 and 10.1  $\mu\text{mol}^{-1}$ , 2.4 and 4.3  $\mu\text{g/g}$ ). No other food showed detectable levels of arsenobetaine.

### 3.5. Conclusion

Quantitatively, the only betaines that are present in the diet at a reasonable level are acetyl carnitine, carnitine, glycine betaine, proline betaine and trigonelline. Acetyl carnitine and carnitine have not been included in this study, because exhaustive data on the content of these betaines in the diet and the physiological effects exists (reviewed in Rebouche 1992). The levels of the remaining betaines in the diet is almost solely attributable to a small number of foods. Investigations into the losses of betaines during cooking showed that betaines are heat stable up to 220 °C. No significant losses of betaines were seen during baking, microwaving or frying, and small losses were seen during steaming. Large losses (60-80%) occurred during boiling, but this was recoverable from the water (Payne and de Zwart, unpublished data).

These data and data from the New Zealand Total Diet Survey by ESR (Cressey 2001) in Christchurch were used to estimate the levels of glycine betaine in a normal diet at 150-200 mg/day. According to published data, the level of glycine betaine intake could potentially be increased by up to 500 mg/day due to the conversion of choline to glycine betaine by gut bacteria and the cells in the gut wall (Zeisel 1994). The average daily intake of trigonelline and proline betaine is 20 and 15 mg/day respectively. It should be reiterated that these values will be variable because of the variation of diets and the variation present in foods.

A diet was designed specifically to maximise the amount of glycine betaine and proline betaine ingested (Price 2001). This gave a daily intake of 620 mg glycine betaine and 140 mg proline betaine. These levels are assumed to be the approximate limit of intake of these betaines through the diet without specific supplementation. The diet that gave these figures is in the appendix 7.3.2.

### 3.5.1. Comparisons with reported data

Trigonelline levels in green coffee has been measured numerous times, and the levels of the two most common varieties are in the range of 6000 to 13000  $\mu\text{g/g}$  (*Coffea arabica*) and 3000 to 11000  $\mu\text{g/g}$  (*Coffea robusta*) (reviewed by Stennert, 1994). Stennert and Maier (1994) examined a range of roasted coffee beans, and reported an average of 6200  $\mu\text{g}$  (range 4300 to 7100  $\mu\text{g}$ ) trigonelline per gram of dry matter. Other reports consistently give the trigonelline level at approximately 1% (10000  $\mu\text{g/g}$ ) (Wu, 1997). The value reported in this study (2000  $\mu\text{g/g}$ ) is lower than these values. The value here does not take into account the water content of roasted coffee beans, which may account for the lower value. The extraction method used for this study was infusion, using a standard household coffee plunger. This may not extract all of the trigonelline in coffee beans, but it more closely resembles what is consumed.

A study of 15 different types of wine was reported by Mar and Zeisel (1999), showing that the glycine betaine content ranged from 2.5  $\mu\text{g/ml}$  to 24.7  $\mu\text{g/ml}$ . The average content was 11  $\mu\text{g/ml}$  (red) and 10  $\mu\text{g/ml}$  (white). The present investigation tested only one wine, and the glycine betaine content found was within the range reported. The glycine betaine content is low compared to other dietary components, so is assumed to be insignificant.

## 3.6. Discussion

The main source of glycine betaine in the New Zealand diet is from flour products (eg, bread, pasta), but shellfish are a very good source (Cressey 2001). Shellfish isn't consumed as much or as often, which means that the glycine betaine intake can be substantially increased by their consumption. A similar case exists with proline betaine. Most proline betaine in the diet originates from citrus fruit, and in most cases it would be simple to increase the consumption of citrus fruit, thus increasing proline betaine intake.

The variation in betaine content is high, often over 50%. If the biological functions of betaines is taken into account, this finding is not surprising. As an osmoprotectant, glycine betaine levels would be expected to be dependent on the stress levels of the organism. For example, an oat grown in a drought would be expected to have a higher level of glycine betaine than one grown in a well irrigated field. DMSP is also an osmoprotectant, and the levels present may depend on the processing (Payne and de Zwart, unpublished data). Also, since trigonelline acts as a signal for rhizobium, the levels would be expected to alter through the life of the legume.

## Chapter 4

### Coffee and Homocysteine

*"Noir comme le diable, chaud comme l'enfer, pur comme un ange, doux comme l'amour ."*

*("Black as the devil, hot as hell, pure as an angel, sweet as love.")*

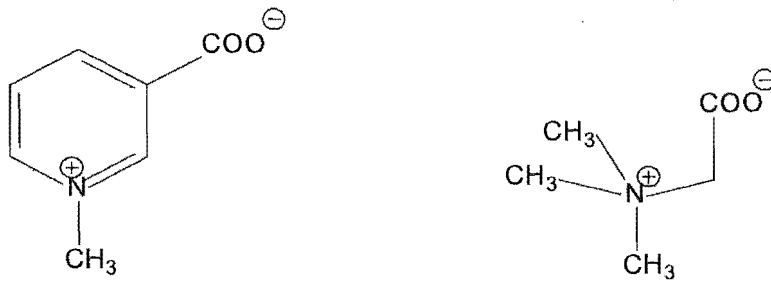
Charles Maurice de Talleyrand-Périgord  
describing his concept of a good cup of coffee

## 4.1. Introduction

Coffee is one of the most popular drinks in the world, with over 400 billion cups of coffee consumed annually. Throughout its history, coffee has been the subject of controversy. Pope Clemente VII was urged by members of the Catholic Church to forbid the consumption of the "devil's beverage," and instead the Pope blessed it (Pettigrew, 1998; Kolpas, 1979). Khair Beg, the corrupt governor of Mecca, tried to ban coffee in 1511, for fear that its influence might foster opposition to his rule. On hearing of this, the Sultan sent word that coffee is sacred and revoked the decision, initiating a chain of events leading to Khair Beg's execution (Kolpas, 1979). In 1675, King Charles II tried to suppress coffeehouses, supposedly because men were neglecting their families to discuss business and politics over coffee. Public outcry caused him to revoke his proclamation (Trager, 1995). Another public outcry in 1775 also caused Frederick the Great to change his mind after he tried to block coffee imports (Kolpas, 1979). Now, however, the controversy surrounding coffee is whether it is good for you. Many would claim that their lives would be much emptier with out their morning cuppa, but there is evidence that coffee consumption has adverse health effects.

Some studies have shown an association between coffee consumption and the risk of ischemic heart disease (Wilhelmsen et al. 2001), but other studies do not (Kleemola et al. 2000; Woodward and Tunstall-Pedoe 1999). There is a significantly higher plasma homocysteine concentration (18.4% in males and 28.9% in females,  $P < 0.001$ ) associated with high coffee consumption (9 cups/day) (Nygård et al. 1997). This remains after adjusting for other factors known to affect plasma homocysteine concentration (age, smoking, diet, activity level), and corresponds to an increase in homocysteine concentrations of 1.9 (male) and 2.3 (female)  $\mu\text{mol}^{-1}$ . No attempt to explain this difference in homocysteine levels was made. Similar observations have been reported by Urgert et al. (1996) and Olthof et al. (2001). It has been estimated that 5  $\mu\text{mol}^{-1}$  increment in plasma homocysteine corresponds to a 50 % increased risk of arteriosclerotic vascular disease (Boushey et al. 1995).

Trigonelline is found in coffee at concentrations of up to 1% by dry weight (Stennert and Maier 1994), and this is the main source of trigonelline in most diets (chapter 3). Trigonelline is structurally related to glycine betaine (figure 4.1), and it was hypothesized that the trigonelline present in coffee interacts with betaine-homocysteine methyl transferase (BHMT) causing an increase in the level of homocysteine in the blood plasma. This hypothesis was tested by



**Figure 4.1:** Structures of trigonelline (left) and glycine betaine (right).

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challenging subjects with a coffee load. Over the course of a day, plasma was collected, and glycine betaine and homocysteine were measured.

## 4.2. Methods

### 4.2.1. Ethical Approval

As any study involving humans requires ethical approval, an application for approval to perform the study was made. The initial protocol was designed with assistance from a member of the local ethics committee. An information sheet, giving the reasons and details of the study and a consent form were created and submitted to the Canterbury Ethics Committee (protocol, information sheet and consent form). After minor recommended changes were made, permission to carry out the study was granted. The consent form and information sheet are in section 7.4.

### 4.2.2. Volunteers

Eight males and two females aged 20-30, non-diabetic, non-smokers and in good health, were recruited. Each subject was asked to abstain from coffee for the three days prior to the trial. No other dietary restrictions were made. One volunteer was withdrawn from the trial due to difficulties with insertion of a functional indwelling catheter for blood sampling.



### 4.2.3. Coffee

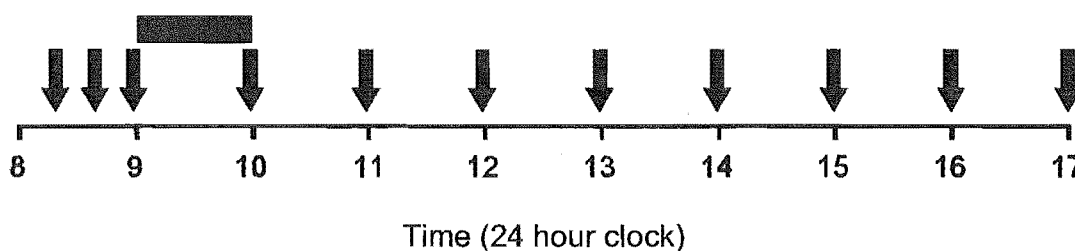
Coffee was produced by brewing 37 g of coarse ground coffee (New York Style roast, Garden City Coffee, Christchurch, New Zealand) in a French Press (plunger) with 1000 ml near-boiling water for 4 minutes.

### 4.2.4. Trial

Participants were given a total of 1000 ml of coffee to be consumed between 9:00 am and 10:00 am. Throughout the trial period, participants had free access to water. Lunch and refreshments (previously tested to confirm it was low in betaines) were supplied. A summary of the trial can be seen in figure 4.2.

### 4.2.5. Sample collection

Baseline blood samples were drawn at 8:20 am, 8:40 am and 9:00 am on the day of the trial. Blood samples were drawn hourly for the next eight hours. Blood samples were collected in heparinized tubes, and put on ice immediately. Samples were separated (centrifuged for 10 min at 6000 rpm) within five minutes of collection, and the plasma was frozen until analysis. This blood collection method is required for accurate homocysteine measurements (IMx, 2000).



**Figure 4.2:** Timing of the trial. Blue arrows represent points at which blood samples were drawn. The red box represents the time when the coffee was consumed. At least one urine was collected prior to coffee ingestion for each participant, and all urine produced during the day (no set times) was collected.

The participants were asked to empty their bladders before the coffee was consumed. Subsequent urine produced during the day, as well as the baseline urine, were collected. All urine volumes were measured, and an aliquot was frozen for analysis.

#### **4.2.6. Biochemical Analysis**

Plasma was analysed for N,N-dimethylglycine, glycine betaine, trigonelline and homocysteine. Urines were analysed for creatinine, glycine betaine, trigonelline, urea and osmolality. N,N-Dimethylglycine, glycine betaine and trigonelline concentrations were assayed using the alumina as described in section 2.5. Creatinine concentrations were analysed using the Jaffe Method (Murray, 1984), and osmolalities were measured by freezing point depression. Homocysteine concentrations were assayed by Canterbury Health Laboratories (IMx, 2000). Urea concentrations were measured by Canterbury Health Laboratories (Aeroset, 1998).

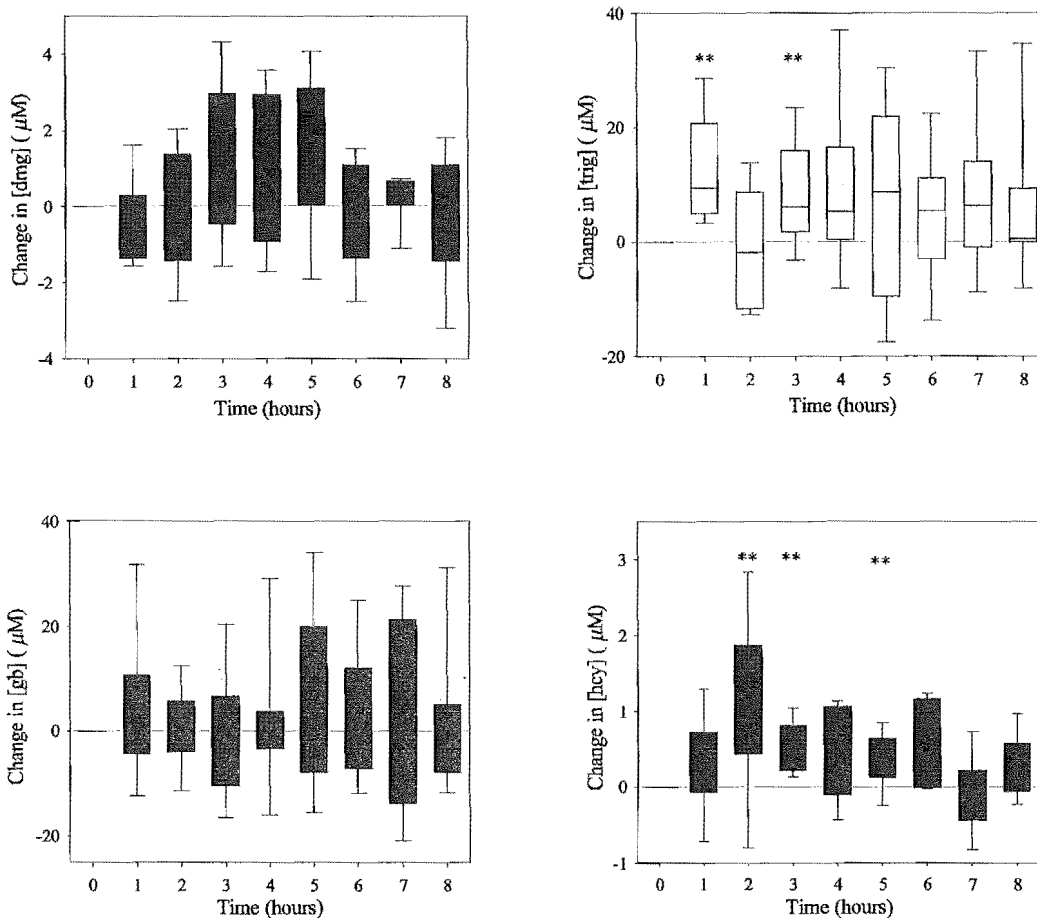
#### **4.2.7. Statistical analysis**

All statistical analyses were carried out using SigmaStat 2.03 (SPSS, 1995).

### **4.3. Results**

#### **4.3.1. Plasma levels**

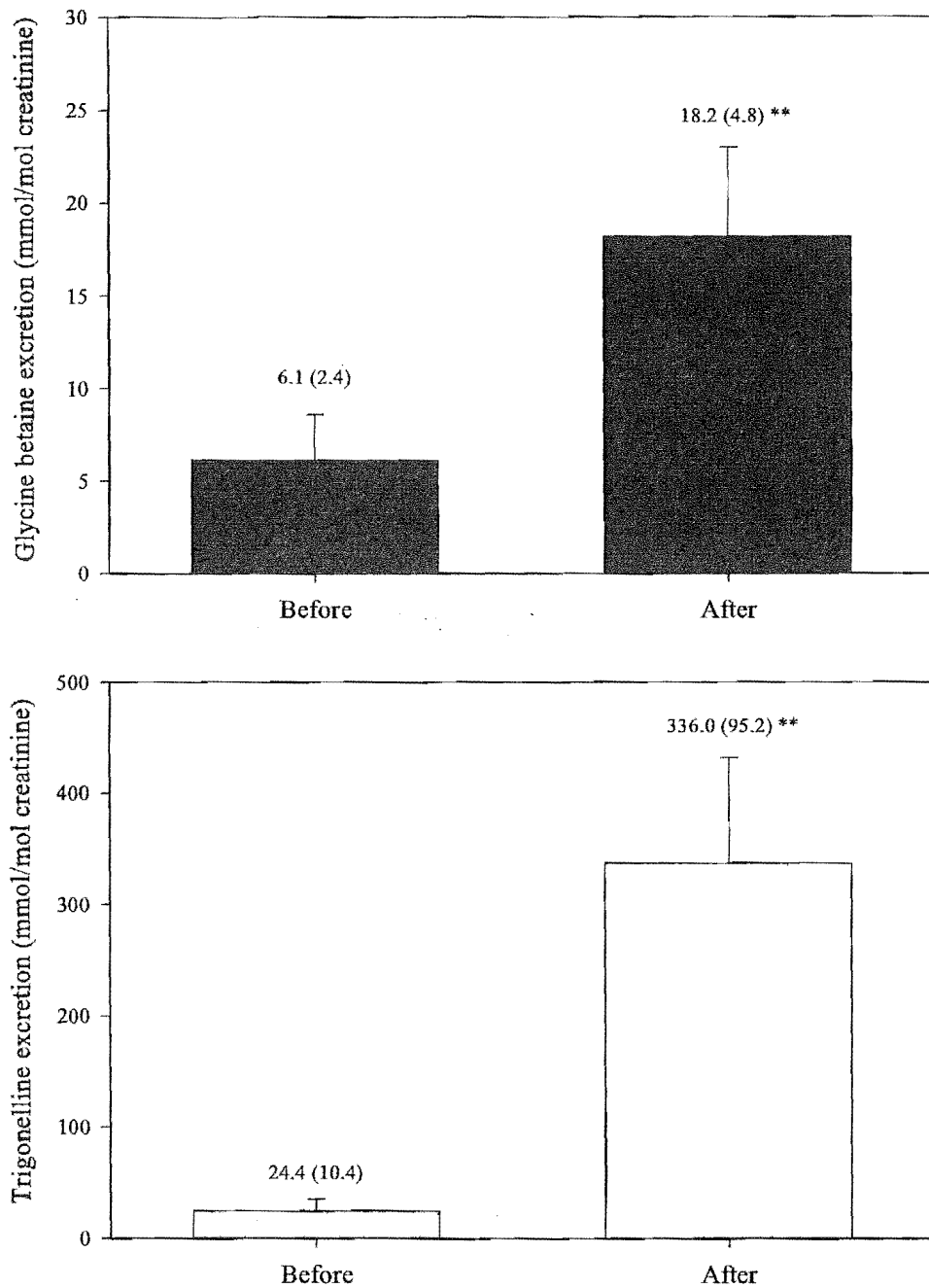
Figure 4.3 shows the changes in the concentrations of plasma metabolite relative to baseline. Significant differences (paired t-tests,  $P < 0.05$ ) have been indicated (\*\*). No significant change in the concentrations of N,N-dimethylglycine (dmg) or glycine betaine (gb) in the plasma were observed. There was a significant increase in plasma trigonelline initially, but no significant differences were seen after 3 hours. A significant increase in plasma homocysteine levels was observed. Raw data can be found in section 7.4.3.



**Figure 4.3:** Change in plasma metabolite levels after the consumption of coffee. Boxes represent interquartile ranges, and error bars represent the 95<sup>th</sup> percentile. Significant differences from the base line ( $P < 0.05$ ) have been marked (\*\*).

#### 4.3.2. Urine levels

Total trigonelline ingested was  $600 \pm 50 \mu\text{mol}$  (mean  $\pm$  SEM), and  $230 \pm 30 \mu\text{mol}$  (38%) was recovered in the urine. Figure 4.4 shows the changes in urinary excretion of glycine betaine and trigonelline. The graphs show the total metabolite (per mol creatinine) before and after coffee consumption. Paired t-tests were carried out, and the increased excretion of both compounds proved to be significant ( $P < 0.05$ ).



**Figure 4.4:** Change in betaine excretion after the consumption of coffee. Values have been adjusted for creatinine excretion. Means (SEM) are shown, and significant differences from the base line are marked (\*\*).

Before values were taken from analysis of the baseline urine (produced before 9 am) and the After values were calculated from analyses of all urines produced after 10 am).

## 4.4. Discussion

Plasma homocysteine is a continuous and graded risk factor for cardiovascular disease (CVD) (section 1.3), so it is of interest that plasma homocysteine concentrations were increased after the acute ingestion of coffee. This result is consistent with the observations of Urgert et al. (1996) that volunteers who drank 1 l of coffee per day for 4 weeks had significantly higher ( $1.5 \mu\text{mol}^{-1}$ ) homocysteine concentrations than when no coffee was consumed. The present study shows an increase of c.  $1 \mu\text{mol}^{-1}$  (base line =  $9.6 \mu\text{mol}^{-1}$ ), 2 h after coffee consumption. A recent study by Olthof et al. (2001) has shown that chlorogenic acid, also present in coffee, causes a moderate, short term increase ( $1.2 \mu\text{mol}^{-1}$ ) in plasma homocysteine levels. The concentration of chlorogenic acid present in the coffee used in this study was not determined, but can be estimated to be 1 g, half the amount of chlorogenic acid given by Olthof et al. and is potentially responsible for the increase in homocysteine concentrations seen.

The increase in trigonelline excretion observed is consistent with previously reported observations. The recovery of trigonelline in the urine in the present study (38%) is higher than the 20% reported by Yuyama et al. (1996). This difference may be due to differences in the doses that were given:  $600 \mu\text{mol}$  (82 mg) in the present study compared with  $288 \mu\text{mol}$  (39 mg) given by Yuyama et al. The increased glycine betaine excretion may be due to diuresis, caused by caffeine and the liquid consumed, but this has not been tested. The change in urinary glycine betaine excretion was compared to a previous study (Sizeland, 1995) in which 20 ml/kg body weight of water was consumed in 10 minutes (ie, 1.4 l for a 70 kg person). The urine was collected for three hours after water was administered, and the glycine betaine excretion increased from 6.2 to 16.2  $\text{mmol}^{-1}$  glycine betaine. In the present study, glycine betaine excretion increased from 6.1 to 18.2  $\text{mmol}^{-1}$  glycine betaine per mole creatinine ( $P=0.014$ ). This increase is slightly higher than published in the previous paper. Full data from the previous study was not available, so a statistical comparison could not be carried out.

The design of the study had limitations, in that it could not prove if trigonelline in coffee causes increased plasma homocysteine concentrations. Ideally, trigonelline would have been given directly, and blood and urine samples collected and analysed. This option was investigated, but the difficulty of obtaining ethical approval, and also the high cost and difficulty in obtaining trigonelline that would be approved for human consumption meant that it was not achievable within the time frame of this thesis.

The changes observed in the homocysteine concentrations varied between individuals. This suggests that coffee affects plasma homocysteine concentrations differently in different people. The differences may be due to many factors, such as protein levels in the diet, absorption of trigonelline, current folate status and genetic determinants. All of these factors are known to affect plasma homocysteine concentrations, and the effect of coffee observed in this and other studies may be dependent on these or other factors. A trial with a much larger number of subjects would be required to demonstrate the effect of coffee consumption with these other factors.

## **4.5. Conclusion**

There is a moderate short term increase in plasma homocysteine associated with the consumption of large amounts of coffee. The causal agent(s) of this could not be identified in this study. A more controlled experiment, using pure substances, is required to pinpoint the component(s) of coffee that are responsible for the observed increase.

As there is no threshold for the risk of CVD associated with homocysteine, any increase in homocysteine has a cumulative effect on the risk of CVD. The change noted here may not be of concern for the individual, but a 10% increase in plasma homocysteine concentrations of coffee drinkers may have major implications for the health of a community.

The observations that both trigonelline and homocysteine are increased in plasma is consistent with the hypothesis that trigonelline, a betaine present in coffee, interacts with homocysteine metabolism and may be a causal agent in the increased homocysteine concentrations associated with coffee consumption.

## Chapter 5

### The Trigonelline Effect

*"If we knew what it was we were doing, it would not be called research, would it?"*

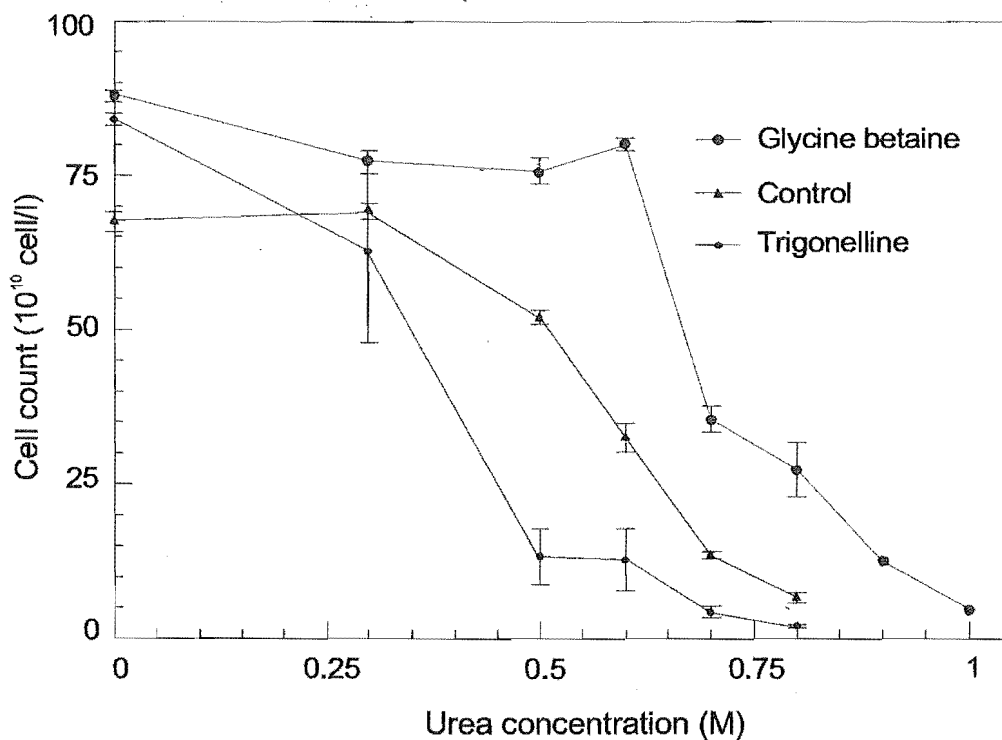
Albert Einstein

## 5.1. Background

Randall (1998) showed that the betaine trigonelline, in the presence of salt, consistently exacerbated the urea intolerance of *Escherichia coli* (ATCC 29522) (Bauer, 1973) in minimal media (figure 5.1). The growth rate in the presence of urea ( $500 \text{ mmol}^{-1}$ ), salt ( $300 \text{ mmol}^{-1}$ ) and trigonelline ( $100 \mu\text{mol}^{-1}$ ) was significantly lower ( $P < 0.001$ ) than the growth rate in the presence of urea and salt (no trigonelline). This effect was not seen with any other related betaines. The mechanism by which trigonelline causes the exacerbation of urea's cytotoxic effect is unknown.

### 5.1.1. Betaine accumulation

Glycine betaine is utilized by *E. coli* as an osmoprotectant. *E. coli* is unable to synthesis glycine betaine, so specific active transporters exist to accumulate glycine betaine from the environment.



**Figure 5.1:** Growth of *E. coli* after 16 hours (37°C, shaking water bath) in minimal media containing urea (0.0 to 1.0  $\text{mol}^{-1}$ ), salt ( $0.3 \text{ mol}^{-1} \text{ NaCl}$ ), and different betaines ( $100 \mu\text{mol}^{-1}$ ). Taken from Randall (1997).



*E. coli* has at least three proteins (ProP, ProU and PutP) that transport glycine betaine across the membrane. ProP and ProU are osmoregulated, and are responsible for the rapid uptake of glycine betaine in hyperosmotic media (reviewed in Randall 1997). These transporters are non-specific, being able to transport betaines including arsonium and sulfonium analogues (Peddie et al. 1994; Randall et al. 1995; Randall et al. 1996), proline betaine (Peddie et al. 1994), ectoine (Jebber et al. 1992), taurine (McLaggan and Epstein 1991) and proline (Wood 1988).

Mammalian cells also accumulate glycine betaine via a number of transporters. Madin Darby Canine Kidney (MDCK) cells transport betaines by osmoregulated and non-osmoregulated ports (Wood 1988). These ports are more selective for different betaines than the bacterial betaine transport systems. (Randall 1997). In mammals, proline betaine and trigonelline are not accumulated in the kidneys (Lever et al. 1994a). Instead, they are excreted into the urine, and glycine betaine is reabsorbed into the blood plasma (Chambers and Lever 1996). This strongly indicates that transporters in mammalian kidney cells are able to differentiate between glycine betaine and other betaines.

### 5.1.2. Urinary tract

Human urine commonly reaches levels of salt and urea that would be inhibitory to *E. coli* and other common urinary pathogens. Despite this, *E. coli* can invade and colonize the urinary tract. Urinary tract infections (UTI) are a major cause of hospitalization, with 50% of all females and 15% of males having at least one symptomatic UTI in their life time (Kunin, 1987). One major mechanism by which urinary pathogens are able to overcome the inhibitory nature of urine is via the accumulation of glycine betaine as a counteracting solute. Glycine betaine is present in low levels in mammalian urine and is an important factor in making the urine a suitable growth medium (Chambers and Kunin 1985).

Conditions in the urinary tract are extremely variable, with the concentration ranging from 50 mosmol<sup>-1</sup> to 1200 mosmol<sup>-1</sup> (Penney 1987) and urea levels reaching 500 mmol<sup>-1</sup> (Asscher 1980). Trigonelline is a dietary betaine (section 2.3.1) and can be found unmetabolized in urine at levels of over 10 mmol<sup>-1</sup> (Lever 1994a; Yuyama 1996; section 4.3.2).

### **5.1.3. Hypothesis**

The urinary tract frequently reaches urea and salt concentrations where the trigonelline effect would be expected to occur. This led to the hypothesis that dietary trigonelline excreted in the urine may inhibit the growth of *E.coli* (Randall 1997). This could be exploited as a treatment of urinary tract infections and reduce the likelihood of recurrence. The plausibility of this was studied in this section.

## **5.2. Methods**

### **5.2.1. Growth media and conditions**

#### **5.2.1.1. Minimal media**

The minimal medium of Davis (Davis 1973) was sterilized by passing through a 0.2  $\mu\text{m}$  filter (Millipore). Other components of minimal media were added as required. Full details can be seen in section 7.5.1. These were incubated at 37°C in a shaking water bath.

#### **5.2.1.2. Urine**

Urines were obtained from volunteers who had abstained from coffee and sprouts (foods known to contain trigonelline) for at least 2 days. Urines were tested, and those low in betaines were pooled and sterilized. Distilled water, glycine betaine solution and/or trigonelline solutions were added to make urines of identical volume and composition except for the betaine content. Full details can be seen in section 7.5.2. These were inoculated and incubated at 37°C in a shaking water bath.

#### **5.2.1.3. Inoculum**

An overnight culture of *E.coli* ATCC 25922 grown in minimal media was used for inoculation of all media. The volume of inoculum was 1% of the final volume, supplying approximately  $10 \times 10^6$  cfu/ml.

## 5.2.2. Bacterial enumeration

Bacterial numbers in minimal media were approximated using formula 5.1 (Randall 1997). Viable counts (plated on blood agar) were used for bacterial enumeration in urine. Plates containing 20-200 cfu were counted.

$$\frac{N}{1000000} = 49.1(\text{OD}) + 45(\text{OD})^2$$

**Formula 5.1:** Formula used to approximate viable cell count using light absorbance. **N:** viable count (cfu/ml). **OD:** absorbance at 620 nm.

---

## 5.2.3. Betaine uptake

### 5.2.3.1. Betaine uptake measurement

The internal betaine content of bacterial cells was measured by washing and separating the cells. The cells were then resuspended in extraction solvent and sonicated. The resultant solution was derivatized and analyzed using the alumina system as described section 2.5. More details can be found in the section 7.5.3.

### 5.2.3.2. Betaine transfer

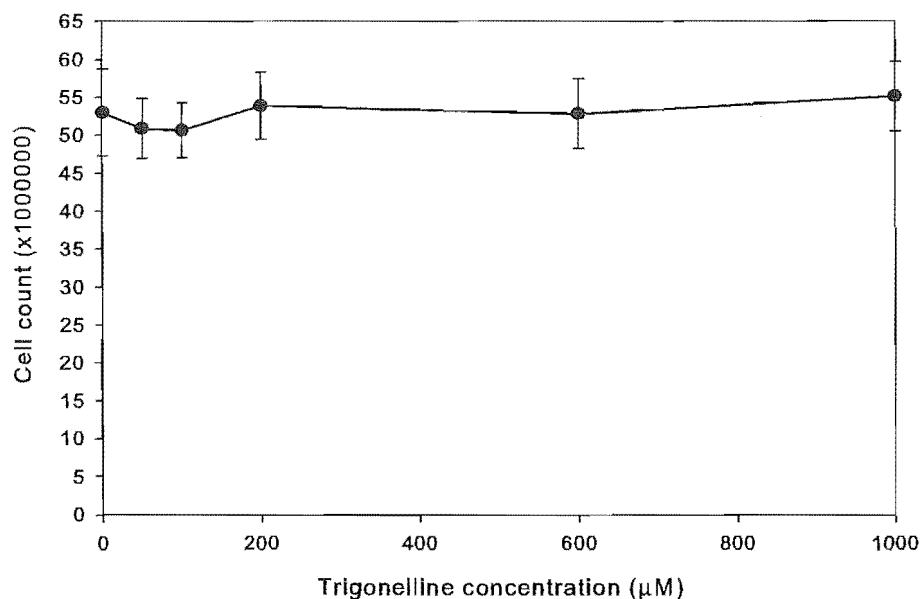
To measure the accumulation of glycine betaine by bacterial cells already loaded with trigonelline, the method used by Randall (1997) was used. The cells were grown in media containing 100  $\mu\text{mol}^{-1}$  trigonelline, separated and rinsed with iso-osmotic saline. The cells were then suspended in iso-osmotic saline solution containing 100  $\mu\text{mol}^{-1}$  glycine betaine. After five minutes, the cells were collected and processed as described in section 5.2.3.1. More details can be found in the section 7.5.4.

### 5.2.4. Analyses

Trigonelline and glycine betaine concentrations were measured as described in section 2.5. Urea concentrations were measured by Canterbury Health Laboratories by urease assay (Abbott, 1998). Salt concentrations were approximated by subtracting the urea concentration from the osmolality, which was measured by freezing-point depression.

### 5.3. Results

Despite using the same strain of *E.coli* ATCC 25922 and identical growth conditions, the trigonelline effect, as described by Randall (1997) was not seen. Trigonelline had no effect in minimal media (figures 5.2 - 5.3) or urine (data not shown) supplemented with salt and urea. Glycine betaine had a protective effect against both salt and urea at all concentrations tested (50



**Figure 5.2:** Graph showing no effect of trigonelline on the growth rate of *E.coli* in the presence of urea ( $500 \text{ mmol}^{-1}$ ) and salt ( $300 \text{ mmol}^{-1}$ ). Bacteria were incubated for 26 hours. Similar graphs were seen after 4, 6, 8 and 10 hours. Error bars show the standard deviation.

$\text{mol l}^{-1}$  -  $1000 \text{ mol l}^{-1}$ ) in minimal media but supplemental glycine betaine did not offer any increased osmoprotection in urine (data not shown).

In the transfer experiments, the net result was that glycine betaine was accumulated quickly if the cells were transferred to a glycine betaine-containing media. No glycine betaine was detected in the cells that were harvested prior to transfer to glycine betaine containing solutions. Trigonelline was seen in all cultures that were incubated in the presence of trigonelline, both before and after transfer.

## 5.4. Discussion

The conditions (media, temperature, source of chemicals, inoculum) were all identical, and the cause of the disappearance of the trigonelline effect is unknown. The most likely change is in the bacteria used. The original culture was stored at  $-40^{\circ}\text{C}$ , and a subculture was grown and kept at  $4^{\circ}\text{C}$ . This subculture was replated from a single colony each week. Although it is unlikely, a mutant may have arisen, and this mutant may have been chosen, resulting in the subculture being a different strain. Another possibility is that the subculture may have been inoculated by an aerosol during preparation, resulting in a completely unrelated strain being present. Subsequent investigations have revealed that the trigonelline effect is strain dependent, with only three out of ten isolates showing the effect (Peddie 2000). Full investigation into this has not been carried out. As was expected, no inhibitory effect of trigonelline was seen in urine.

With the transfer experiments, the concentrations of glycine betaine and trigonelline measured are not consistent between replicates, so it is impossible to tell if glycine betaine replaces trigonelline, or if glycine betaine is accumulated in conjunction with trigonelline.

## 5.5. Conclusion

The trigonelline effect as noted by Randall (1997) cannot be exploited for the treatment or prevention of UTI for two reasons.

First and foremost, glycine betaine accumulation and trigonelline accumulation appear to be competitive, with the  $K_m$  of glycine betaine being much lower than the  $K_m$  of trigonelline. If *E.coli* is in a medium with both glycine betaine and trigonelline in it, the glycine

betaine is accumulated first. Trigonelline is accumulated to a similar degree, but only after the glycine betaine has been accumulated (Randall, 1997). As glycine betaine is constantly being added to urine in the urinary tract, it can be predicted glycine betaine never becomes limiting, so very little trigonelline will be accumulated. This applies even if the cell has accumulated trigonelline and then is exposed to glycine betaine. Glycine betaine is always found in urines, although dilute urines have very low levels. It must be noted that dilute urines also have very low levels of trigonelline if any trigonelline is present. Any urine with trigonelline present also has glycine betaine, and the glycine betaine present is accumulated to the detriment of trigonelline accumulation.

The second reason for which this claim can be made is that not all strains show the trigonelline effect. In fact, half of the clinical strains (strains that were isolated from UTI) tested did not show the trigonelline effect (Peddie 2000).

## Chapter 6

### Discussion

*"Questions are what drives science, not answers."*

Robert S. Root-Bernstein

## 6.1. Glycine betaine

Glycine betaine is emerging as an important metabolite in humans. It plays vital roles as an osmolyte and counteracting solute in kidneys, and as a methyl donor in the reaction catalyzed by BHMT. Increasingly, it is becoming apparent that glycine betaine is essential for cell volume regulation in other tissues also (reviewed in Somero and Yancey 1997). The concentration of glycine betaine in blood is homeostatically controlled about individual set points (Lever et al. 1994a), and disturbances can be seen in some diseases, including diabetes mellitus (both type 1 and type 2) and renal disease (Dellow et al. 1999). Supplementation with glycine betaine has been shown to have a variety of beneficial effects (reviewed in chapter 1), and no studies reporting an adverse effect have been published. In the human body, glycine betaine can be synthesised from choline by bacteria or endothelial cells in the gut or by the liver, or it can be absorbed directly from the diet (reviewed in chapter 1).

The data presented in this thesis allows the dietary intake of glycine betaine to be estimated at 150-200 mg/day in the average New Zealand diet (section 3.5), most of which comes from wheat and flour products. Shellfish, beetroot and spinach are also good sources (tables 3.3-3.5), but do not contribute significantly to the average New Zealand diet (Cressey 2001). The small number of relatively concentrated sources of these glycine betaine means that it is relatively simple to increase the intake, and a diet supplying 620mg/day is realistically obtainable (section 3.5). Choline is present in all foods (as an essential component of biological membranes), and can be oxidized to glycine betaine by bacteria in the gut and by the liver (Zeisel 1981). It has been estimated that the intake of glycine betaine in the form of choline could be as high as 500 mg/day (Zeisel 1994). However this figure is an estimate, and a database of choline content in foods, as well as data relating to the absorbance and metabolism of choline is required, to allow evaluation of the amount of glycine betaine that is synthesized under various metabolic states. This research was recommended by the report to the (USA) Food and Nutrition Board (1999), so this data would be expected to be available in the near future. If 0.5 g/day of glycine betaine (synthesized from choline) can be achieved, in conjunction with a high glycine betaine diet, a daily intake of 1 g or more of glycine betaine is not unrealistic.

Given that a change in physiological markers can be seen with glycine betaine supplementation of 2-6 grams per day (reviewed in chapter 1), there is potential that dietary glycine betaine and choline are sufficiently high to of benefit to human health. To evaluate the data



presented in this thesis fully, a clinical trial measuring the dose-response of glycine betaine is recommended. It is hard to know what would be best used as a marker of adequate glycine betaine intake, but homocysteine, vitamin B status, or the dm<sub>g</sub>/gb ratio could be considered. Studies looking at glycine betaine supplementation with folate deficient and non-folate deficient subjects are required. There is evidence that the BHMT pathway may act as an "overflow" pathway, and be much more active in folate deficient patients. Thus, patients with a functional folate deficiency may respond to glycine betaine supplements differently from patients with out folate deficiency.

Although the dietary intakes of betaines are reported here, the bioavailability is unknown. How much betaine in the diet is absorbed into the circulatory system? It is probable that glycine betaine is absorbed in the gut probably by the non-selective IMINO porter (Stevens 1985), and increased blood concentrations can be seen after supplementation. Other betaines that can be found in the diet, and are not required in human metabolism, can also be found in blood and urine, indicating that they are absorbed (Lever et al. 1994a). The extent of this is also unknown. It is possible that the procedures used in this project (chapter 2) measures betaines that are unavailable to humans in the diet, and this needs to be addressed.

## 6.2. Other betaines

This thesis has established that only two betaines, other than glycine betaine, can be found in the diet at levels high enough to have potential metabolic effects; proline betaine and trigonelline. Coffee is the predominant source of trigonelline, with small amounts coming from legumes (section 3.3). Legumes also contribute to proline betaine, but most is obtained from citrus fruit. The relatively concentrated sources of these compounds means that it is relatively simple to alter their intake (section 3.3). Neither of these betaines are homeostatically controlled in the body, and both are highly variable in the urine and blood (Lever et al. 1994a). A strong correlation between plasma glycine betaine concentrations over three years was seen (figure 1.3A), while no correlation was seen between plasma proline betaine concentrations over the same time (Lever et al. unpublished data). There is no evidence that either trigonelline or proline betaine have a metabolic role in normal human physiology, although trigonelline is a metabolite of nicotine (Budavari 1989). Both proline betaine and trigonelline are cleared efficiently from the blood into the urine by the kidney, and neither are accumulated by the kidney, unlike glycine betaine, which

is resorbed (fractional clearance = 2-5%), and is accumulated by cells in the kidney (Sizeland et al. 1993). There is a weak association between urinary glycine betaine and urinary proline betaine levels (Lever et al. 1994a), implying a metabolic effect of proline betaine in the kidney. Also, Dellow et al. (1999) reported a weak ( $R = -0.28$ ) but significant negative correlation between glycine betaine and trigonelline in the plasma. Again, this shows that there is a metabolic interaction of some sort between dietary betaines and glycine betaine. Trigonelline may also have a slight anti-diabetic effect (Al-Habori and Raman 1998). These strongly suggest that dietary betaines do have metabolic effects. The extent of these metabolic effects is unknown, and are of great interest because of the potential implications for human health.

There is potential that trigonelline is responsible for the increase in homocysteine concentrations seen after consumption of coffee (chapter 4), although Olthof et al. (2001) reported that chlorogenic acid, present in coffee, is at least partially responsible for the increase in plasma homocysteine concentrations associated with coffee. Another potential cause of the rise in homocysteine concentrations after the consumption of coffee is the large increase in urinary excretion of glycine betaine reported in this thesis. However, this effect may be due to diuresis, caused by the consumption of water (in coffee) and caffeine (a diuretic). Diuresis can also be suggested as the mechanism by which diabetics have high plasma homocysteine concentrations.

Given that proline betaine is a weak substrate for BHMT (Mulligan et al. 1998), there is the potential for it and other dietary betaines to act as inhibitors in physiological conditions. Further studies on purified BHMT are recommended, to see which substances in the diet and in the body act as inhibitors or substrates at physiological concentrations. It is common for supplementations of glycine betaine or methionine to be given with orange juice, a rich source of proline betaine. The observations reported in many studies may in fact be a result of proline betaine instead of the compound given, or the effect of these compounds may be altered by proline betaine. If studies with purified BHMT show that proline betaine interacts significantly in physiological conditions, then a clinical study into the physiological effect of proline betaine is recommended. If there proline betaine does affect activity or regulation of BHMT, then the conclusions drawn from studies using orange juice as an inert carrier may need to be revised. The kinetics of other betaines, especially trigonelline, also need to be elucidated.

There are a number of other betaines that can be found in nature (figure 1.1) and would be expected to be found in foods. The data presented here shows that the levels of these betaines in foods are low, and thus are unlikely to have any metabolic effects.

### **6.3. Conclusion**

This thesis has laid the foundations for the study of the effect of dietary betaines on human metabolism. With the data presented here, the dietary betaines that are potentially important, and foods in which they are present, have been identified. The foods which contain high levels of betaines have also been identified, and the relevance of studies supplementing glycine betaine can be put into perspective.

## Chapter 7

## Appendix

*"The end is nigh!"*

Unknown

## 7.2. Chapter 2

### 7.2.1. Recovery of dichloromethane extraction

A solution containing glycine betaine and trigonelline ( $1000 \mu\text{mol}^{-1}$ ) was extracted with dichloromethane as described in section 2.5.1.5. The dichloromethane layer was separated and evaporated at room temperature under a stream of  $\text{N}_2$  gas. This was resuspended in  $500 \mu\text{l}$   $\text{H}_2\text{O}$ . The resuspended solution, the original aqueous standard and the extracted aqueous standard were derivatized.

### 7.2.2. Derivatization reaction at the start of this project

A  $20 \mu\text{l}$  aliquot of sample was added to 1 ml of 10% methanol in acetonitrile. A heaped spatula of anhydrous  $\text{Na}_2\text{SO}_4$  (c. 350 mg) was added, and the mixture was shaken for an hour. The mixture was then centrifuged (5 min, 11000 rpm) to remove the  $\text{Na}_2\text{SO}_4$  and any other solids, such as denatured protein. A  $250 \mu\text{l}$  aliquot of the supernatant was transferred to a fresh microfuge tube. Derivatizing reagent ( $50 \mu\text{l}$ ,  $100\text{mmol}^{-1}$  2-naphthacyl trifluoromethanesulfonate in acetonitrile) was added, and the mixture was shaken for five minutes.  $\text{Mg}(\text{OH})_2$  solution ( $5 \mu\text{l}$ , 0.1g/ml  $\text{MgO}$  suspended in distilled water) was added, and the solution was shaken for a further five minutes. This mixture was then centrifuged (5 min, 11000 rpm) and the supernatant was transferred to an HPLC vial for analysis. Vials were capped immediately to stop concentration by evaporation.

### 7.2.3. Testing of derivatization solvents

Acetonitrile, 10% methanol in acetonitrile, monoglyme (1,2-dimethoxyethane), 5% water in monoglyme, diglyme (2-methoxyethyl ether), propan-2-ol, methanol and acetone were tested using a standard betaine solution (containing  $1 \text{mmol}^{-1}$  acetyl carnitine, carnitine, creatinine, *N,N*-dimethylglycine, glycine betaine, trimethylamine, and trigonelline). A  $100 \mu\text{l}$  aliquot of the test solution was added to 1 ml of 10% methanol in acetonitrile (once for each solvent being tested). This was evaporated to dryness at room temperature under a stream of  $\text{N}_2$  gas. The residue was

resuspended in 250  $\mu\text{l}$  of the appropriate test solvent and was shaken for 15 minutes, ensuring all of the betaines were in solution. Derivatization was then carried out as described (2.5.2), using a derivatizing solution made in acetone instead of acetonitrile. The experiment was also repeated with a standard containing 1  $\text{mmol}^{-1}$  of arsenobetaine, betonicine, butyrobetaine, *N,N*-dimethylglycine, glycine betaine, and trigonelline. These were separated on the Zorbax HPLC system as described in section 2.5.3.2. The HPLC traces were compared to show the relative efficiencies of derivatization.

#### 7.2.4. Effect of water on derivatization

A 1  $\text{mmol}^{-1}$  betaine solution containing acetyl carnitine, carnitine, creatinine, *N,N*-dimethylglycine, glycine betaine, trimethylamine oxide and trigonelline was derivatized (as described in section 2.5.2) in acetone containing varying amounts of water (0, 5 and 10% v/v), and the resultant HPLC traces were compared.

#### 7.2.5. Order of reactants

A sample containing 100  $\mu\text{mol}^{-1}$  of acetyl carnitine, arsenobetaine, betonicine, butyrobetaine, carnitine, creatinine, glycine betaine, *N,N*-dimethylglycine and trigonelline, and a water blank were used. Derivatization was carried out in acetone, using 5  $\mu\text{l}$  of sample. Derivatizing reagent and base were prepared as described in section 2.5.2. In treatment A, base was added, and derivatizing reagent was added 5 minutes later. In treatment B, derivatizing reagent was added, and base was added five minutes later, and in treatment C, derivatizing reagent and base were added at the same time. Each sample was shaken for 5 minutes, and then centrifuged. These were then analysed using the Zorbax system, and the chromatograms were compared.

#### 7.2.6. Phenosphere

A standard containing 100  $\mu\text{mol}^{-1}$  each of acetyl carnitine, carnitine, glycine betaine, *N,N*-dimethylglycine and trigonelline was derivatized. The same mobile phase as the Zorbax system but with half the buffer concentration (3.5  $\text{mmol}^{-1}$  glycolic acid, 7  $\text{mmol}^{-1}$  tetramethyl

ammonium hydroxide, 5% water in acetonitrile) was used for the blue trace. The buffer concentration was doubled to 7 mmol<sup>-1</sup> glycolic acid and 14 mmol<sup>-1</sup> tetramethyl ammonium hydroxide while the water concentration was kept at 5% to increase the elution times. The water concentrations was raised to 10% (v/v), resulting in the green trace.

## 7.3. Chapter 3

### 7.3.1. Foods that had inconsistent chromatography

#### 7.3.1.1. Apples

The chromatogram from the Zorbax system gave glycine betaine and trigonelline concentrations of 2.5 and 3.4  $\mu\text{g/g}$  respectively. No peaks were seen on the alumina system with the low sensitivity detector. Less than 5  $\mu\text{g/g}$  of glycine betaine and trigonelline has been reported.

#### 7.3.1.2. Avocado

Proline betaine was measured at 5  $\mu\text{g/g}$  using the Zorbax system, but was not seen the other two. Less than 1  $\mu\text{g/g}$  has been reported.

The glycine betaine concentration in avocado was measured at 7.4  $\mu\text{mol}^{-1}$  (Zorbax), 12  $\mu\text{mol}^{-1}$  (Phenosphere) and 46  $\mu\text{mol}^{-1}$  (alumina). These values correspond to concentrations of less than 10  $\mu\text{g/g}$  (for Zorbax and Phenosphere) and 16  $\mu\text{g/g}$  (for the alumina). The peak shape on the alumina system was distorted, indicating that one or more compounds co-eluted with glycine betaine. The difference between the two remaining results is within the error of the assay, and less than 10  $\mu\text{g/g}$  has been reported.

Trigonelline was measure at 34  $\mu\text{mol}^{-1}$  (14  $\mu\text{g/g}$ ) on the Phenosphere system, but was not seen with the other systems. However, the retention time of this peak is 25.5 minutes, while the trigonelline standards eluted at 24.5 minutes. The difference in retention times is great enough to conclude that the peak is due to another compound. Trigonelline has been reported as less than 1  $\mu\text{g/g}$ .

#### 7.3.1.3. Banana

The concentration of proline betaine, glycine betaine and trigonelline in the banana extract was

9, 25 and 95  $\mu\text{mol l}^{-1}$  respectively. None of these peaks were visible with the low sensitivity UV detector. The average of the results from the chromatograms using the good UV have been accepted.

#### **7.3.1.4. Broccoli**

There was significant interference in one chromatogram at the time at which trigonelline would run, so no quantification was possible. The other two chromatograms showed no trigonelline present, so less than 1  $\mu\text{g/g}$  has been reported.

The concentration of glycine betaine obtained on the Zorbax system was 7.8  $\mu\text{g/g}$ . No glycine betaine was detected in the chromatograms which were recorded with the UV detector with lower sensitivity. The concentration of glycine betaine has been reported as less than 10  $\mu\text{g/g}$ .

#### **7.3.1.5. Brussel Sprouts**

The trigonelline values obtained range from 28 to 93  $\mu\text{g/g}$  in brussel sprouts. The values from the alumina system have been accepted, because they are consistent and lower than the Phenosphere values. The trigonelline peak on one of the alumina chromatograms was asymmetrical and was ignored, although the peak height was consistent with the other chromatograms.

#### **7.3.1.6. Cauliflower**

Trigonelline was unquantifiable in one system due to interference. Only the values from the other system were used.

#### **7.3.1.7. Cheese**

The cheese extract showed a very large glycine betaine peak, corresponding to over 2000  $\mu\text{mol l}^{-1}$ , on the alumina system. No peak of that size was seen anywhere on the Phenosphere system. This indicates that the compound either eluted early on the Phenosphere, or it was retained on the column. If the first applies, then the compound would elute in the initial peak, so would not be detected. If the compound was retained on the column, then it may appear during latter chromatograms, or it may be retained on the column indefinitely. Evidence of either of these scenarios was not seen. No investigations were carried out to identify the compound. Since the



peak was not seen on the Phenosphere system, the level of glycine betaine in cheese has been reported as less than 1  $\mu\text{g/g}$ .

#### 7.3.1.8. Cod

Arsenobetaine was detected in about half of the chromatograms, at levels up to 25  $\mu\text{g/g}$ , but no arsenobetaine was detected in the remaining chromatograms and no arsenobetaine has been reported.

Proline betaine was detected using the Zorbax system at 38  $\mu\text{g/g}$ , but in the other eight chromatograms less than 5  $\mu\text{g/g}$  was detected. This value has been reported.

Trigonelline was detected using the alumina system, but was not observed using the other systems, so less than 1  $\mu\text{g/g}$  has been reported.

#### 7.3.1.9. Groper

Proline betaine was detected in three chromatograms (9, 50 and 228  $\mu\text{mol}^{-1}$ ), but was not seen in the remaining three chromatograms. Less than 1  $\mu\text{g/g}$  has been reported.

Glycine betaine was quantifiable in three of the samples, but there was interference in the remaining three samples. The value reported is the mean of three concentrations.

Two large peaks were detected on two of the chromatograms. One of these has a retention time that is different from any of the known betaine derivatives. No further investigation was made. The other peak has a retention time corresponding to ergothionine, but this was only seen on the alumina system. No peak corresponding to ergothionine was detected on the Phenosphere system. No ergothionine has been reported, and no further investigation of these compounds were made.

#### 7.3.1.10. Gurnard

Proline betaine was detected in one system, at a concentration of 11  $\mu\text{mol}^{-1}$ , corresponding to a concentration of 4.5  $\mu\text{g/g}$ . No proline betaine was detected on the other systems, and has been reported as less than 1  $\mu\text{g/g}$ .

Glycine betaine was detected at four different levels. Using the Zorbax system, the glycine betaine concentration was measured at 90 and 1550  $\mu\text{g/g}$ . The alumina system gave a result of 6.0  $\mu\text{g/g}$ , and glycine betaine was not detected using the Phenosphere system. The glycine betaine concentration was reported as less than 10  $\mu\text{g/g}$ .

Trigonelline was detected at a concentration of 65  $\mu\text{g/g}$  in one chromatogram, but no trigonelline was detected in the other chromatograms. This was reported as less than 1  $\mu\text{g/g}$

A small amount (4.1, 5.8, 18.7  $\mu\text{mol l}^{-1}$ ) of arsenobetaine was detected in three different systems, corresponding to between 2 and 10  $\mu\text{g/g}$ . This was reported as less than 10  $\mu\text{g/g}$ .

#### **7.3.1.11. Kiwifruit**

The values for proline betaine and glycine betaine were 0.7 and 4.6  $\mu\text{g/g}$  respectively in one system. The other system did not detect any betaines, but was recorded using the UV detector with low sensitivity. The concentration of these betaines has been reported as less than 1 and less than 5  $\mu\text{g/g}$  respectively.

#### **7.3.1.12. Kumara**

A substantial amount of interference occurred on one chromatogram. The remaining chromatograms gave consistent results, and the mean of these has been accepted.

#### **7.3.1.13. Leek**

Glycine betaine was detected at 0.6  $\mu\text{g/g}$  in one system, and was not detected in the other systems. Glycine betaine in leek has been reported as less than 1  $\mu\text{g/g}$ .

The concentration of proline betaine detected on the alumina was 2.9  $\mu\text{g/g}$ , and no proline betaine was observed using the Phenosphere system. The proline betaine concentration has been reported as less than 1  $\mu\text{g/g}$ .

#### **7.3.1.14. Lettuce**

The lettuce sample on the alumina system gave a glycine betaine and a trigonelline concentration of 7.5 and 4.9  $\mu\text{g/g}$ . Neither were detected on the Phenosphere system, and are reported as less than 1  $\mu\text{g/g}$ .

On the Phenosphere system, a peak corresponding to arsenobetaine was seen. The peak was very small, and no arsenobetaine was detected using the alumina system. No arsenobetaine has been reported.

#### 7.3.1.15. Marmite

A high level (14.3  $\mu\text{g/g}$ ) of arsenobetaine was detected in one derivatization when assayed on the alumina system. Repeats on the alumina system and on the Phenosphere system yielded no detectable arsenobetaine present. It can be assumed that there was a problem with that particular derivatization, highlighting the importance of repeating samples and injections on the HPLC. No arsenobetaine has been reported.

Proline betaine was detected at concentrations up to 9.8  $\mu\text{g/g}$  in some chromatograms, but was undetectable on other chromatograms. This has been reported as less than 1  $\mu\text{g/g}$ .

Glycine betaine was detected at concentrations ranging from 4.4 to 9.1  $\mu\text{g/g}$ , except for one chromatogram that showed a glycine betaine concentration of 60  $\mu\text{g/g}$ . It was assumed that this result was due to interference, so less than 10  $\mu\text{g/g}$  has been reported.

Trigonelline was not present in all of the chromatograms except one. That result was 73.7  $\mu\text{mol l}^{-1}$  (12  $\mu\text{g/g}$ ), but this is likely to be due to interference. The value reported is less than 1  $\mu\text{g/g}$ .

#### 7.3.1.16. Mushroom

The results from three different systems gave very similar results (c. 23  $\mu\text{g/g}$ ) for glycine betaine. Repeats of the mushroom extract were made, yielding undetectable levels of glycine betaine. This discrepancy cannot be readily explained. Less than 10  $\mu\text{g/g}$  has been reported.

#### 7.3.1.17. Onion

Both glycine betaine and proline betaine were detected at a low levels, but were not seen when the low-sensitivity detector was used. Both have been reported at less than 5  $\mu\text{g/g}$ .

#### 7.3.1.18. Parsnip

Proline betaine and glycine betaine were detected at 2.6 and 4.5  $\mu\text{g/g}$  respectively on the Zorbax system. These were not detected on other systems with the low-sensitivity detector, and have both been reported as less than 5  $\mu\text{g/g}$ .

### 7.3.1.19. Pepper

A high arsenobetaine concentration was detected using the Phenosphere system, but a repeat of this on the same system showed no arsenobetaine. None of the other chromatograms showed any arsenobetaine, and none has been reported.

Glycine betaine was also detected in some systems at low levels, but were not detected in other systems. These were all below 10  $\mu\text{g/g}$ , and this value has been reported.

### 7.3.1.20. Plum

Small amounts of glycine betaine were detected in some chromatograms, but none was seen in the others. These peaks correspond to a concentration of less than 5  $\mu\text{g/g}$  so have been reported as that.

Trigonelline was detected on one chromatogram (22  $\mu\text{mol l}^{-1}$ , 2.8  $\mu\text{g/g}$ ), was not detected in the other chromatograms, including a repeat on the same system. The trigonelline concentration has been reported as less than 1  $\mu\text{g/g}$ .

Proline betaine was detected at low levels (c. 10  $\mu\text{mol l}^{-1}$ , 1.3  $\mu\text{g/g}$ ) in some chromatograms, but none was seen in others. This has been reported as less than 1  $\mu\text{g/g}$ .

### 7.3.1.21. Potato

A small amount of glycine betaine was seen in two different systems. There was a considerable difference in the values (18 and 38  $\mu\text{mol l}^{-1}$ ), but both of these correspond to concentrations of less than 10  $\mu\text{g/g}$  in potato, so that has been reported.

### 7.3.1.22. Perch

Arsenobetaine was detected at 5.6 and 10.1  $\mu\text{mol l}^{-1}$  (2.4 and 4.3  $\mu\text{g/g}$ ) using the Zorbax system, but was not detected in alumina or Phenosphere systems. This has been reported as less than 5  $\mu\text{g/g}$ .

Proline betaine was detected at 1 and 61  $\mu\text{g/g}$  using the Zorbax system but was not detected in either the alumina or Phenosphere systems. This has been reported as less than 1  $\mu\text{g/g}$ .

Glycine betaine was detected at three different levels. Using the Zorbax system, the glycine betaine concentration of was recorded at 360 and 8750  $\mu\text{mol l}^{-1}$  (100 and 2440  $\mu\text{g/g}$ ). The alumina system gave a result of 93  $\mu\text{mol l}^{-1}$  (26  $\mu\text{g/g}$ ). There was a peak corresponding to glycine betaine detected on the Phenosphere system, but this was unquantifiable. The result from the

alumina system was accepted, and the glycine betaine concentration has been reported as 26  $\mu\text{g/g}$ .

Trigonelline was detected in one of the Zorbax chromatograms, but was absent from the remaining three chromatograms, so has been reported as less than 1  $\mu\text{g/g}$ .

#### 7.3.1.23. Salmon

Arsenobetaine was detected at levels up to 25  $\mu\text{mol l}^{-1}$  (up to 15  $\mu\text{g/g}$ ) using the Phenosphere system, but no arsenobetaine was observed using the alumina system. This has been reported as less than 1  $\mu\text{g/g}$ .

Proline betaine was observed in one chromatograms using the Phenosphere system, and on one chromatogram using the alumina system. These chromatograms were from the same derivatization. The values obtained were 40 and 27  $\mu\text{mol l}^{-1}$  (18 and 12  $\mu\text{g/g}$ ) respectively. Proline betaine was not detected in repeats using the same systems, so it appears that the peaks observed were artifacts from that derivatization. The proline betaine concentration has been reported as less than 1  $\mu\text{g/g}$ .

The glycine betaine peaks on three of the chromatograms were asymmetric, suggesting that another compound co-eluted with it. The remaining four chromatograms gave consistent glycine betaine concentrations, and the mean of these values has been reported.

Trigonelline was detected in one chromatogram, at a level of 50  $\mu\text{mol l}^{-1}$  (22  $\mu\text{g/g}$ ), but was absent in the other six chromatograms. This has been reported as less than 1  $\mu\text{g/g}$ .

#### 7.3.1.24. Soy sauce

Proline betaine was detected at levels between 15.9 and 37.1  $\mu\text{mol l}^{-1}$  (2.3 and 5.3  $\mu\text{g/g}$ ). This has been reported as less than 5  $\mu\text{g/g}$ .

#### 7.3.1.25. Terakihi

Arsenobetaine was detected at 13, 6 and 10  $\mu\text{g/g}$ . The concentration reported is the mean of these values.

Proline betaine was detected on one chromatogram (10  $\mu\text{mol l}^{-1}$ , 3.4  $\mu\text{g/g}$ ), but not in the other three chromatograms. This has been reported as less than 1  $\mu\text{g/g}$ .

The glycine betaine concentrations range from 1.4 to 28  $\mu\text{g/g}$ . The chromatograms recorded on the alumina and Zorbax, give similar results (1.4 and 3  $\mu\text{g/g}$ ), and less than 5  $\mu\text{g/g}$  has been reported.

Trigonelline was detected at 13, 6, and 0  $\mu\text{g/g}$ . These data are consistent with a coeluting compound on the Zorbax system. The trigonelline concentration has been reported as less than 1  $\mu\text{g/g}$ .

#### 7.3.1.26. Tuna

In four of the chromatograms, the values of arsenobetaine ranged between 1.7 and 2.5  $\mu\text{g/g}$ . The remaining two chromatograms have interfering peaks at this time, and were unquantifiable. The arsenobetaine concentration has been reported as less than 5  $\mu\text{g/g}$ .

Proline betaine was observed in one chromatogram, at a concentration of 4.6  $\mu\text{g/g}$ , but was not detected in any of the remaining five chromatograms. This has been reported as less than 1  $\mu\text{g/g}$ .

Glycine betaine was detected in all systems. The four chromatograms also gave differing results. The Phenosphere chromatograms gave results of 7.7 and 7.8  $\mu\text{g/g}$ , where as the results from the alumina chromatograms were 36 and 28  $\mu\text{g/g}$ . It is most likely that the glycine betaine derivative coelutes with something else on the alumina system, and the Phenosphere results were accepted. This has been reported as less than 10  $\mu\text{g/g}$ .

#### 7.3.1.27. Vegemite

The chromatograms of Vegemite extracts were virtually identical to those of Marmite.

Proline betaine was detected in high quantities in some chromatograms, but was not detectable in others, so less than 1  $\mu\text{g/g}$  has been reported.

Glycine betaine was measured in concentrations ranging from 1.9 to 11  $\mu\text{g/g}$ , except for one chromatogram which gave a concentration of 87  $\mu\text{g/g}$ . The value reported is less than 10  $\mu\text{g/g}$ .

Trigonelline was not detected in seven of the chromatograms, but was detected at a high concentration (140  $\mu\text{mol}^{-1}$ , 21  $\mu\text{g/g}$ ) in one chromatogram. This is assumed to be contamination, and the trigonelline concentration has been reported as less than 1  $\mu\text{g/g}$ .

#### 7.3.1.28. Wine

The trigonelline peak on the alumina system is symmetrical, but the trigonelline peak on the Phenosphere system is asymmetric, indicating coelution of another compound. The height of the peak on the Phenosphere chromatogram is consistent with the alumina system result, and the value obtained with the alumina system has been accepted.

#### 7.3.1.29. Yam

The yam extract was assayed with both the Phenosphere and the alumina system twice. This gave three different results for the trigonelline content (0, 20 and 50  $\mu\text{mol}^{-1}$ ). These all correspond to a concentration of less than 10  $\mu\text{g/g}$ , so this has been reported.

Glycine betaine was recorded at four different concentrations ranging from 4 to 38  $\mu\text{mol}^{-1}$ . All of these correspond to less than 10  $\mu\text{g/g}$ , so this has been reported.

A peak corresponding to arsenobetaine was detected in one run on the Phenosphere system, but this was not seen on either the Phenosphere or the alumina column. No arsenobetaine has been reported.

### 7.3.2. High betaine diet

#### Breakfast

Porridge (30 g oats, 500 ml milk)  
Grapefruit, one (236 g)  
Bread, one thick slice (40 g, assume flour = 75 %)  
Margarine/butter (10 g)  
Marmite (0.5 tsp, 3.25 g)  
Tea (180 ml, with 30 ml milk)

#### Morning tea

Orange, one (128 g)

#### Lunch

Bread, wholegrain, two medium slices (56 g, assume flour = 75%)  
Margarine/butter (25 g)  
Lettuce leaves, two (16 g)  
Tomato, one (127 g)  
Tuna (90g)  
Beetroot (60 g)  
Muffin (80 g, assume flour = 25 %)  
Tangelo, one (90 g)  
Yoghurt (150 g)  
Orange juice (250 ml)

#### Afternoon tea

Tea (180 ml, with 30 ml milk)  
Wine biscuits, two (16 g, assume 80 % flour)

#### Dinner

Pasta (300 g)  
Mussels (110 g)  
Tomato Sauce (125 ml)  
Spinach (84 g)  
Cheese (25 g)  
Crackers, four (18 g, assume 90% flour)  
Coffee (180 ml, with 30 ml milk)

#### Supper

Bread, one thin slice (28 g, assume flour = 75 %)  
Marmite (0.5 tsp, 3.25 g)  
Milo (5 g milo, 30 ml milk)



## 7.4. Chapter 4

### 7.4.1. The consent form for the coffee trial

#### Consent Form

#### **Can coffee and bean sprouts combat bacteria in the urinary tract and increase the risk of cardiovascular disease?**

I have read and understood the information sheet dated 2nd November 1998 for volunteers taking part in the study designed to see whether the consumption of food or drink containing the compounds trigonelline and/or proline betaine would produce urine which inhibited the growth of bacteria and also to discover the achievable levels of these compounds and of homocysteine and dimethylglycine in plasma and urine. I have had the opportunity to discuss this study and I am satisfied with the answers I have been given.

I understand that taking part in this study is my own choice. I may withdraw at any time and this will not affect my future health care in any way.

I understand that my participation in this study is confidential and that no material which would identify me will be used in any reports on the study.

I understand the ACC compensation provisions for this study.

I have had time to consider whether to take part.

I know who to contact if I have any side effects to the study, or if I have any questions about the foodstuffs being used in the study.

I consent to the researchers storing specimens of my blood and urine for their later use as a part of this study. YES/NO

I wish to receive a copy of the results YES/NO

I \_\_\_\_\_ (full name) hereby consent to take part in this study.

Date \_\_\_\_\_

Signature \_\_\_\_\_

## 7.4.2. Information sheet for the coffee trial

<p>INFORMATION SHEET 2nd November 1998</p>	<p>1</p>
<p><b>Can coffee and bean sprouts combat bacteria in the urinary tract and increase the risk of cardiovascular disease?</b></p>	
<p><b>Principal Investigator:</b> Dr Barbara A Peddie, Scientific Officer, Department of Nephrology, Christchurch Hospital. Phone: 364 0617 (or 364 0640 ext 80617)</p>	
<p><b>Introduction</b></p> <p>You are invited to take part in a study which will measure the amounts of certain chemical compounds which appear in urine and blood after consumption of certain foods or liquids. We will be looking for eight to ten volunteers. Please read this information sheet carefully and take all the time you require to decide whether to take part.</p>	
<p><b>THE STUDY</b></p> <p><b>A)</b> Urinary tract infections (UTI) are common causes of consultations in general practice and hospital admissions. In the younger age group, women have more uncomplicated UTI. The infection rate is higher for Polynesian than Caucasian women. As age increases, the infection rate in males rises, and UTI are a significant cause of illness in the elderly population. Urine itself can inhibit bacterial growth. This phenomenon is variable and incompletely understood. Urea is thought to be urine's principal antibacterial component, its effects augmented by concentrated and acidic urines. Our work with betaines suggests that trigonelline, which is abundant in the diet (especially in coffee and legumes), may also play a role in concentrated urine (usually the first morning urine), <i>Escherichia coli</i>, the major urinary tract pathogen, accumulates betaines, including trigonelline. Trigonelline, unlike other betaines tested, exacerbates the toxic effects of urea. This project aims to establish the conditions in which this property might be used in treating UTI and whether this may explain some of the antibacterial properties of urine. Antibacterial effects of trigonelline will be determined over a range of physiological conditions found in the urinary tract and in the presence of other naturally-occurring betaines like glycine betaine and proline betaine.</p> <p><b>B)</b> In addition, there has been recent data indicating that in diabetic patients, the plasma levels of the compound homocysteine are affected by the amount of glycine betaine and possibly also of trigonelline. We wish to see whether this interrelationship also occurs in healthy individuals and we propose to collect blood samples to measure the above betaines, homocysteine and dimethylglycine. Homocysteine is known to be an independent risk factor for cardiovascular disease.</p>	

Eight to ten people will be asked to take part in the study. They will be selected by the researchers. No-one who is not already a coffee drinker will be considered for the study; nor will anyone who feels that to go for a week without coffee is a great hardship!

The range of achievable levels of trigonelline and proline betaine will be measured in urine and blood following ingestion of foods containing these compounds. Homocysteine and dimethylglycine will also be measured. Baseline low levels will be established by restricting participants to a diet free of foods known to contain high levels of trigonelline and proline betaine for 5 days. Participants will then be given coffee (four cups) over one hour. Urine and blood samples will be collected immediately before ingestion of the food to establish baseline levels. Three blood samples will be taken before ingestion, and eight hourly samples will be taken after ingestion. Each blood sample will be 5 ml. Urine will be sampled hourly as the participants are able.

Antibacterial activity will be determined by measuring the growth of E coli in the urines, using optical density.

To obtain the blood samples, a heparinized catheter will be inserted into the participant's arm for the day. Blood will be taken by Dr Steve Chambers or by a registered nurse. All work on the blood and urine samples will be done either in the Clinical Pathology Laboratory of the Nephrology Department, or in the Biochemistry Laboratory, Canterbury Health Laboratories.

The entire study should be completed within one year, although the work done with volunteers' urine and blood samples is not expected to occupy more than half of that allotted time.

**Time involved:** The entire study will involve the participants for one working day. Participants will spend the day in a clinic room in Christchurch Hospital. Food, entertainment and refreshments will be supplied. The participants will be asked to report on the following day for a final blood and urine sample.

**Samples:** All urines will be midstream urines. These are collected by letting the first few mls of the stream go (in order to flush out skin bacteria) and then passing urine into the container provided. For the study measuring the rates of excretion of the test substances, one early morning urine and eight hourly samples will be collected for each of the four test substances. Blood samples will be drawn from an indwelling heparinized catheter. 5 ml of blood will be drawn each time (total of 55 ml).

## RISKS AND BENEFITS

### 1. Risks and/or inconveniences.

There may be some risk that a true coffee addict may suffer withdrawal symptoms (headache). If you can't start the day without your coffee 'fix' please don't volunteer for this study.

### 2. Benefits.

This is a scientific study and there are no direct benefits to the subjects taking part.

### 3. Therapeutic or non-therapeutic?

This study will measure the levels of certain chemicals which appear in urine and blood after eating or drinking certain substances. These may or may not prove to have activity against bacterial infections in urine or to have some implication in modifying homocysteine levels. This is not a study in which these substances will be used to treat any infection in the participants.

In the event that a preliminary baseline urine shows infection, the person will not be used further in the study but will be advised to seek treatment from their GP in the regular way.

**4. Costs and/or payments.**

There will be no cost to any participant - other than time given in collecting samples. Payment of \$50 to cover any inconvenience is to be offered to each participant. All food and refreshments required will be supplied by the researchers.

**PARTICIPATION**

Your participation is entirely voluntary. You do not have to take part in this study, and whether or not you do it will make no difference to any future care or treatment. If you do choose to take part, you are free to withdraw from the study at any time and you do not have to give any reasons for doing so. If you do withdraw, again it will make no difference to any future care or treatment you may need. If any harmful effects appear, the researcher will stop your participation in the study immediately.

**GENERAL**

At the end of the study, all the results from your urine and blood samples will be available to you, and you are welcome to talk to the researchers about the project at any time.

If you have any questions about the study, you are invited to contact the researchers at any time.

If you have any queries or concerns about your rights as a participant in this study, you may wish to contact a Health and Disability Services Consumer Advocate, telephone 3777501

**CONFIDENTIALITY**

No material which could possibly identify you will be used in any reports on this study. Records of work done will be kept in the Laboratory workbooks which are only available to the researchers and all samples will be coded (by number, not by name).

**RESULTS**

The results of this work will be published in one of the scientific journals, and participants will be sent a summary of results if they wish.

**COMPENSATION**

In the unlikely event of a physical injury as a result of your participation in this study, you will be covered by the accident compensation legislation with its limitations. If you have any questions about ACC please feel free to ask the researcher for more information before you agree to take part in this trial.

7.4.3 Raw data from the coffee trial

Time (hrs:mins)	Subject								
	A	B	D	E	F	G	H	I	J
-0:40	9.9	6.7	4.4	10.3	8.6	20.4	7.0	7.3	11.4
-0:20	9.4	6.3	7.0	10	8.6	20.7	7.6	7.3	11.2
0:00	8.4	5.9	5.9	10.1	8.2	20.8	7.6	6.6	11.1
1:00	9.1	6.3	7.3	10.5	7.5	20.2		7.8	11.8
2:00	10.0	6.5	6.7	10.8	9.6		7.5		11.9
3:00	9.9	6.5	6.9	10.4	8.7	20.6		7.9	12.0
4:00	10.0	6.3	6.9	9.7	9.5		7.1		11.8
5:00	9.8	6.8	6.7	10	9.1	18.8		7.7	11.6
6:00	9.6	6.6	7.0	10.1	9.6		7.1		11.6
7:00	9.4	6.3	6.7	9.2	7.9	20		7.2	11.5
8:00	10.2	6.5		9.9	8.9				11.5

**Table 7.1:** Raw plasma homocysteine results. All values are in  $\mu\text{mol}^{-1}$ . Coffee was consumed between Time 0:00 and Time 1:00.

Time (hrs:mins)	Subject								
	A	B	D	E	F	G	H	I	J
-0:40	31.7	53.7	109	79.2	77.2	42	42	43.5	43.4
-0:20	21.4	56.1	123	83.1	70.5	40.6	61	53.8	40.7
0:00	24.4	64	102	91	70.7	45.9	68.1	86.6	40.2
1:00	26.5	74.9	99	82.1	77.4	44.3	45.8	73.9	38.9
2:00	26.8	59.5	107	82.1	82.4	63.2	48.4	58.8	46.3
3:00	28.8	67.9	102	72.9	67	58.8	52	58.6	44.5
4:00	36.9	56.7	114	72.9	75.3	51.1	46.8	53.6	42.6
5:00	40.7	6.8	122	75.7	65.2	64.1	44.3	67.4	51.1
6:00	34.4	6.6	101	84.8	80.1	53.5	44.1	54.8	56.5
7:00	38.8	6.3	122	67.7	59.2	47.1	41.8	7.2	55.6
8:00	45.1	6.5		72.4	67.2	39.8		65.3	46.8

**Table 7.2:** Raw plasma glycine betaine results. All values are in  $\mu\text{mol}^{-1}$ . Coffee was consumed between Time 0:00 and Time 1:00.

Time (hrs:mins)	Subject								
	A	B	D	E	F	G	H	I	J
-0:40	29.8	29.8	15.3	20.3	19.8	24	29.3	41.1	48.9
-0:20	16	18.4	16.9	18.9	12.9	30.3	25.4	45.2	45.7
0:00	24.7	20.2	25.5	27.3	21.8	20.8	22.7	48.7	60.3
1:00	15.5	29.1	22.5	25.3	29.4	18.4	26.4	48.0	61.0
2:00	27.6	26.6	16.8	18.9	25.7	21.5	35.1	32.0	48.6
3:00	20.3	28.2	21.6	18.1	18.3	17.3	28.9	52.1	53.3
4:00	18.4	30.9	13.6	22.6	15.7	18.5	25.5	47.4	94.7
5:00	24.1	23.1	22.9	15.4	25.6	20.5	17.1	38.0	68.5
6:00	22.8	26.1	19.5	18.4	30	18.3	20.9	52.1	65.1
7:00	19.9	20.1	14.3	22.7	39.4	18.7	22.8	49.8	59.5
8:00	19.1	22.8		19.1	25	26.3		85.3	51.5

**Table 7.3:** Raw plasma trigonelline results. All values are in  $\mu\text{mol}^{-1}$ . Coffee was consumed between Time 0:00 and Time 1:00.

Time (hrs:mins)	Subject								
	A	B	D	E	F	G	H	I	J
-0:40	4	5.4	0.7	2.5	4.3	3.7	2.1	5.1	5.1
-0:20	2	2.8	3.7	4.2	4.2	0.9	1.9	1.8	0.0
0:00	2.9	4.3	0	3.5	6.2	3.4	4.4	0.2	5.6
1:00	3.3	2.9	3.4	2	3.3	2.8	2.3	14.0	5.2
2:00	2.7	1.5	1.7	1.6	6.3	3.9	5	5.9	5.2
3:00	6.4	4.4	2.9	2.7	3.1	7.2	4.3	10.2	4.9
4:00	4.8	2.8	3.6	6.6	3.1	3.1	6.5	2.6	4.2
5:00	6.4	4.2	1.5	4.7	2.5	6.9	4.9	3.5	4.9
6:00	4.2	1.5	1.7	1.5	5.5	2.9	4.4	8.2	4.5
7:00	3.5	2.8	1.4	4.1	5.2	3.4	3.2	4.8	4.2
8:00	3.7	3.3		5.2	1.7	3.5		10.1	7.0

**Table 7.4:** Raw plasma *N,N*-dimethylglycine results. All values are in  $\mu\text{mol}^{-1}$ . Coffee was consumed between Time 0:00 and Time 1:00.

Time (hr:min)	GB ( $\mu\text{mol}^{-1}$ )	Trig ( $\mu\text{mol}^{-1}$ )	Creatinine ( $\text{mmol}^{-1}$ )	Volume (ml)
-0:55			8.4	
-0:20	17.0	1.0	8.6	40
1:40	12.5	1.0	0.1	1000
2:07	5.5	150.1	0.1	480
4:27	26.1	587	2.8	205
6:45	20.0	201.6	5.6	135

**Table 7.5:** Raw urine data, subject A

Time (hr:min)	GB ( $\mu\text{mol}^{-1}$ )	Trig ( $\mu\text{mol}^{-1}$ )	Creatinine ( $\text{mmol}^{-1}$ )	Volume (ml)
-0:48	30.8	473.6	11.1	40
-0:05	33.3	504.6	10.0	20
1:50	12.1	166.4	0.7	500
2:25	21.7	538.0	2.4	90
7:05	13.5	381.9	6.8	300

**Table 7.6:** Raw urine data, subject B

Time (hr:min)	GB ( $\mu\text{mol}^{-1}$ )	Trig ( $\mu\text{mol}^{-1}$ )	Creatinine ( $\text{mmol}^{-1}$ )	Volume (ml)
-0:05	4.6	1.0	4.7	na
2:05	16.3	36.0	0.3	na
3:15	15.0	147.3	0.1	670
4:50	24.4	266.4	0.4	430
7:10	20.5	287.5	1.0	450

**Table 7.7:** Raw urine data, subject D

Time (hr:min)	GB ( $\mu\text{mol}^{-1}$ )	Trig ( $\mu\text{mol}^{-1}$ )	Creatinine ( $\text{mmol}^{-1}$ )	Volume (ml)
-0:02	46.5	71.1	16.1	28
2:20	20.3	65.7	1.9	280
4:15	25.1	480.2	3.8	210
6:47	24.4	356.2	3.3	244

**Table 7.8:** Raw urine data, subject E

Time (hr:min)	GB ( $\mu\text{mol}^{-1}$ )	Trig ( $\mu\text{mol}^{-1}$ )	Creatinine ( $\text{mmol}^{-1}$ )	Volume (ml)
0:22	13.9	10.0	7.2	na
2:10	18.2	36.3	1.2	420
4:10	9.3	214.4	0.8	450
8:03	6.5	224.0	0.8	540

**Table 7.9:** Raw urine data, subject F

Time (hr:min)	GB ( $\mu\text{mol}^{-1}$ )	Trig ( $\mu\text{mol}^{-1}$ )	Creatinine ( $\text{mmol}^{-1}$ )	Volume (ml)
-0:02	11.9	35.6	4.2	182
3:20	3.2	122.2	0.1	700
6:23	8.2	256.4	0.7	556
7:55	5.3	120.3	0.1	578

**Table 7.10:** Raw urine data, subject G

Time (hr:min)	GB ( $\mu\text{mol}\cdot\text{l}^{-1}$ )	Trig ( $\mu\text{mol}\cdot\text{l}^{-1}$ )	Creatinine ( $\text{mmol}\cdot\text{l}^{-1}$ )	Volume (ml)
-0:10	14.9	52.8	1.0	250
0:45	6.7	17.3	0.1	380
1:53	6.3	22.7	0.1	790
2:35	3.3	19.7	0.2	576
3:40	4.0	95.0	0.1	455
4:50	6.2	88.9	0.1	520
6:10	0.1	94.7	0.1	325
7:22	0.1	107.9	0.1	608

**Table 7.11:** Raw urine data, subject H

Time (hr:min)	GB ( $\mu\text{mol}\cdot\text{l}^{-1}$ )	Trig ( $\mu\text{mol}\cdot\text{l}^{-1}$ )	Creatinine ( $\text{mmol}\cdot\text{l}^{-1}$ )	Volume (ml)
-0:15	39.1	76.6	4.9	68
2:25	1.0	97.7	0.2	530
4:30	20.6	244.9	0.2	330
5:42	1.0	49.5	0.2	495
7:03	7.2	95.6	0.6	560

**Table 7.12:** Raw urine data, subject I

Time (hr:min)	GB ( $\mu\text{mol}\cdot\text{l}^{-1}$ )	Trig ( $\mu\text{mol}\cdot\text{l}^{-1}$ )	Creatinine ( $\text{mmol}\cdot\text{l}^{-1}$ )	Volume (ml)
0:13	61.8	76.9	8.2	82
2:45	10.1	63.4	4.0	200
4:15	16.5	195.5	6.1	102
5:55	34.4	310.4	1.2	130

**Table 7.13:** Raw urine data, subject J



## 7.5. Chapter 5

### 7.5.1. Minimal media

The minimal medium of Davis (Davis, 1973) consisting of  $K_2HPO_4$  (7 g),  $KH_2PO_4$  (3 g), sodium citrate (0.5 g),  $(NH_4)_2SO_4$  (1 g),  $MgSO_4$  (0.1 g) and 2.9 g of glucose made up to one litre (osmolality = 175 mosmol/kg), and neutralized (pH = 7) with dropwise addition of 1 M NaOH or HCl as required was used. This was sterilized by passing through a 0.2  $\mu m$  filter (Millipore). Other components of minimal media were added as required. The addition of filter-sterilized urea or NaCl to the solution was done volumetrically. Filter sterilized glycine betaine and trigonelline standards (10 mmol $^{-1}$ ) were and added to make the desired solution.

### 7.5.2. Urine

Urines were obtained from volunteers who had abstained from coffee and sprouts (foods known to contain trigonelline) for at least 2 days. These were filter sterilized, and betaine content was analysed. Urines with detectable levels of trigonelline were discarded, as were urines containing  $>10 \mu mol l^{-1}$  glycine betaine. Urines were pooled to ensure identical growth conditions. Distilled water, 10 mmol $^{-1}$  glycine betaine and 10 mmol $^{-1}$  trigonelline (all filter sterilized) were added to make urines of identical volume and composition except for the betaine content. The glycine betaine and trigonelline additions ranged from 0 to 1000  $\mu mol l^{-1}$ .

### 7.5.3. Betaine uptake measurement

An overnight culture (10 ml) was centrifuged (15 min @ 3000 rpm), and the pellet was washed with isotonic solution (containing either NaCl or NaCl and urea depending on the growth medium). This was centrifuged, and the supernatant was removed. The pellet was resuspended in 2 ml of 10% methanol in acetonitrile and sonicated (5 min). This solution was then derivatized (section 2.5.2) analysed using the alumina system (section 2.5.3.2).

#### 7.5.4. Betaine transfer

To test the accumulation of glycine betaine when cells were already loaded with trigonelline, 20 ml of overnight culture (with trigonelline) was passed through a 0.45  $\mu\text{m}$  filter (Millipore). Iso-osmotic NaCl (or NaCl/urea) solution (5 ml) was passed through the filter to wash the cells. The cells were washed off the filter using 20 ml iso-osmotic NaCl (or NaCl/urea) solution containing 100  $\mu\text{mol l}^{-1}$  glycine betaine. After 5 minutes, the cells were collected and processed as described in section 5.2.3.1.

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