TRAPPING OF METHYLGLYOXAL BY DIETARY COMPOUNDS *IN VITRO*

by

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ABSTRACT OF THE DISSERTATION

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Dissertation Director: Professor Chi-Tang Ho

The importance of reactive dicarbonyl speices has been raising because of its link to many health complications. Diabetes, with the high prevalence and continued investigation on its link to methylglyoxal interested us to initiate this study. We believe that MG is involved in a vicious circle in which the elevated methylglyoxal is caused by the hyperglycemic condition and also by the consequent oxdative stress. On the other hand, the increase of methylglyoxal and oxidative stress deplete antioxidative system including glutathione and its related enzymes. This will then not only worsen the oxidative stress, but also change the route of glycolysis and other metabolisms, ketone body, fructose, etc. The vicious circle is thus turned on. We, therefore, state here that controlling the intra and extra sources and causes of methylglyoxal is of significance.

Our study first established the reliable and sensitive analytical methods for methylglyoxal determination. The combinations of proper derivatization reagents for methylglyoxal and analytical methods, GC or HPLC have been examined. Secondly, we have investigated the methylglyoxal trapping efficiency and mechanisms of dietary compounds. The kinetic studies were carried out and reaction products were characterized by LC/MS.

Tea polyphenol compounds, firstly, have shown the potential health benefits in many aspects including prevention diabetes which has the impact with methylglyoxal and

methylglyoxal related AGEs. They did show the excellent trapping activity of methylglyoxal. The kinetic study and identification of EGCG-methylglyoxal products provides the preliminary understanding of the trapping mechanism by tea.

The second group, dipeptides and tripeptides, are having higher bioavailability compared to tea polyphenol compounds. The chosen dipeptides may trap methylglyoxal via formation of pyrazinone or *S*-(carboxyethyl)cysteine derivatives. With different side chain, most dipeptides represented different reactivity to methylglyoxal and kinetic trends. Moreover, the difference observed between triglycine and diglycine, and the one between Gly-Cys and Cys-Gly make the studying of mechanisms more essential. This varied reactivity combined with the high bioavailability, peptides may be potent to target the methylglyoxal generated at different conditions. Further consideration of their bioavailability and kinetic trends may provide the diverged and useful ways to trap methylglyoxal *in vitro* or *in vivo*.

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ABBREVIATIONS

2,3-DAN	2,3-diaminonaphthalene
AGEs	advanced glycation end products
AMC	7-amido-4-methylcoumarin
CEC	*S*-(carboxyethyl)cysteine
CID	collision-induced dissociation
CMC	S-(carboxymethyl)cysteine
DG	diglycine
EC	(-)-epicatechin
ECG	(-)-epicatechin-3-gallate
EGC	(-)-epigallocatechin
EGCG	(-)-epigallocatechin gallate
ESI	electron spray ionization
FID	flame ionization detector
GA	gallic acid
GAPDH	glyceraldehydes-3 phosphate dehydrogenase
GO	glyoxal
GSH	glutathione
IS	internal standard
LOQ	limit of quantification
MG	methylglyoxal
MQ	2-methylquinoxaline
MS	mass spectrometry
MSD	mass detector
N-Ac-Cys	*N*-acetylcysteine
PFBHA	hydroxylamine hydrochloride
PY	pyrogallol
RCS	reactive carbonyl species
SHR	spontaneously hypertensive rat
SOD	superoxide dismutase
SSAO	semicarbadize-sensitive amine oxidase
TF1	theaflavin
TF2s	theaflavin-monogallates
TF3	theaflavin-3,3′-digallate
TG	triglycine
WKY	Wistar-Kyoto
TLC	thin-layer chromatography

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. Introduction

Methylglyoxal (MG), is one of the reactive carbonyl species (RCS) which is being drawn more and more attention. The regulation of MG levels and its close link to many chronic health disease is potent. Among all, diabetes and its complications may be the most considered one. Hyperglycemia has been confirmed as the most important factor in the onset and progress of diabetic complications from epidemiological and large prospective clinical studies. More and more evidence indicates the increase in reactive carbonyl intermediates is a consequence of hyperglycemia in diabetes. Several studies have shown that higher levels of glyoxal (GO) and MG were observed in diabetic patients' plasma than those in healthy people's plasma. GO and MG, the two major α-dicarbonyl compounds found in humans, are very reactive dicarbonyl species and will lead to the nonenzymatic glycation *in vivo*. Glycation is a complex series of reactions between reducing sugars and amino groups of proteins, lipids and DNA, and will lead to the formation of advanced glycation end products (AGEs). Both AGEs and the dicarbonyl species link to the possible clinical significance in chronic and age-related diseases, which raised the importance and interests for this study.

The endogenous and exogenous sources of MG have been under study. MG in food and beverages may come from sugars, the intermediates of Maillard reaction and lipids. In our body, MG is primarily formed during glycolysis. However, it can be from not only glucose, but also fructose or ethanol. On the other hand, the elevated MG may be from the metabolism of aminoacetone or acetone from lipolysis, and the metabolisms of amino acid, glycine and threonine. Its generation and metabolisms are highly regulated. However, it was shown that under hyperglycemic condition like diabetes, the cells that cannot reduce the glucose insides the cells efficiently, capillary endothelial cells, for

example are easily damaged. MG seems to be involved in the vicious circle initiated with the hyperglycemic and the consequent oxidative stress. The induced increase of MG can then deplete glutathione and its related enzyme destroying the antioxidant system and changing the metabolic routes. As that the oxidative stress cannot be ameliorated as that the circle will be on and on. Moreover, the increase of AGE produced from MG and other RCS may change the signaling and cellular dysfunction, and modify the circulating proteins. In turn the modified proteins are able to bind to the AGE receptors and activate them, which may influence the gene expression directly or indirectly.

Many synthetic organic compounds were demonstrated as the excellent AGE inhibitors. For our purpose, we would like to find dietary compounds to minimize the safety concerns. It is well-known that tea is rich in polyphenolic compounds and with the potential health benefits including prevention of cancer, heart disease, and diabetes. Certain flavonoids have shown more effective inhibition of AGEs formation than aminoguanidine, a well-known AGEs inhibitor. More recently, researchers had documented the effects of added natural phenolic compounds on the generation of reactive carbonyl intermediates from Maillard reaction including MG. It has been reported that drinking Oolong tea significantly decreased the plasma glucose and fructosamine concentrations of diabetes. With the linkage between MG and diabetes as mentioned above, the importance of tea polyphenols in the reduction of MG levels under physiological conditions is illustrated in our study.

Dipeptides and tripeptides were also investigated in our study because of its relatively high bioavailability. It was based on the previous studies on formation of pyrazinone and the *S*-(carboxymethyl)cysteine (CMC). Formation of pyrazinone between

dipeptides and glyoxal was firstly observed at the cooking condition, 100 ^{o}C and pH 5.0. But it was also formed between tripeptide and glyoxal and methylglyoxal at physiological conditions recently. Formation of CMC or CEC (*S*-carboxyethylcysteine) between thiol group and GO or MG would be more reactive and important since thiol group provides the better nucleophilic properties than amine groups. The diversity of the peptides should be able to provide the useful information to design the MG trapping reagents.

We hypothesize that it is possible to trap MG *in vivo* by suitable dietary components such as polyphenols and peptides. So the objectives of this research are:

1) To develop a quantitative method to measure the methylglyoxal in model and food systems;

2) To test the efficiency of tea polyphenol compounds on MG trapping;

3) To investigate the trapping mechanism between MG and selected tea polyphenols;

4) To search for dipeptides and tripeptides which can effectively trap methylglyoxal under physiological conditions;

5) To understand the mechanism of the reaction of methylglyoxal with selected dipeptides and tripeptides.

2. Literature review

2.1 Methylglyoxal and Health Concerns

2.1.1 Role of Methylglyoxal in Diabetic Complications and Hypertension

Diabetes is a heterogeneous disorder and generally accompanied with multiple complications. It involves resistance of glucose and lipid metabolism in peripheral tissues to the biological activity of insulin and inadequate insulin secretion by pancreatic β cells, Epidemiological and large prospective clinical studies have confirmed that hyperglycemia is the most important factor in the onset and progress of diabetic complications, both in Type 1 (insulin-dependent) and 2 diabetes mellitus (The Diabetes Control and Complications Trial Research Group, 1993; UK Prospective Diabetes Study Group, 1998a,b). Increasing evidence identifies the formation of AGEs as the major pathogenic link between hyperglycemia and diabetes related complications (Singh *et al.*, 2001).

Nonenzymatic glycation is a complex series of reactions between reducing sugars and amino groups of amino acids, peptides and proteins, lipids and DNA. As the first step of AGEs formation, proteins in the tissues are modified by reducing sugars (e.g., glucose) through the reaction between a free amino group of proteins and a carbonyl group of the sugars, leading to the formation of fructosamines via a Schiff base by Amadori rearrangement. Then, both Schiff's base and Amadori product further undergoes a series of reactions through dicarbonyl intermediates [e.g., glyoxal (GO), methylglyoxal (MG) and 3-deoxyglucosone], to form AGEs (Singh *et al*., 2001). GO and MG, the two major α-dicarbonyl compounds found in humans, are extremely reactive and readily modify lysine, arginine, and cysteine residues on proteins (Nagaraj *et al*., 2002). Reactive

carbonyl compounds such as GO and MG have recently attracted much attention because of their possible clinical significance in chronic and age-related diseases. They are considered to accumulate in body fluids and tissues mainly by accelerated oxidative stress, and modify proteins, DNAs and phospholipids to form biologically active adducts such as AGEs (Baynes and Thorpe, 1999; Onorato, Thorpe, and Baynes, 1998; McLellan, *et al*., 1994). It has been shown that dicarbonyl compounds are more reactive than reducing sugars and are more important for cross-linking proteins in the glycation process (Baynes and Thorpe, 1999; Onorato, Thorpe, and Baynes, 1998; McLellan *et al.*, 1994). More and more evidence indicates the increase in reactive carbonyl intermediates is a consequence of hyperglycemia in diabetes. Carbonyl stress leads to increased modification of proteins, followed by oxidant stress and tissue damage (Baynes and Thorpe, 1999; Onorato *et al.*, 1998; McLellan *et al*., 1994). Several studies have shown that higher levels of GO and MG were observed in diabetic patients' plasma than those in healthy people's plasma (Odani *et al.,* 1999; Lapolla *et al*., 2003; Khuhawar *et al*., 2006). In a most recent report (Khuhawar *et al*., 2006), the amount of MG from diabetic patients was found to be 16-27 μg/dL as compared with the normal subjects of 3.0-7.0 μg/dL. Several dicarbonyl-derived products in proteins from diabetic individuals have been identified; these include imidazolium crosslinks, imidazolysine (Nagaraj *et al*., 1996; Frye *et al.*, 1998), carboxymethyllysine (Reddy *et al*., 1995), GOLD (glyoxal-lysine dimer) (Odani *et al*., 1998), carboxyethyllysine (Ahmed *et al*., 1997), and argpyrimidine (Shipanova *et al*., 1997; Oya *et al*., 1999). Thus, decreasing the levels of GO and MG will be a useful approach to prevent the formation of AGEs. Some therapeutic agents such as aminoguanidine, L-arginine, OPB-9195, tenilsetam, pyridoxamine, and

metformin have been reported to trap reactive carbonyl compounds, thereby preventing the formation of AGEs and protein crosslinks (Voziyan *et al.*, 2002).

Hyperglycemia-induced tissue damage focuses on capillary endothelial cells in the retina, mesangial cells in the renal glomerulus, and neurons and Schwann cells in peripheral nerves. All of those cells that cannot reduce the glucose inside the cells efficiently are easily damaged (Brownlee, 2005). Hyperglycemia causes the increased polyol pathway flux in which aldose reductase plays an important role. Normally, this enzyme is used to detoxify the aldehydes in the cells to inactive alcohols, but it also reduces glucose to sorbitol consuming the cofactor NADPH when the glucose concentration is high. The consumption of NADPH will then deplete the glutathione and also increase the susceptibility to oxidative stress. Moreover, hyperglycemia leads to the increase of AGE precursors, such as methylglyoxal (MG), which may change the signaling and cellular dysfunction, and may modify the circulating proteins. In turn the modified proteins are able to bind to the AGE receptors and activate them. The binding to the receptors then increases the production of the inflammatory cytokins and a series of pathological events. On the other hand, MG can bind to the glutathione forming lactaldehyde which can be a better substrate for aldose reductase to further reduce it to propanediol (Vander Jagt *et al.*, 1992, 2001). However, at low glutathione concentrations, aldose reductase can convert methylglyoxal to acetol, which was found to accumulate in diabetic patients (Vander Jagt *et al*., 1992). Even though the second reduction is hard, aldose reductase seems to play a more important role than glyoxalase, as the glutathione concentration is low.

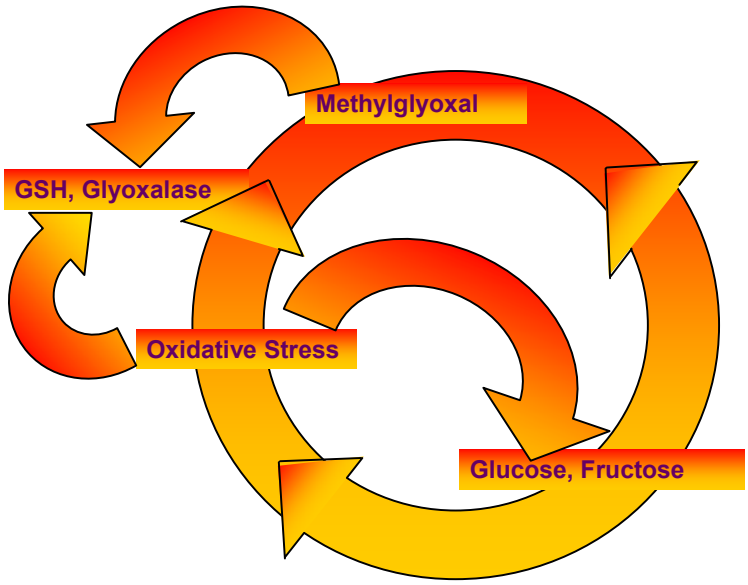


Figure 1. The proposed vicious circle caused by MG.

2.1.2 MG and Other Health Complications

Hypertension can be induced by MG or a diet high in sucrose or fructose sweeteners (Hallfrisch *et al.,* 1983; Israel *et al.,* 1983; Reaven 1991; Reiser *et al.*, 1989). In addition, treating Wistar-Kyoto (WKY) rats with 0.2-0.8% MG in drinking water for a week increased their blood pressure (Vasdev *et al*., 1998). An elevated level of MG has been reported in the plasma and aorta of spontaneously hypertensive rat (SHR) compared to the normotensive WKY rats (Wang *et al*., 2004; 2005) but there is no difference in the blood glucose level. The blood pressure of 8-week old SHR was significantly higher than 8-week old WKY rats, and was associated with the increased MG and MG-induced AGEs. One of the possible explanations is that MG can directly bind arginine residue of glutathione reductase in a NADPH independent way (Vander Jagr *et al.*, 1997). This reaction then decreases the enzyme scavenging efficiency for the free radicals. This again provides the linkage between glutathione and MG levels.

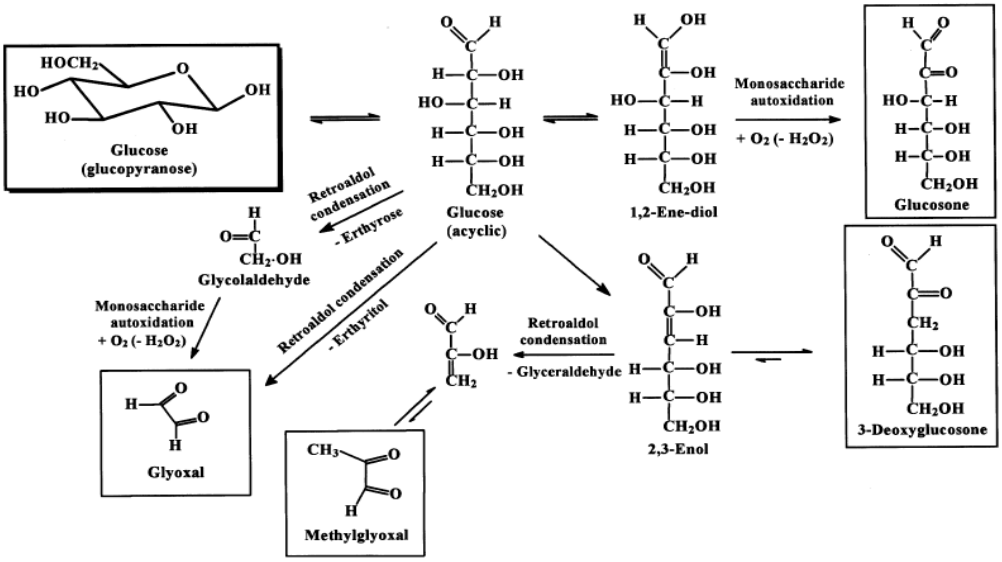
2.2 Methylglyoxal

Methylglyoxal (MG), also called pyruvaldehyde, is one of the reactive carbonyl species. It is present in three forms in aqueous solution in rapid equilibrium as shown below (Creighrom *et al.*, 1988; Rae *et al.*, 1990) Among all, monohydrate is the most (71%) followed by dihydrate (28%) as anhydrated form is only about 1%.

$$\underset{\text{Unhydrated}}{CH_3COCHO} \overset{+H_2O}{\underset{-H_2O}{\rightleftharpoons}} \underset{\text{Monohydrate}}{CH_3COCH(OH)_2} \overset{+H_2O}{\underset{-H_2O}{\rightleftharpoons}} \underset{\text{Dihydrate}}{CH_3C(OH)_2CH(OH)_2}$$

2.2.1 Sources of Methylglyoxal in Foods

MG in food and beverages may come from sugars, the intermediates of Maillard reaction and lipids. In addition, microorganisms growing during the processing and/or storage might generate MG as well (Nemet *et al.,* 2006). MG is generated from carbohydrates by auto-oxidation, or fragmentation and retro-aldol reaction through Maillard reaction intermediates (Fig. 2 and Fig. 3).



Minhas *et al.* Biochem. J. (1999) 344, 109-116

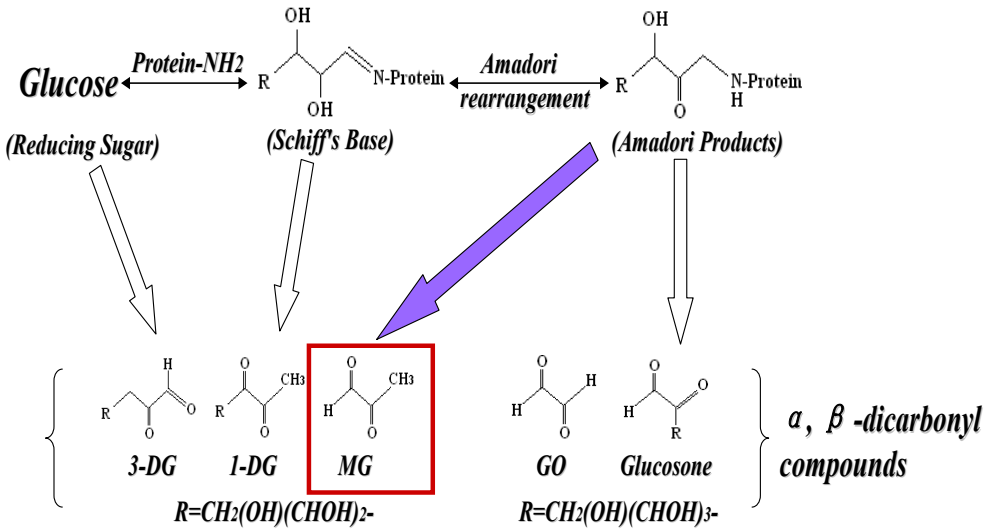


Figure 3. The generation of MG from Maillard reaction.

2.2.2 Formation and Mechanism of Methylglyoxal *in vivo*

Methylglyoxal is primarily formed during glycolysis in cells (Fig. 4). It may be degraded from triose phosphate intermediate such as dihydroxyacetone phosphate and glyceraldehydes 3-phosphate. This process can be non-enzymatic one, or be catalyzed by triosephosphate isomerase (Pompliano *et al.*, 1990) and by methylglyoxal synthase (Ray and Ray, 1981). Except being transformed from glucose, fructose or ethanol, the elevated MG may be from the metabolism of aminoacetone or acetone from lipolysis catalyzed by semicarbazide-sensitive amine oxidase (SSAO) or acetol mono-oxygenase (Wu, 2006; Kalapos, 1999; Brownlee, 2005; Nemet *et al*., 2006). The metabolisms of amino acid, glycine and threonine, can also produce MG as the intermediate.

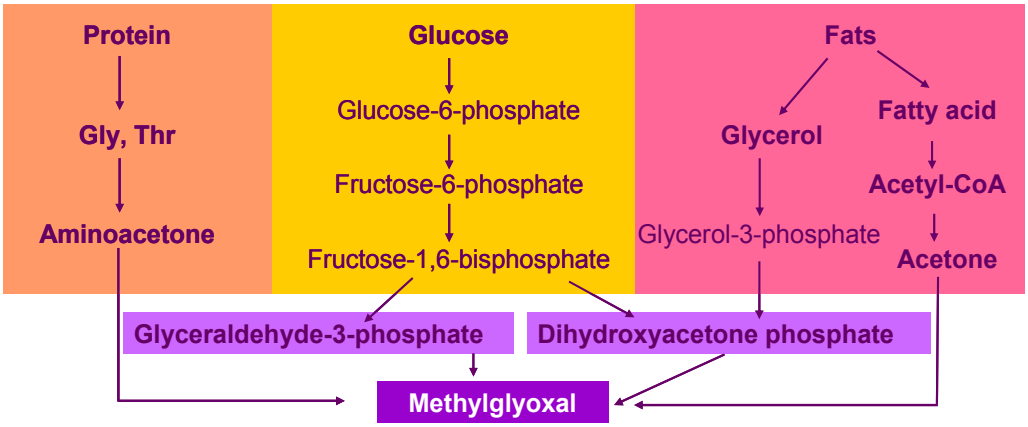


Figure 4. The sources and generating pathways of MG *in vivo*.

In culture cells, the major source of MG is from the nonenzymatic fragmentation of triose phosphates (Thornalley, 1996). In human red blood cells, for example, MG generation would be increased by adding glucose, fructose, dihydroxy-acetone and D-glyceraldehydes, and acetone, which stimulates the flux of triose phosphates (Thornalley, 1988; Phillips and Thornalley, 1993b).

2.3 Detoxification of Methylglyoxal *in vivo*

The rate of MG formation is approximately 120 μM/day which is about 0.1% of the flux of glucose under normal conditions measured *in vitro* red blood cells (Thornalley, 1988; Phillips and Thornalley, 1993). Even with such a small fraction, MG is of importance and of threaten because of its high reactivity. Detoxification of MG is thus playing an important protecting function. MG can be detoxified by the enzymatic systems. At least four systems have been known (Vander Jagt and Hunsaker, 2003). One is the glyoxalase system which converts MG to S-D-lactoylglutathione and D-lactate in the cytosol of all kinds of cells. D-lactate is then metabolized to pyruvate by 2-hydroxyacid

dehydrogenase in the mitochrondria in mammalian systems (Thornalley, 1993). This system composes of two enzymes, glyoxalase I and II, and is GSH dependent. At the first step, reduced GSH binds to MG forming hemithioacetal which is then catalyzed to S-D-lactoyl-glutathione by glyoxalase I. Glyoxalase II is involved at the second step, in which S-D-lactoyl-glutathione is catalyzed to D-lactate. The concentration of glyoxalase I was measured as 0.2 μg/mg protein in most human tissues (Larsen *et al*., 1985). In addition, K_M and k_{cat} values of glyoxalase I were 120 μM and 1.8×10^{1} S^{-1} (Allen *et al*., 1993) with the Keq of the formation of hemithioacetal being of 333 M^{-1} (Vander Jagt *et al*., 1975) and saturated NADPH conditions.

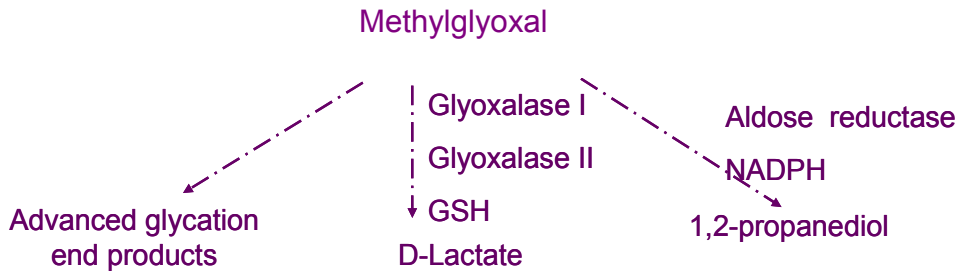


Figure 5. The metabolism of MG *in vivo*.

The second system is NADPH-dependent aldose reductase (Grimshaw, 1992). MG is converted to hydroxyacetone mostly (95%) and D-lactaldehyde in small portion (Vander Jagt *et al.*, 1992). The former will be further reduced to L-propane-1,2-diol, while the later will be catalyzed as D-propane-1,2-diol. This system might only be more

important than glyoxalase system in kidney medulla because it only appears relatively high attribution there. The K_M value of MG was 2.4 μM, k_{cat} as 1.7 S^{-1} and k_{cat}/K_M was 4.2×10^4 $M^{-1}S^{-1}$ (Cook *et al.*, 1995). Mathematically calculated, glyoxalase I is normally 10-40 times more efficient in human tissues than aldose reductase except in the kidney where they are estimated to function equally (Allen *et al*., 1993).

Besides, 2-betaine aldehyde dehydrogenase is able to catalyze MG to pyruvate (Izaguirre, Kikonogo, and Pietruszko, 1998). Although its efficiency is much lower than its own substrate, betaine aldehyde. Among all the studied aldehyde dehydrogenases, MG has its specific one as 2-oxoaldehyde dehydrogenase which is primarily in liver (Dunkerton and James, 1975; Vander Jagt and Hunsaker, 2003). It was suggested that a vicinal amine-alcohol or an amine like glycine was needed (Vander Jagt and Hunsaker, 2003)

In the streptozotocin-induced diabetic rats study, the concentration of MG was increased in most tissues, and D-lactate was increased in the lens and blood of diabetic rats (Phillips *et al*., 1993). Application of the aldose reductase inhibitor, Statil, prevented these MG increases in most tissues except in the kidney cortex compared to normal controls, and D-lactate increase was partially prevented in blood but not in the lens. The influence of MG formation by the inhibitor relates all the NADPH-dependent steps including the inhibited sorbitol pathway influx to triosephosphate leading to the decrease of MG formation, the prevention of the depletion of glutathione (GSH) associated with NADPH oxidation, and surely the function of the enzymatic conversion of MG to hydroxyacetone and D-lactaldehyde. The increase of D-lactate may support the increase burden of glyoxalase system.

The increased glyoxalse I activity was observed in diabetic patients (McLellan *et al*., 1994). Elevated MG induced by high glucose (25 mM) in diabetic mouse lenses was accompanied with an increased level of mRNA amounts and the activity of glyoxalase I (Staniszewske and Nagarag, 2006). However, the depletion of GSH seemed not to affect MG level in this study. In other study, two months of low dose intake (1% MG in drinking water) decreased the GSH content and GSH-S-transferase activity in the tested mice (Ankrah and Appiah-Opong, 1999). In addition, the glucose tolerance and the red blood cell capacity to retract oxidative stress were impaired compared with the controls. All these suggested the activity of glyoxalase I is upregulated by MG level, however, the system cannot compensate the dramatic increases due to the other systems that regulate the redox statue. Some study has shown that MG did bind to the cysteine residue in the enzyme. On the other hand, MG's binding to GSH has been revealed and may be more critical.

2.4 The Concentration of Methylglyoxal *in vivo*

In a most recent study, the concentration of MG is ranged between 0.160 and 0.270 μg/mL with coefficient of variation (C. V.) 2.5-4.6% in diabetic and ketosis patients' serum (n=15) (Khuhawar and Kandhro, 2006), and is 0.030-0.070 μg/mL (C.V. 1.6-4.8) in the normal subjects' (n=15) (Table 1). The MG was derivatized by Meso-stilbenediamine and measured by HPLC in this study. Other study derivatized MG with 2,3-diaminonapththalene (2,3-DAN) and detected MG by ESI/LC/MS (Odani *et al*., 1999). MG is 158 ng/mL in diabetic patients, 110 ng/mL in uremic pateints, and 47 ng/mL in normal subjects. Another study using GC followed by derivatization with

PFBHA evaluated ten diabetic patients (29.3 μg/mL) and control (8.5 μg/mL) (Lapplla *et al*., 2003).

Human plasma MG level in different studies			
MG (μg/dL)		**Quantifying method†**	**Source**
Patients	**Control**		
15.8±4.6 (n=20)	4.7 ± 1.2 (n=15)	2,3-diaminonaphthalene; 3,4-hexanedione; ESI/LC/MS	Odani, Hinzato, and Matsumoto, 1999
2930±550 (n=10)	850 ± 50 (n=20)	Ethanol; PFBHA; *o*-chlorobenzaldehyde; GC/MS	Lapolla *et al*., 2005
20.6±3.8 (n*=15)	4.9 ± 1.2 (n=15)	Methanol; Meso-stilbenediamine; HPLC (358 nm)	Khuhawar and Kandhro, 2006

† Quantifying method is listed with the sequence of deproteinization agents, derivatization agents, internal standard, and equipment.
* This study included both diabetes and ketosis patients.

Table 1. The human plasma MG level in different studies.

The MG concentration in patients is about three times as high as in the normal subjects although the patients' diseases were not indicated clearly in Khuhawar and Kandhro's study (Khuhawar and Kandhro, 2006). This was consistent as other studies (Odani *et al*., 1999).

2.4.1 Animal study

The concentration of MG in blood and tissue samples from streptozotocin-induced diabetic rats was measured by Phillips *et al.* (1993). It showed that MG in the kidney cortex and medulla, lens and blood were increased in diabetic rats, compared to normal

controls. The MG concentration in blood was 0.22 nmol/mL in the diabetic mice and was 0.16 nmol/mL in the control. The difference was not as big as in the human subjects, however, the p value ANOVA of 0.003 showed the significant difference. The most significant difference between two groups among all tissues tested was in the kidney cortex with the P value of 0.0001.

2.5 Methylglyoxal and Advanced Glycation Endproducts

Increased MG increases the AGE production. AGEs are found in plasma, cells, and tissues and accumulate in the arterial wall, the kidney mesangium, and glomerular and other basement membranes (Gugliucci, 2000). Some examples of MG related AGE were showed at figure 6. Accumulation of AGEs in long-lived proteins contributes to the age-related increase in brown color and fluorescence, poor solubility of lens crystallins, and to the gradual cross-lining and decrease in elasticity of connective tissue collagens with age (Singh *et al.,* 2001*;* Jakus and Rietbrock*,* 2004*;* Gugliucci*,* 2000*)*. These processes are enhanced in patients with diabetes. Formation of AGEs increases at a great rate than the increase in blood glucose; this suggests that even moderate elevations in diabetic blood glucose levels result in substantial increases in AGEs accumulation (Singh *et al*., 2001; Jakus and Rietbrock, 2004; Gugliucci, 2000).

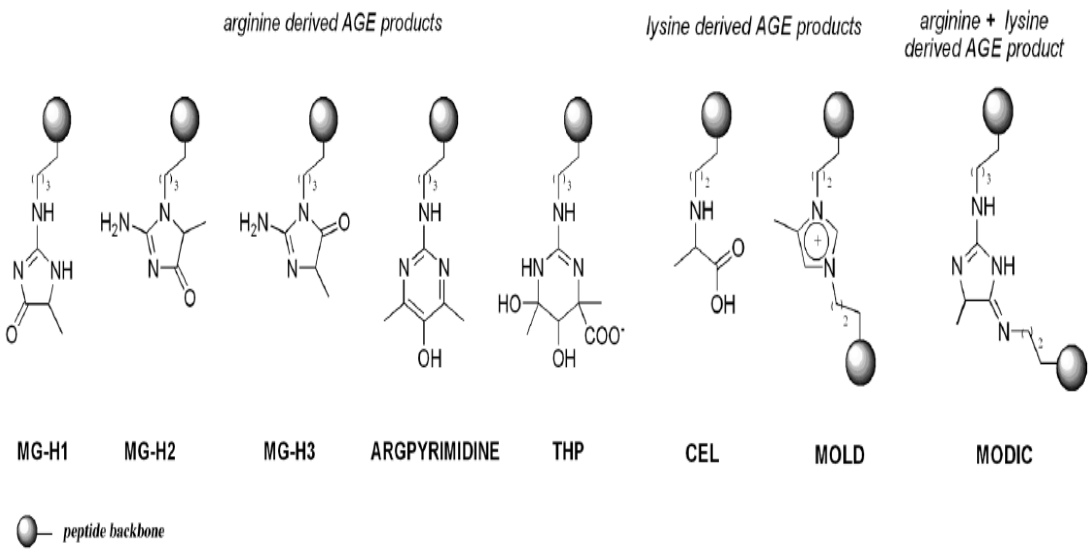


Figure 6. Examples of MG related AGE. (Nemet, *et al.*, 2006)

Besides AGE itself, some studies found that the receptor of AGE (RAGE) also applied to other diabetes complications including inflammation, thrombosis, angiogenesis and tissue injury. In addition, RAGE is related to the depletion of glutathione, which leads to oxidative stress. This contributes to MG, AGE and other oxidative stress related reactions. This breaks down defensive enzyme systems, such as glyoxalase. It is because glyoxalase needs glutathione to complete the detoxification of reactive dicarbonyl compounds and RAGE. This combined effect will not only hinder the detoxification of MG but also stimulate all pathways to generate MG, and consequently to generate AGE.

The hyperglycemia-induced pathogenic mechanism has been linked to the overproduction of superoxide by mitochondrial electron transport chain (Fig. 1) (Brownlee, 2005). More glucose oxidized in the TCA cycle pushes more electron donors into the electron transport chains in the mitochondria. Reaching the threshold will finally

block the electrons back to coenzyme Q and generate superoxide which needs to be converted to water and oxygen by superoxide dismutase (SOD). The overproduction of superoxide will impair DNA and release the poly(ADP-ribose) polymerase from the nucleus, which decrease glyceraldehydes-3 phosphate dehydrogenase (GAPDH). It then activates all the hyperglycemia-induced subsequences. All these provide the linkage between glycemic and oxidative stress, and also reveal the importance of MG as the active intermediate of the glucose metabolism.

2.6 Analytical Methods for Quantification of Glyoxal and Methylglyoxal

To quantify MG, derivatization process is needed before the chromatographic analysis. Several derivatization agents listed in Table 1 including diamino derivatives of benzene and naphthalene, react with MG to form the quinoxalines. Quinoxalines have been analyzed and quantified with HPLC and can be monitored by UV detector at 300-360 nm, by fluorescent detector at 300-360 nm with excitation wavelengths and 380-450 nm with emission wavelengths, or mass detector (MS). Other agents like 6-hydroxy-2,4,5-triaminopyrimidine forming pteridin derivative, and cysteamine forming 2-acetylthiazolidine, have also been analyzed by HPLC. The reverse phase HPLC column is often applied.

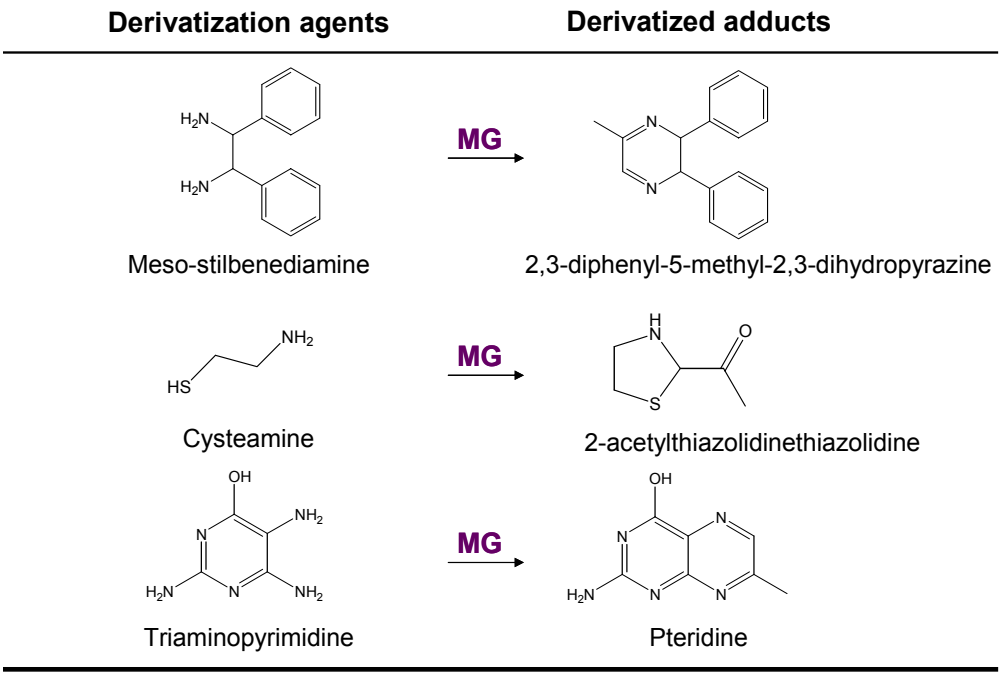


Fig 7. Examples of derivatization reagents for MG analysis and their adduct products.

For GC method, *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine can be detected either on MS/SIM detector, electron-capture detector, or flame photometric detector. 1,2-diaminobenzene derivatives of MG can be analyzed with MS/SIM or on specific nitrogen phosphorus detector.

For the biological samples, extra procedure might be needed to separate MG from proteins. More than 90% of MG was demonstrated to be bound to proteins as that perchloric acid was necessary to be used as a deproteinization agent (Nemet *et al*., 2006). Another benefit of the use of perchloric acid is to keep the samples in low pH, which can prevent degradation of dihydroxyacetone phosphate and glyceraldehydes 3-phosphate to MG.

Table 2 Examples of derivatization methods			
	Derivatization reagent	Derivatives products	Detector
HPLC	6-hydroxy-2,4,5-triaminopyrimidine cysteamine mesostilbenediamine	Pteridin 2-acetylthiazolidine 2,3-diphenyl-5-methyl-2,3-dihydropyrazine	
GC	1,2-diaminobenzene	Quinoxaline	MS-SIM NPD
	O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA)	Oxime	MS/SIM EDC NPD FPD
EDC: Electron-capture detector NPD: nitrogen phosphorus detector FPD: flame photometric detector			

Table 2. Examples of derivatization methods for MG analysis. (Nemet, *et al*., 2006)

2.7 Effects of Polyphenols on Diabetes and Related Complication

It is well-known that tea is rich in polyphenolic compounds. Many studies in humans, animal models, and cell lines suggest potential health benefits from the consumption of tea, including prevention of cancer, heart disease, and diabetes (Yang *et al*., 2002; Higdon and Frei, 2003). It has been reported that drinking Oolong tea could significantly decrease the plasma glucose and fructosamine concentrations of diabetes patients (Hosoda *et al.*, 2003). A more recent study showed that green tea promoted glucose metabolism in healthy human volunteers at 1.5 g/body in oral glucose tolerance tests and lowered blood glucose levels in diabetic db+/db+ mice and streptozotocin-diabetic mice (Tsuneki *et al*., 2004). Rutter *et al.* reported that green tea extract was able to delay collagen aging in C57BL/6 mice by blocking fluorescent AGEs formation and collagen crosslinking (Rutter *et al*., 2003).

Many synthetic organic compounds were demonstrated to have high values in AGE inhibitions (Rahbar *et al.*, 2000). Certain flavonoids have shown more effective

inhibition of AGEs formation than aminoguanidine, a well-known AGEs inhibitor (Wu and Yen, 2005). More recently, researchers had documented the effects of added natural phenolic compounds on the generation of reactive carbonyl intermediates from Maillard reaction including MG (Peterson and Totlani, 2005; Totlani and Peterson, 2006). In the present study, the importance of polyphenols in the reduction of MG levels under physiological conditions is illustrated.

2.8 MG Trapping and Peptides

2.8.1 Formation of Pyrazinone between α-Dicarbonyl Compounds and Peptides

The formation of pyrazinone between dipeptides and glyoxal was firstly proposed by van Chuyen *et al.* (1973). It was observed that a series of pyrazinones, 2-(3′-alkyl-2-oxo-pyrazin-1′-yl) alkanoic acid, were generated at the cooking condition, 100 ^{o}C and pH 5.0. Recently, the pyrazinones produced between tripeptide and glyoxal and methylglyoxal have also been reported at physiological conditions (pH 7.4, 37 ^{o}C) (Krause *et al*., 2004). The reaction scheme between MG and dipeptides is shown in Figure 8. MG adds to the primary amino group forming a Schiff base. After tautomerization, the amido nitrogen of the first peptide bond attacks the remaining carbonyl C-atom. It is finally followed by an elimination of a second molecule of water. It has been observed that 5-methyl-pyrazinone derivative is the main product in the reaction between Gly-Ala-Phe and MG (Krause *et al*., 2004). It is supported with the observation that the aldehyde group of methylglyoxal is hydrated in aqueous solution (Thornalley, 1996), as it is the ketone group and not the aldehyde group that is targeted by the primary amino group at the first step. It was also mentioned that the compound

was relatively stable as it was examined at pH 2.0 to 9.1 at 60 ^{o}C for 3 days. In van Chuyen *et al.*'s work, they observed that the browning degree of triglycine was higher than diglycine which was higher than glycine. This finding indicated that the pK_2 of a peptides rather than the availability of free amino acids or peptides was important to react with the carbonyl compounds. The reactivity of the amino group turned out to be more important. The pK_2 of Gly-Gly is 8.25 while triglycine is 7.91, which means triglycine has the higher reactivity than diglycine.

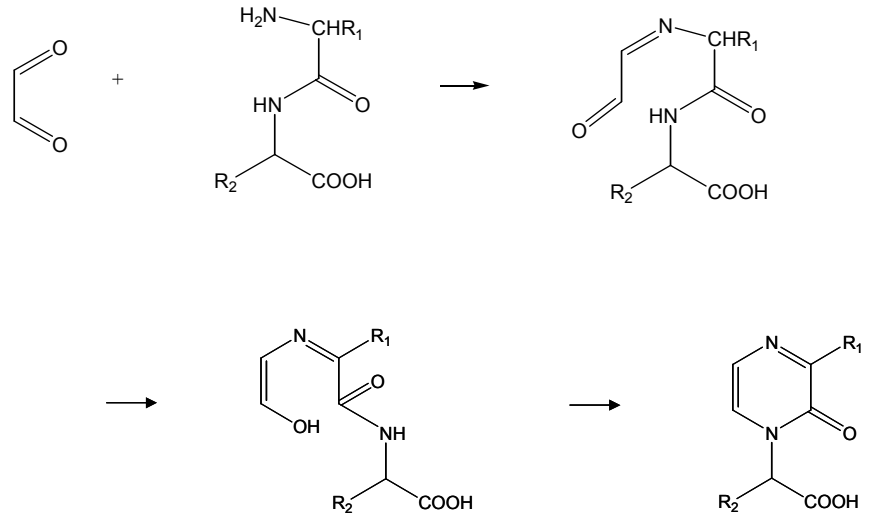


Figure 8. Mechanism of pyrazinone formation from glyoxal and dipeptides proposed by van Chuyen, 1973.

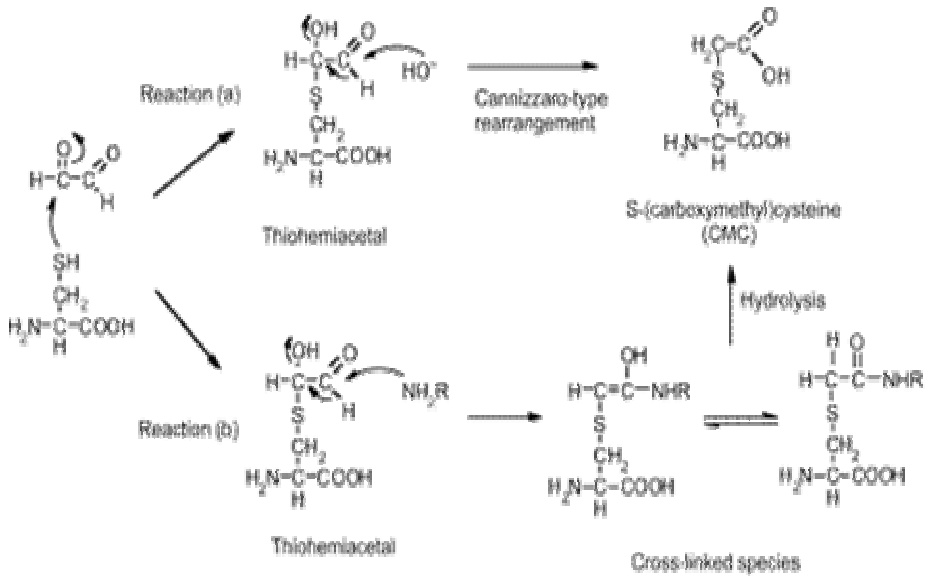
It was shown that the reactivity of trapping MG by *N*-terminal of the peptide and the quanidino function of arginine were similar under both separate and mixing incubation (Krause *et al*., 2004). The author suggested the *N*-termini of peptides are as

important as arginine for reactions with α-dicarbonyl compounds. In addition, the study showed the preferred formation of the pyrazinone structure at the B-chain (5.9±0.6%) than at the A chain (2.0±0.4%) of insulin. The reaction rate was suggested to be influenced by the unprotonated amino groups. So the lower pKs value of the α-amino group of *N*-terminal phenylalanine at B-chain (pKs=7.1) compared to glycine at the A-chain (pKs=8.4) provided the higher reactivity supported the author's observation. In addition, the effect of neighboring groups should be considered as well, but both reacting residues should be accessible for GO according to the structure study. On the other hand, the reaction of GO and MG might differ as that we cannot conclude if MG will react with insulin at the same phenomena.

2.8.2 Reaction of α-Dicarbonyl Compounds with Thiol Groups

S-(carboxymethyl)cysteine (CMC) derivatives were identified as the adducts of the reaction of glyoxal and thiol groups on amino acids, peptides, and proteins by Zeng and Davies. (2005). Formation mechanisms were also proposed as shown in Figure 9. There were two pathways proposed here. At the reaction a, formation of thiohemiacetals occurs first reversibly. The thiohemiacetal can then undergo an intramolecular Cannizzaro rearrangement to form the CMC. At the reaction b, considering there is free amine existing like GSH or peptides, the formation of a cross-linked species followed by the hydrolysis might also lead to CMC. The *N*-acetyl-Cys (*N*-Ac-Cys) reacting with MG at 37 ^{o}C for 2 hours formed the adduct at m/z = 236 (163+72+1). In addition, the similar adduct was detected when peptide *N*-Ac-Leu-Val-Cys-Asp incubated with either MG or glyoxal. However, CMC derivatives was not generated when the *N*-terminal of peptides

was blocked by 7-amido-4-methylcoumarin (AMC), while there was only a single major product, the modification of thiol group of peptide by dicarbonyl compounds, was found. It was also shown that more CMC was generated in enzyme (creatine kinase) than peptide Val-Thr-Cys-Gly. This can be explained by which the anion thiol is the better nucleophile than the parent thiol, and the pKa of the Cys residue in enzyme is always lower, pKa = 5.6 for creatine (Wang *et al.*, 2001), than the free amino acid (Dawson *et al.*, 1986).



Zeng and Davies, 2005

Figure 9. Formation of S-(carboxymethyl)cysteine from glyoxal and cysteine proposed by Zeng and Davies, 2005.

In vivo study CMC has also been detected more in diabetes than in normal subjects (Ubuku, Kodama, and Mizubara, 1967; Alt *et al*., 2004; Thorpe and Baynes, 2003). CMC was detected when incubation of high concentration glucose (1 M) with BSA for 1 week (Zeng and Davies, 2005). CMC might be concerned as an AGE which

might lead to the biological dysfunction, on the other hand, it might provide another possibility in designing the MG trapping reagents. Free CMC, or CMC hydrolyzed from proteins has been shown stable at 37 ^{o}C for one day (Dubruc *et al.*, 1987) and 4 ^{o}C for 30 days (Suntornsuk, 2001).

3. Materials and Methods

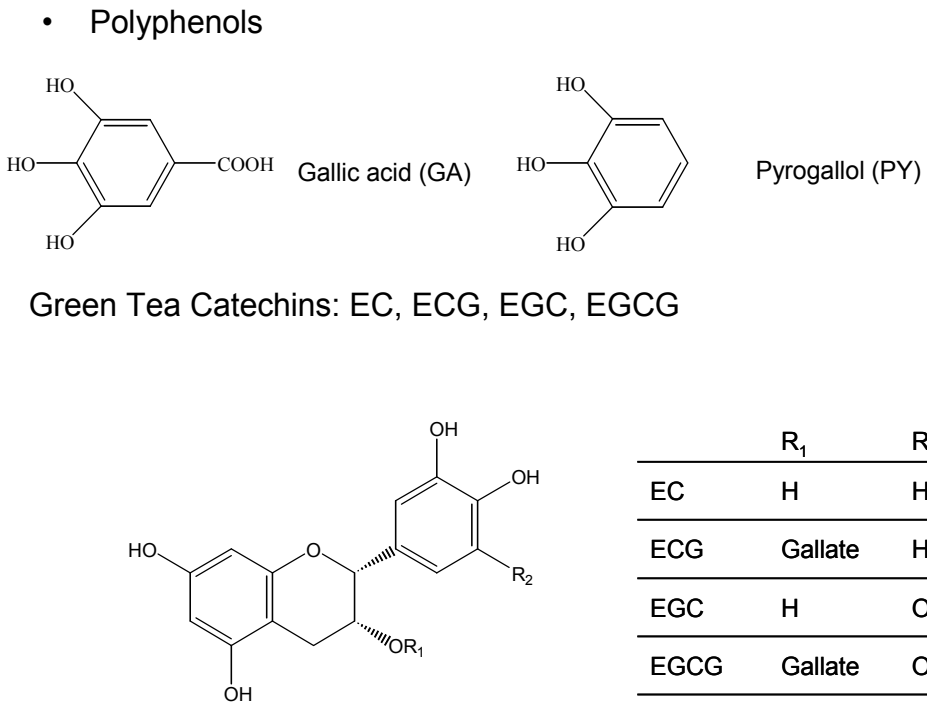
3.1 Materials

The dicarbonyl molecule used in this study was methylglyoxal (MG). MG, 40 wt. % in water, phosphate buffered saline (PBS; pH 7.4), *O*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride (PFBHA), gallic acid (GA), pyrogallol (PY), (-)-epicatechin (EC), 1,2-diaminobenzene and 2-methylquinoxaline were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO). 2-Chlorobenzaldehyde (internal standard; IS) and tannase from *Aspergillus Ficuum* were purchased from Fluka (Milwaukee, WI). 22.2 mL glass threaded vials (23 x 85 mm; O.D. x H), absolute ethanol, hexanes, ethyl acetate, acetonitrile, water (HPLC grade), and methylene chloride were purchased from Fisher Scientific (Fairlawn, NJ, USA). 95% Ethanol was purchased from VWR Scientific (South Plainfield, NJ). (-)-Epigallocatechin gallate (EGCG; 100% pure) was provided by Mitsui Norin Co. Ltd. (Shizuoka, Japan). Thin-layer chromatography (TLC) was performed on 250 μm thickness, 2-25 μm particle size TLC plates (Sigma-Aldrich). Theaflavins 28% crude extract was obtained from Hunan Kinglong Bio-resource Co., Ltd. (Hunan, China).

Cys, *N*-acetylcysteine, Gly-Gly, Gly-Leu, Gly-Phe, Gly-Pro, Gly-Ser, Gly-Gly-Gly were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO). Cys-Gly, Gly-Cys, and γ-Glu-Cys were gifts from Kohjin Co., Ltd (Tokyo, Japan).

3.1.1 Preparation of (-)-Epigallocatechin (EGC), (-)-Epicatechin-3-gallate (ECG), Theaflavin (TF1), Theaflavin-monogallates (TF2s) and Theaflavin-3,3′-digallate (TF3)

In the reaction of EGCG hydrolysis reaction: 300 mg EGCG was dissolved in 10 mL, 0.05 M, pH 5.0 phosphate-citrate buffer solution. 100 mg tannase was added to the solution and stirred continuously for 20 min. The reaction solution was dried under vacuum. After applying reaction mixtures onto a Sephadex LH-20 column which was eluted with 95% ethanol, pure EGC was collected for study.



	R_1	R_2
EC	H	H
ECG	Gallate	H
EGC	H	OH
EGCG	Gallate	OH

Figure 10. Structures of gallic acid, pyrogallol and green tea catechins.

Ethyl acetate fraction of theaflavin extract was subjected to a Sephadex LH-20 column and eluted with acetone solution (40%; v/v). According to their elution sequences, four fractions were collected. They are catechins, TF1, TF2 and TF3. ECG was obtained from catechins fraction followed by the same procedure as EGC purification. The emphasis on TF2s composition was further pointed out here. TF2s was a mixture of theaflavin-3-monogallate (TF2) and theaflavin-3′-monogallate (TF2′). They cannot be isolated to pure compounds under LH-20 chromatography. Their composition ratio for TF2: TF2′ was 3:1 after quantified by HPLC method (Huang *et al.*, 2006).

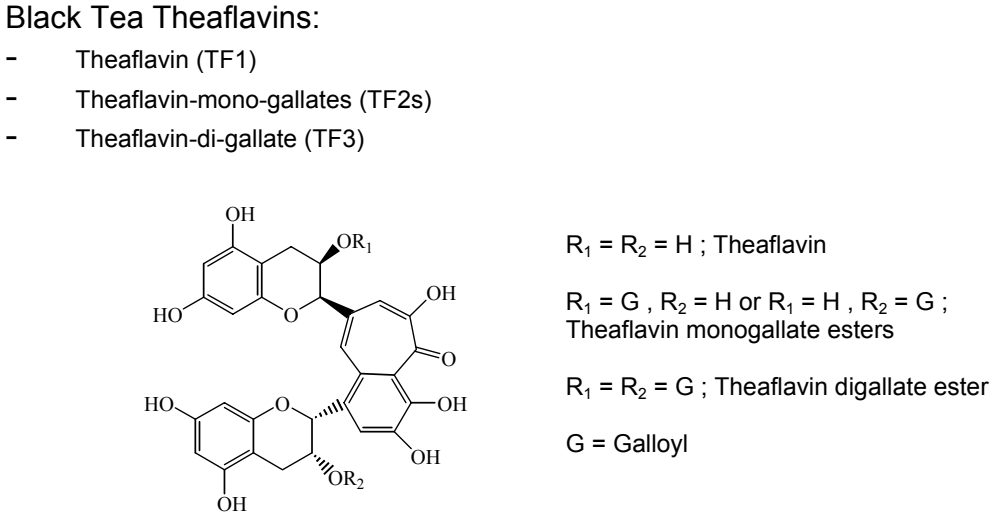


Figure 11. Structure of black tea theaflavins

3.2 Reaction of MG with Tea Polyphenols

3.2.1 Experimental Protocol for GC Method

All the experiments were carried in the 0.01 M PBS which contains NaCl-0.138 M; KCl-0.0027 M. The condition was mimicking the physiological condition: pH 7.4 and

37 ^{o}C. 2.00 mM MG and 5.33 mM of each individual polyphenol compound, PFBHA and IS (2-chlorobenzaldehyde) in PBS were freshly prepared before the experiment.

MG residue was monitored by GC method for the preliminary test, in which 8 mL MG was mixed with 1 mL PBS or a polyphenol PBS solution. After capping the vials, the sample was stirred vigorously for 5 s. Control samples, MG and PBS only, were placed in the salt/ice bath and other vials were placed in the 37°C water bath incubator and shaken at 40 rpm speed. After 1 h incubation, appropriate amount of PFBHA and internal standard (IS) were added to the samples. Samples were stirred vigorously for another 5 s before derivatization.

For the derivatization reaction between MG, IS and PFBHA, the shaking speed was increased to 50 rpm instead of 40 rpm but the temperature was kept the same at 37°C. After the reaction was completed, the *O*-PFB-oximes derivatives were extracted with 4 mL methylene chloride three times in the preliminary comparison of all polyphenol compounds, and were extracted with 1.5 mL methylene chloride two times in the rest of the experiments. The organic phase was dried over anhydrous sodium sulfate. The volume was reduced to around 0.5 mL under gentle nitrogen flow before GC/FID analysis. The flow chart indicating the composition and treatment procedure before GC/FID analysis of sample is shown in Figure 12. The entire procedure repeated three times to get triplicate results for analyses. The amount of decreased oximes in triplicate samples was expressed as mean ± SD.

3.2.2 Experimental Protocol for HPLC Method

The procedure of further investigation on time course study of TF3 and MG was adjusted, in which MG residue was monitored by HPLC system. Smaller volume (2 mL) of each reactant was used, and the initial concentration was also changed to 1.15 mM each. After the reaction was stopped by settling the reaction vials in the ice bath, the sample was divided into two. One was added with HCl to stop further reaction of TF3 on trapping MG before the measurement by HPLC. The other was to quantify MG with HPLC equipped with UV detector at 313 nm after derivatization with 1,2-diaminobenzene with the available standard, 2-methylquinoxaline.

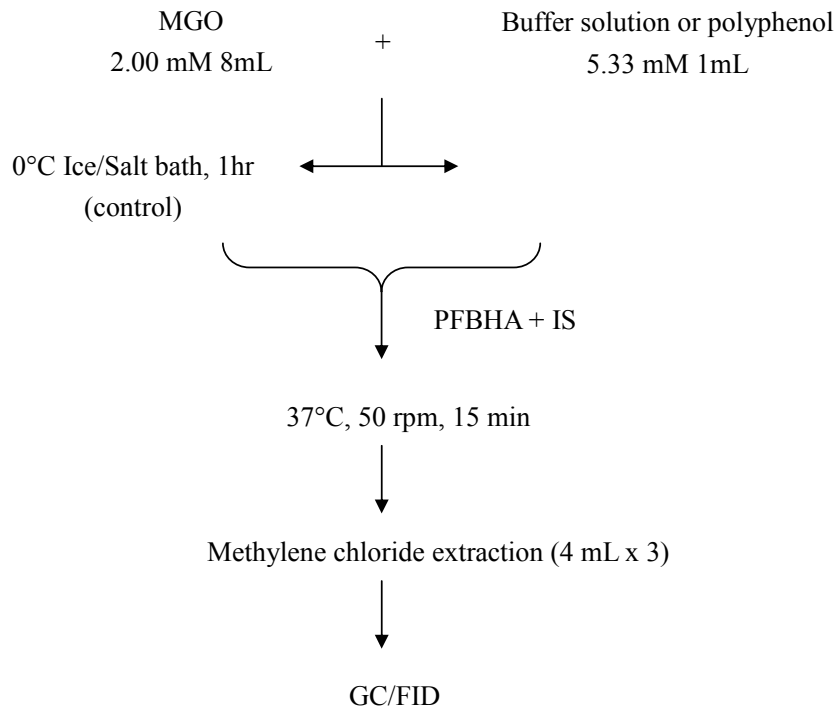


Figure 12. Sample composition, incubation conditions and derivatization reaction procedure before GC analysis.

3.3 Reaction of Methylglyoxal with Dipeptides and Tripeptide

MG of 1.15 μmole in 1 mL, pH 7.4 phosphate buffer was mixed with different dipeptides and tripeptides of 1.15 μmole in 1 mL, pH 7.4 phosphate buffer in the glass vial. The reaction was carried out under physiological conditions at 37°C, and was stopped by putting the vials in the ice bath. MG is quantified by HPLC equipped with UV detector at 313 nm after derivatization with 1,2-diaminobenzene. The amounts of MG are calculated in absolute value with the available standard, 2-methylquinoxaline.

3.4 Quantification of MG

3.4.1 GC Method

The analyses of derivatized volatiles were performed with an Agilent Gas Chromatograph (Agilent Technologies, Inc., Palo Alto, CA, USA). The Agilent Gas Chromatograph (6850 Series) was equipped with an Agilent autosampler (7683 Series Injector) and a flame ionization detector (FID). 1 μL samples were analyzed on a HP-1 MS dimethylpolysiloxane silica capillary column (part number 19091S-733E, 30 m x 0.25 mm i.d., film thickness 1.00 μm (Agilent, Wilmington, DE, USA). The injector temperature was 250°C and detector temperature was 300°C with hydrogen, air and makeup gas (helium) flow rate at 30.0, 300.0 and 5.0 mL/min, respectively. The injector was in 1:1 split mode. The 1.0 mL/min constant carrier gas (helium) flow rate was set. The GC oven temperature was programmed as followed: the initial oven temperature 40°C held for 0 min and increased to 208°C at the rate of 4°C/min held for 0 min. Then the temperature was increased to final temperature (260°C) at the rate of 40°C/min and held for 3 min. The total run time was 46.3 min.

The concentrations of MG were obtained based on the internal standard. The decrease of MG was the difference between the amount of MG at the indicated time point and the initial time point (zero min). The decrease of MG was divided by the amount of MG at the initial time point as the decrease percentage of MG.

3.4.2 HPLC Method

HPLC analysis was performed on a Dionex UHiMate 3000 with UV detector with a C18 column of 150 ×4.60 mm i.d. (Phenomenex luna 3u C18 100A). The column elution started at the constant flow rate of 0.8 mL/min with 8.0% of B and 92% of A (water with 0.2% acetic acid), followed by progressive, linear increases in B (100% acetonitrile) to 40% at 10 min, 48% at 12 min, 60% at 13 min, 28% at 70 min. The mobile phase was then re-equilibrated to 8% of B for 5 minutes. The injection amount was 15 μL. The wavelength used for MG detection was 313 nm. The compounds were detected at peak of 11 min.

MG concentrations were calculated according to the standard curve generated from 0.05 mM to 3 mM 2-methylquinoxaline (MQ) (Fig. 13). The equation is y=0.0147x+0.0083 with the R square equals to 0.997, while y represents the area under curve (mAU*min) and the x represents the concentration of 2-methylquinoxaline (mM). The amounts of MG were the concentrations of MG time the total volume of reactant which were mostly 2.5 mL.

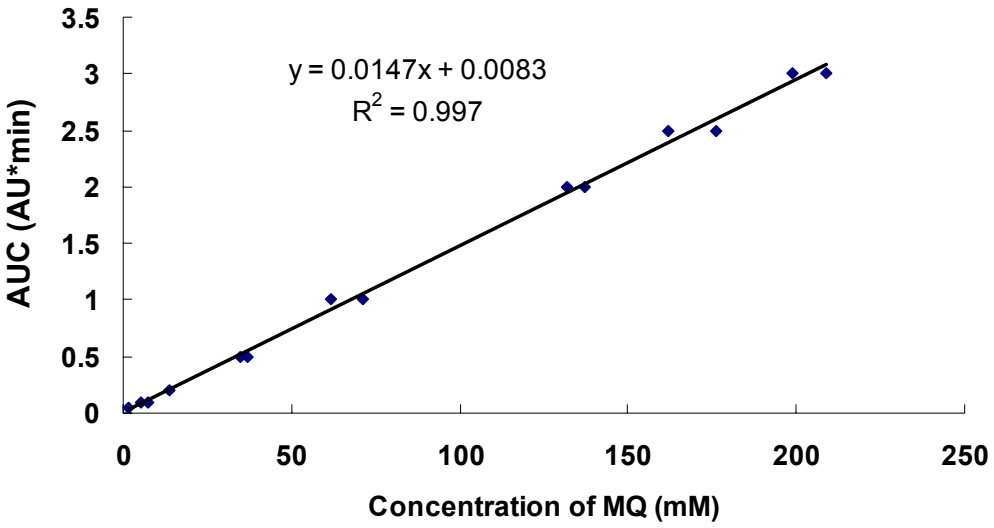


Figure 13. Standard curve of MQ for MG quatification in HPLC method.

3.5 Quantification of TF3

HPLC analysis was performed on a Dionex UHiMate 3000 with UV detector with a C18 column of 150 ×4.60 mm i.d. (Phenomenex luna 3u C18 100A). The column elution started at the constant flow rate of 0.8 mL/min with 8.0% of B and 92% of A (water with 0.2% acetic acid), followed by progressive, linear increases in B (100% acetonitrile) to 12% at 10 min, 18% at 40 min, 21% at 41 min, 28% at 70 min. The mobile phase was then re-equilibrated to 8% of B at 71 min for 5 minutes. The injection amount was 15 μL. The wavelength used for TF3 detection was 280 nm. The compounds were detected at peak of 33 min.

3.6 Quantification of Pyrazinone

Quantification of pyrazinone was calculated and represented as the area under the curve. The same HPLC program was perform as MG described as followed. HPLC analysis was performed on a Dionex UHiMate 3000 with UV detector with a C18 column of 150 ×4.60 mm i.d. (Phenomenex luna 3u C18 100A). The column elution started at the constant flow rate of 0.8 mL/min with 8.0% of B and 92% of A (water with 0.2% acetic acid), followed by progressive, linear increases in B (100% acetonitrile) to 40% at 10 min, 48% at 12 min, 60% at 13 min, 28% at 70 min. The mobile phase was then re-equilibrated to 8% of B for 5 minutes. The injection amount was 15 μL. The wavelength used for MG detection was 313 nm. The compounds were detected at peak of 7 min.

3.7 Identification of LC/MS/MS Analyses of Peptide-methylglyoxal Adducts

Most of the identification of the peptide-MG adducts were analyzed by LC/MS/MS on a Finnigan LTQ ion trap mass spectrometer equipped with a Finnigan electron spray ionization (ESI) interface. A 50 × 2.0 mm i.d., 3 μm Phenomenex Gemini C18 column was used for separation with a flow rate of 0.2 mL/min. The column elution started with 3-min isocratic phase of 100% solvent A (5% aqueous acetonitrile with 0.2% acetic acid), followed by progressive, linear increases in B (95% aqueous acetonitrile with 0.2% acetic acid) to 40% at 33 min and 100% at 38 min for 4 mins. The mobile phase was then re-equilibrated to 100% A at 43 min for 7 mins. The LC elute was introduced into the ESI interface. The positive ion polarity mode was set for ESI ion source with the voltage on the ESI interface maintained at approximately 5 kV. Nitrogen gas was used as the sheath gas at a flow rate of 40 arb and the auxiliary gas at 5 arb, respectively. The structural information of the test compounds was obtained by tandem

mass spectrometry (MS/MS) through collision-induced dissociation (CID) with a relative collision energy setting of 30%.

For the comparison of adducts from methylglyoxal reacted with diglycine and triglycine, respectively, another LC-ESI-MSMS was also applied as described below. It was performed on a TSQ Quantum tandem mass spectrometry (Thermo-Electron Company, USA) equipped with an ESI interface and Agilent 1100 capillary LC system. The capillary pump was set under the micro-flow mode. The LC separation was achieved by using a luna NH2-silica column (Phenomenex, USA). The flow rate was 100 μL/min and the injected volume was 1.0 μL. The column was equilibrated under each experimental condition for at least 30 minutes. The TSQ Quantum was operated in the negative ion mode under the following conditions: (nitrogen (>99.99%) was used for sheath gas and auxiliary has at pressure of 35psi and 5units, respectively. ESI spray voltage was set at 3.0 kV in ESI interface. The temperature of the heated capillary was maintained at 350 °C. A collision induced dissociation was achieved using argon as the collision gas at the pressure adjusted to more than 0.8mTorr above the normal, and the applied collision offset energy was set from -35 to -45 eV for individual analyte. Identification was accomplished by the selected reactant monitoring (SRM) analysis. The quantitative m/z: from 167.2 (molecular ion) to 122.9 (major fragment) was set for diglycine-methylglyoxal adduct, and from 224.2 (molecular ion) to 122.9 (major fragment) was set for triglycine-methylglyoxal adduct. Data was acquired with Xcalibur software system (Thermo-Electron Company, USA)

4. Results and Discussion

4.1 Detection and Quantification of Methylglyoxal with GC system

Methylglyoxal (MG) is a highly reactive alpha-dicarbonyl formed endogenously in numerous enzymatic and nonenzymatic reactions. MG is very volatile and often coeluted with solvent peak when the mixture is prepared for GC analysis. Hence, many papers focused on environmental, clinical or food studies have been published on its detection and quantification by the advantage of further chemical derivatization (Nemet *et al*., 2006). After derivatization, it provides the specificity on analyte, which carries a unique functional group and is able to be detected with conventional detectors such as FID. In the present study, PFBHA was used as a carbonyl derivatization reagent. The chemical reaction between PFBHA and MG is illustrated in Figure 14. The *O*-PFB-oximes are the derivative compounds utilized in MG quantification.

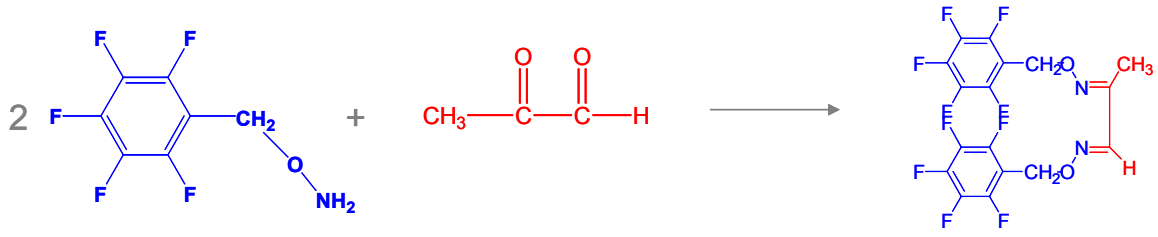


Figure 14. Reaction between PFBHA and MG forming PFBHA-MG oximes.

Figure 15 shows the chromatogram for derivatized MG samples. There were six *O*-PFB-oximes peaks found after GC/FID analysis. Their retention times were at 35.19, 36.35, 38.18, 39.37, 39.78 and 40.18 min. In order to confirm their authentic identifications, the control sample was analyzed by GC/SIM-MS . The mass spectra for the *O*-PFB-oximes are identical to those previously reported (Lapolla *et al*., 2003). Thus,

peaks 1 and 2 were the syn and anti *O*-PFB-oxime stereoisomer derivatives of IS (2-chlorobenzaldehyde) and peaks 3, 4, 5 and 6 were the syn+syn, syn+anti, anti+syn and anti+anti *O*-PFB-oxime stereoisomer derivatives of MG. The quantification of MG-*O*-PFB-oxime compounds was based on the sum of these signals. The limit of quantification (LOQ) was determined as 5.1 μg/L. The linear relationship between MG concentrations used in this experiment and its *O*-PFB-oximes FID signals was confirmed.

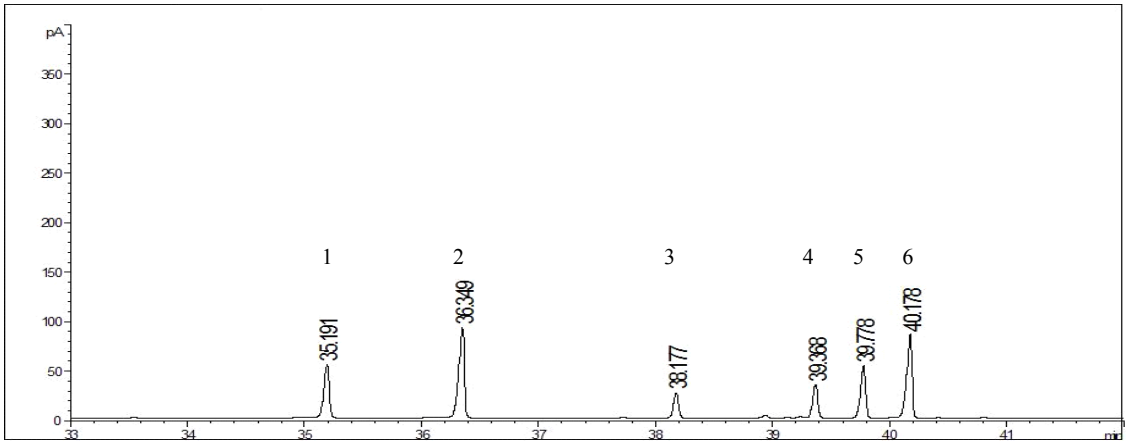


Figure 15. GC/FID chromatogram of methylglyoxal control sample. Sample composition and treatment were shown in Figure 12). Peaks 1 and 2 were due to syn and anti *o*-chlorobenzaldehyde (internal standard) *O*-PFB oximes. Peak 3, 4, 5 and 6 were due to the syn+syn, syn+anti, anti+syn and anti+anti methylglyoxal *O*-PFB oximes.

4.2 Trapping of Methylglyoxal by Tea Polyphenolic Compounds

In the polyphenol adducts investigation, the molar ratio of MG to each specific polyphenol was three and MG decrease percentage (%) was compared with control sample at 0°C on ice/salt bath for 1 h. After one hour, 37°C incubation, the MG was very stable. Only 5.8% MG decrease was shown in Figure 16. All the tea polyphenolic compounds showed the scavenging ability to MG. Nevertheless, the partial catechin moiety, GA and PY, showed 17.1 and 27.8%, respectively. On one hand, among four catechins, the MG decrease percentages were relatively close between ECG and PY.

ECG was the lowest decrease in catechins. Most tea catechins decreased MG about 33% which indicated that one catechin molecule react with one MG molecule since the initial molar ratio of MG and tea polyphenolic compounds is 3 to1. On the other hand, EGC showed the highest decrease amount of MG. From the result (45.7% decrease), which is more than one third, it is obvious that the active sites on EGC were more than one.

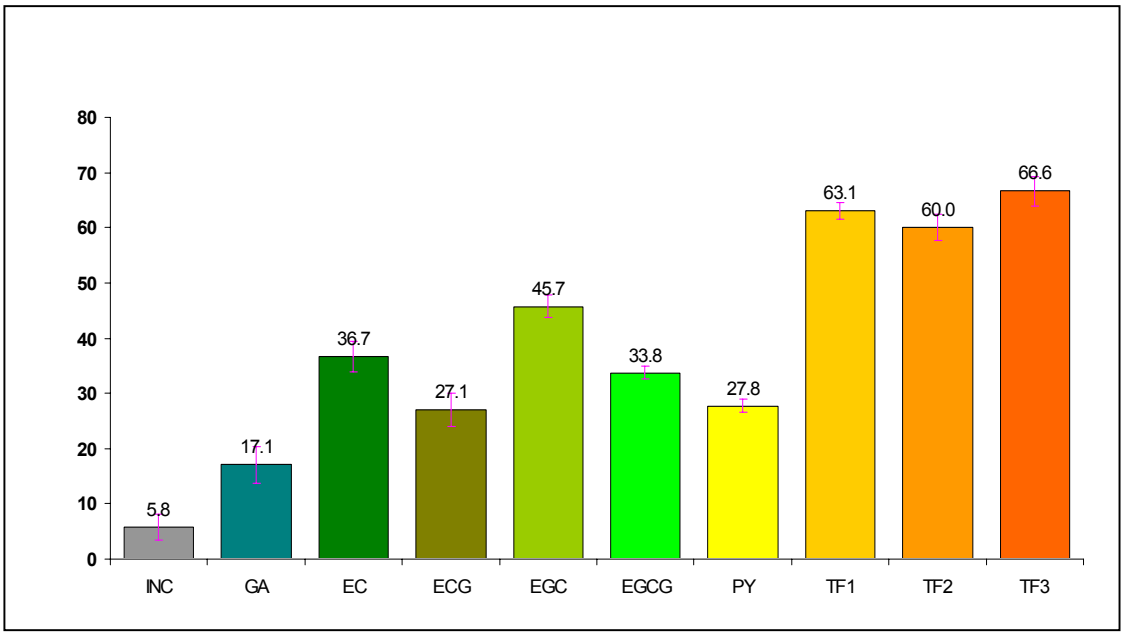


Figure 16. The comparison of polyphenol compounds on trapping efficiency of MG. Decrease percentage of MG were detected among MG only or added with each different polyphenol. Reactions were carried under physiological condition at 37°C, 40 rpm shaking speed water bath for 1hr. Methylglyoxal control sample was in salt added ice bath. The decrease percentage values were expressed in mean ± SD (n=3). (INC: methylglyoxal only incubation sample, GA: gallic acid, EC: (-)-epicatechin, ECG: (-)-epicatechin gallate, EGC: (-)-epigallocatechin, EGCG: (-)-epigallocatechin gallate, PY: pyrogallol, TF1: theaflavin, TF2: theaflavin-3 or -3′-gallate, and TF3: theaflavin-3,3′-digallate)

Theaflavins, the main black tea components, showed to be more reactive with MG than other polyphenols tested here (Fig 16). Theaflavins showed the high levels of MG reduction in respect to control samples, which indicated that theaflavins will be the

excellent candidates in treatment of MG scavenging in future *in vivo* studies. The decreased amounts of MG in TF1, TF2 and TF3 were 63.1%, 60.1% and 66.7%, respectively. All tested theaflavins decreased MG about 66%, which implied that one theaflavin molecule can trap two MG molecules as that the decrease percentage was greater than 33%. This was supported by another study in our lab. The primary adduct between EGCG and MG has been identified (Lo, *et al.* 2006). It is concluded the reaction between EGCG and MG dominantly occurs at the C8-position in the A ring of EGCG. Figure 17 shows the structures of two peracetylated derivatives of adducts between EGCG and MG. The proposed mechanism of reaction between MG and EGCG was illustrated in figure 18. Whether theaflavin compounds react with MG follow the similar mechanism with the further oxidation products of catechins need to be further clarified.

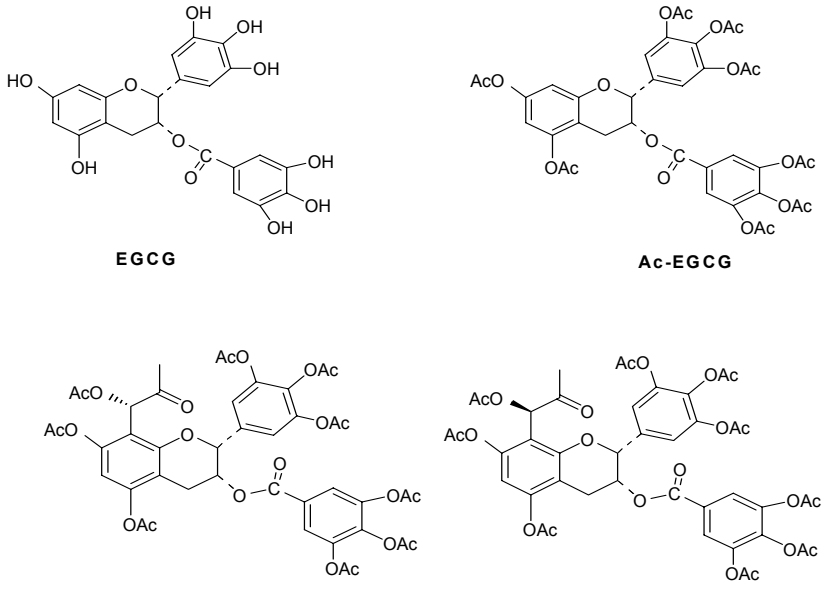


Figure 17 Structures of EGCG, Acetylated EGCG (Ac-EGCG) and adducts C and D

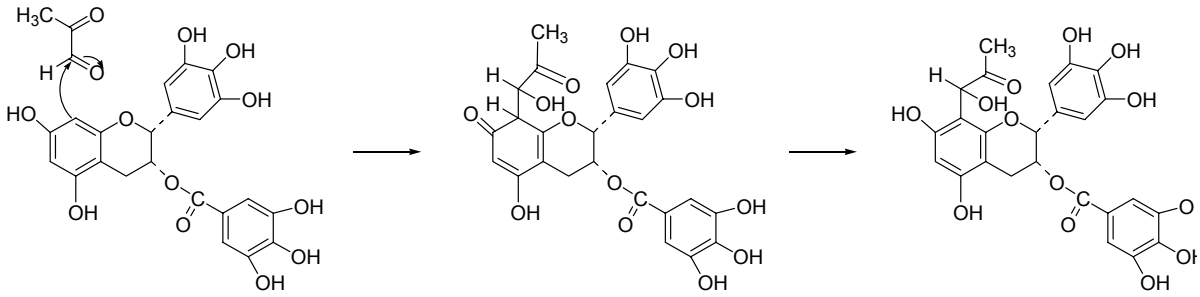


Figure 18 Proposed mechanism of formation of EGCG-MG adducts.

4.2.1 Time Course Study of Theaflavin-3,3'-digallate on Methylglyoxal Trapping

To better understand the reaction trend of TF and MG, time course study was carried out with the same molar amount of TF and MG in an hour. In addition, the derivatization and analytical method was modified. Firstly, the GC method was changed to HPLC method described before. Secondly, the derivatization reagent was changed to 1,2-diaminobenzene. This adjustment facilitates the experimental procedure and minimizes the possible loss of MG residue by eliminating the necessary extraction process for GC analysis. In addition, the HPLC chromatogram for the adduct product between MG and 1,2-diaminobenzene, MQ, shows only one single peak (Figures 19 and 20). Furthermore, MQ is commercially available as that the absolute concentration value can be obtained by conversion from the area under curve with the standard curve (Fig. 13).

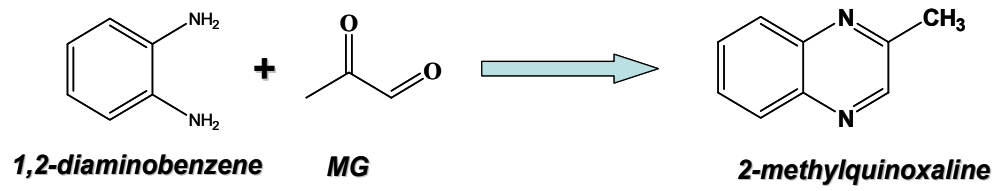


Figure 19. Reaction between MG and 1,2-diaminobenzene.

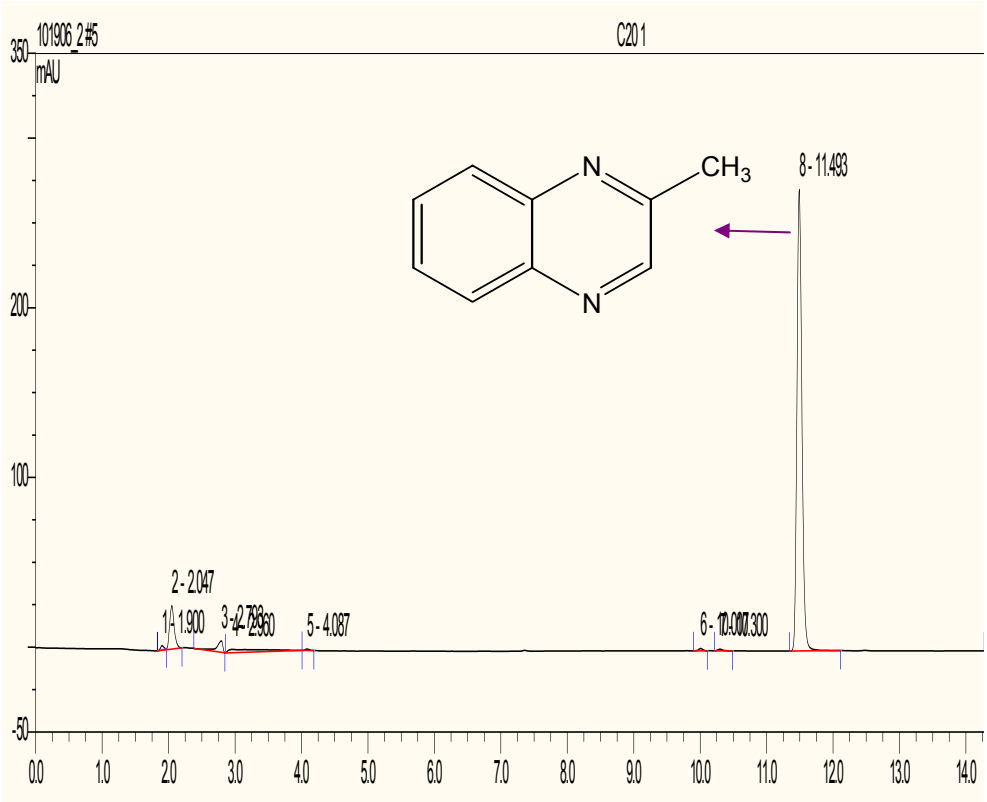


Figure 20. HPLC chromatogram of methylglyoxal control sample. The area under curve of the peak at 11.20 min which is 2-methylquinoxaline was used to calculate the MG concentration in the experiments carried with HPLC method.

The result showed that TF3 started the MG scavenging reaction rapidly (Fig. 21). At the first measurement at three minute, more than one third of MG was trapped. The reaction of MG with TF3 has almost been completed during the time of our experiment. In addition, TF3 was decreasing simultaneously as MG was decreasing (Fig. 22). The decline of TF3 was even sharper than MG, which may be due to one molecule of TF to be able to react with two molecules of MG as observed in our early finding. Nevertheless TF3 is very unstable in an aqueous solution, which may also contribute the sharp decline of TF. Different molar ratios and the identification of the adduct products should be

investigated in the future to better understand the trapping mechanism. Based on our results, the first molecule of MG might attack TF3 at the rate much faster than the attack of the second molecule of MG.

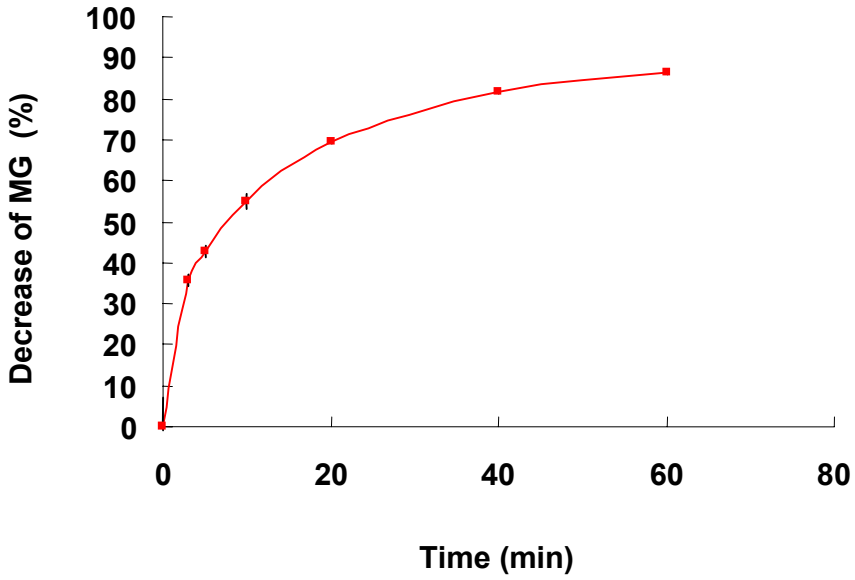


Figure 21. The time course study of TF3 and MG in 1:1 molar ratio within 60 minutes. The decrease percentages of MG compared to the initial point were presented, and the values were expressed as mean ± STDEV (n=3).

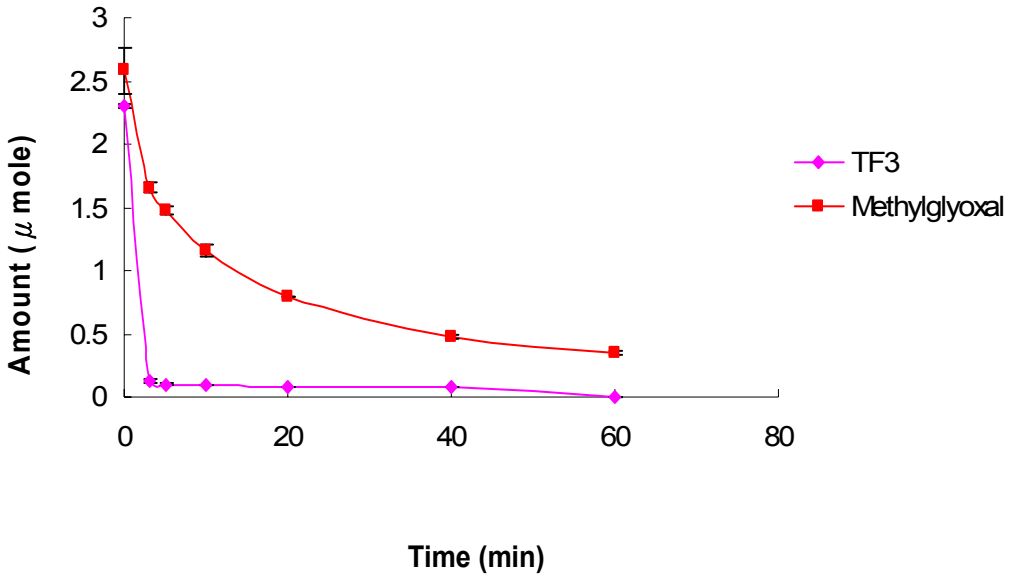


Figure 22. The time course study of TF3 and MG in 1:1 molar ratio within 60 minutes. The amounts of TF and MG were presented in unit of μmole, and the values were expressed as mean ± STDEV (n=3).

4.3 Trapping of Methylglyoxal by Dipeptides and Tripeptides

Selected dipeptides with the N-terminal glycine and triglycine were primarily tested as shown in Fig. 23. In one day incubation, Gly-Cys has a much higher scavenging efficiency (53.42%) than all the other peptides we tested, which may indicate a very different mechanism behind. Moreover, triglycine (TG) showed relatively high trapping efficiency (47.0) compared to most dipeptides including diglycine (12.3%) may imply other elements than the properties of side chain group influence the trapping of MG.

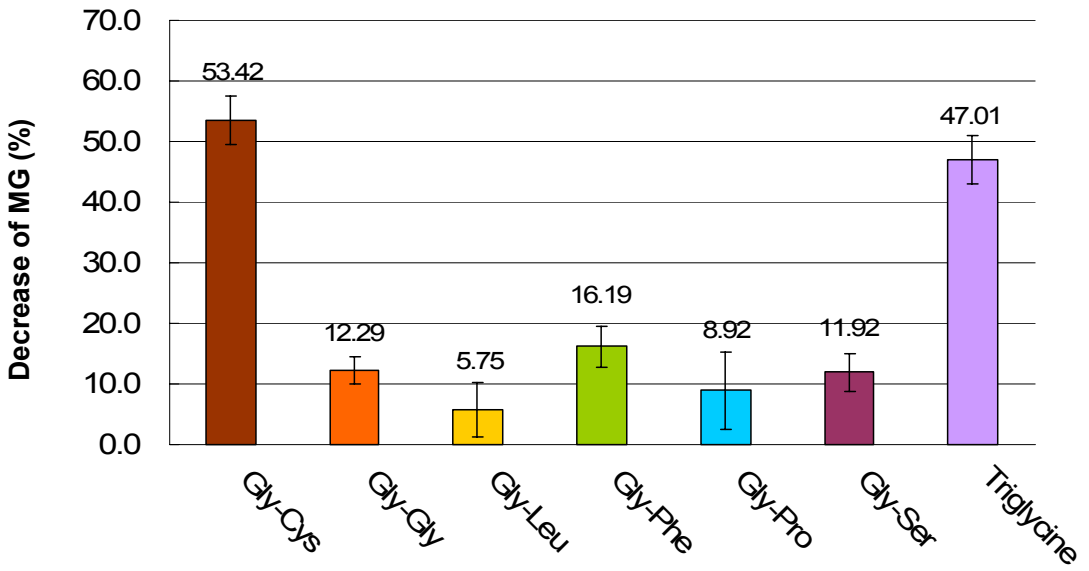


Figure 23. The comparison of MG trapping efficiency among dipeptides and triglycine. Six Gly *N*-terminal dipeptides and trigylcine were compared under physiological condition at one day incubation.

4.3.1 The Reaction between Cysteine Containing Peptides and MG

The thiol group is more nucleophilic than the primary amine group, and it was shown to react with dicarbonyl group competitively when it is judged by the formation of CMC (Zeng and Davies, 2005). The cysteine-containing peptides were then investigated to reveal the mechanism of trapping MG in our study. Cys-Gly was used besides Gly-Cys to compare the influence of sequence on the trapping efficiency and kinetic trend. Furthermore, Cys, *N*-Ac-Cys, γ-Glu-Cys were also included to study the mechanism. The results showed that Cys consumed the MG most rapidly; more than 50% of MG was decreased by 2 hours (Fig. 24). Cys-Gly was trapping MG faster than Gly-Cys, 39.6% compared to 12.3% in 2-hr incubation and 82.5% compared to 61.9% in 1-day incubation. γ-Glu-Cys seemed to react with MG more efficiently than Gly-Cys as well, which was believed to trap MG by forming the Schiff base. The results showed that MG was

depleted by all Cys-containing reagents toward the end of the incubation.

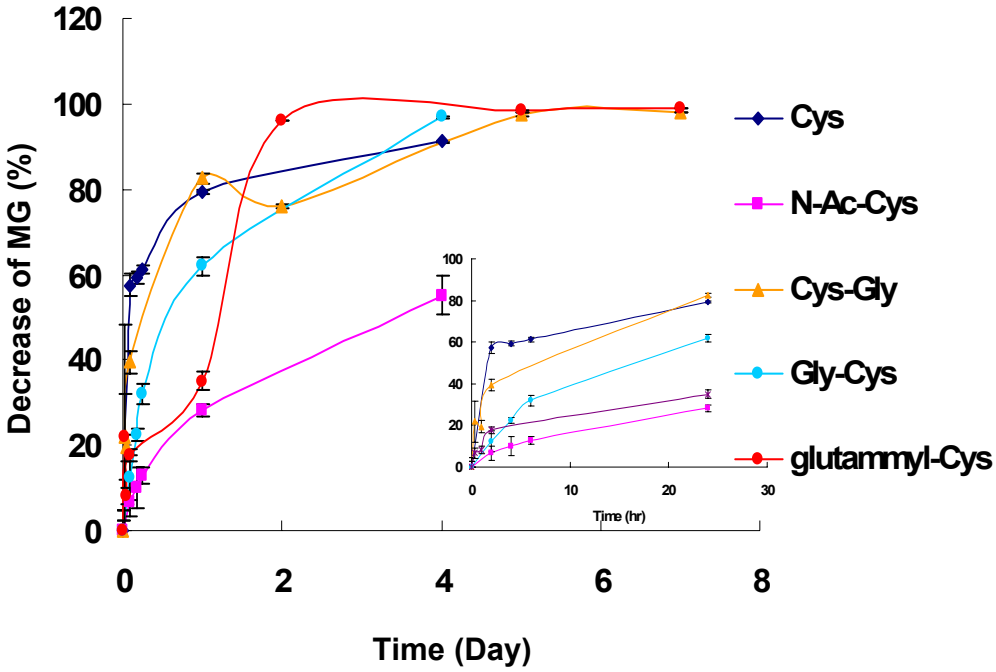


Figure 24. Time course study of trapping of MG among Cysteine-containing compounds. Cys, *N*-Ac-Cys, Cys-Gly, Gly-Cys and γ-Glu-Cys were included to reveal the kinetic of trapping of MG by Cys-containing peptides.

With the nucleophilic addition of sulfhydryl group of Gly-Cys to the carbonyl carbon of methylglyoxal, there are three possible addition products as shown in Figure 25. It was shown that the thiol concentration decreased and the CMC increased more when GO is incubated with *N*-Ac-Cys than with Cys (Zeng and Davies, 2005). Furthermore, the authors found that the decrease amount of thiol is much higher than the amount of CMC generation. In addition, when the N-terminal blocked peptide N-Ac-Leu-Val-Cys-Asp-AMC (AMC=7-amido-4-methylcoumarin) was reacting with the dicarbonyl

compounds, only one species were detected. The author thus concluded it is because the thiol group was the only point to be modified in this condition, which is different than other Cys-containing materials used in their study. If CMC was the only product between glyoxal and Cys materials, their result would have been against ours in which more MG decreased as it was incubated with Cys than with *N*-Ac-Cys. However, all the observation showed that there should be other products than CMC generated in the reaction. Zhen and Davies (2005) also indicated that the trends of thiol decrease were different between reactions with MG and with GO no matter which Cys-containing materials (*N*-Ac-Cys, GSH, and Val-Thr-Cys-Gly) were used to react with these two dicarbonyl species. This might be because there are two aldehydic groups available in GO in aqueous solution while there is only one aldehydic group in MG. This is assumed with the observation that the dicarbonyl species exist in a hydrate form as they are in aqueous solution (Creighrom *et al.*, 1988; Rae *et al.*, 1990). The reactivity of an aldehyde is much higher than a keto group for thiol group attacks. So the CMC production is higher when GO presented than the production of CEC when MG presented.

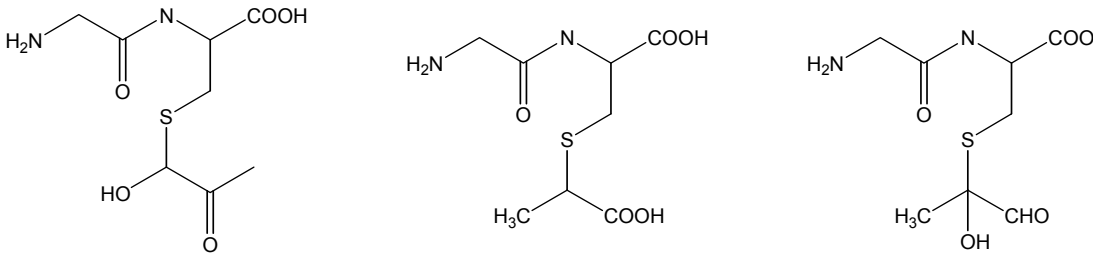


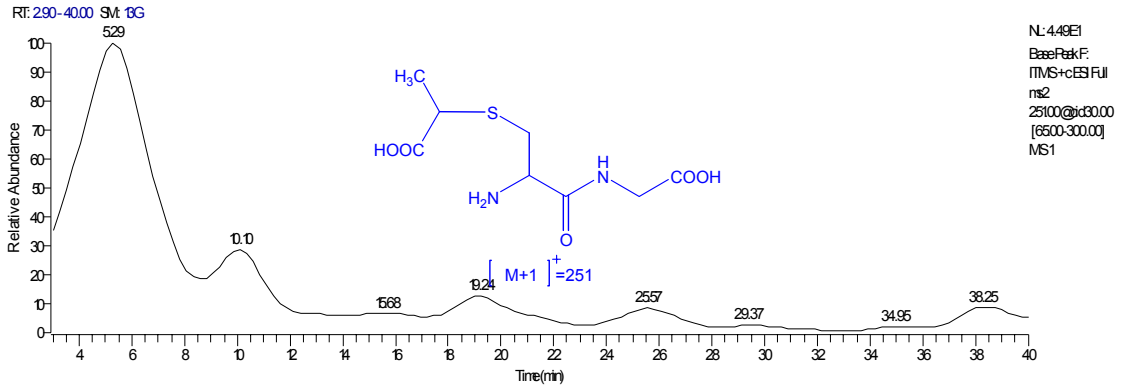
Figure 25. The possible adducts from Gly-Cys and MG

4.3.2 Identification of Adducts from the Reaction of Methylglyoxal with Cys-containing Dipeptides

To explain the diverged kinetic trends in trapping MG, LC/MS was applied to investigate the adduct products. All the samples were made of mixing methylglyoxal with indicated dipeptide in aqueous solution. Mixtures had been incubated at 37 ^{o}C for a week and then store at room temperature for 4 weeks. The resulting reaction mixtures were subjected to LC-MS to look for the expected adducts.

The potential formation of pyrazinone derivatives from the dipeptides, Cys-Gly and Gly-Cys was investigated. Figure 26 shows the LC-MS of the reaction product between MG and Cys-Gly scanned at the [M+1] ion of 251. From the MS fragmentation pattern of this m/z 251 ion as shown in Figure 26A, it is believed that the most probable structure is the CEC derivative. For Gly-Cys as shown in Figure 27, the peak at the retention of 10.10 min could be the expected CEC derivatives from Gly-Cys and MG as well. Figure 28 shows the possible mass fragmentation mechanism of the CEC product from Gly-Cys. The one for Cys-Gly is similar and not shown here. The loss of a molecule of water from [M+1] ion will lead to ion at m/z 233. On the other hand, an ion of m/z 205 can be obtained from a neutral loss of a molecule of formic acid from [M+1] ion. A small fragment ion at m/z 187 can be a further loss of a molecule of water from ion at m/z 205. This peak may well be an indication that the original molecule contains two carboxylic acid moieties. Figure 29 shows the possible mechanism for the formation of CEC derivative from the addition of Gly-Cys or Cys-Gly to MG. This is very similar to the reported mechanism of Zhen and Davies (2005) which was shown in Fig. 9 before.

A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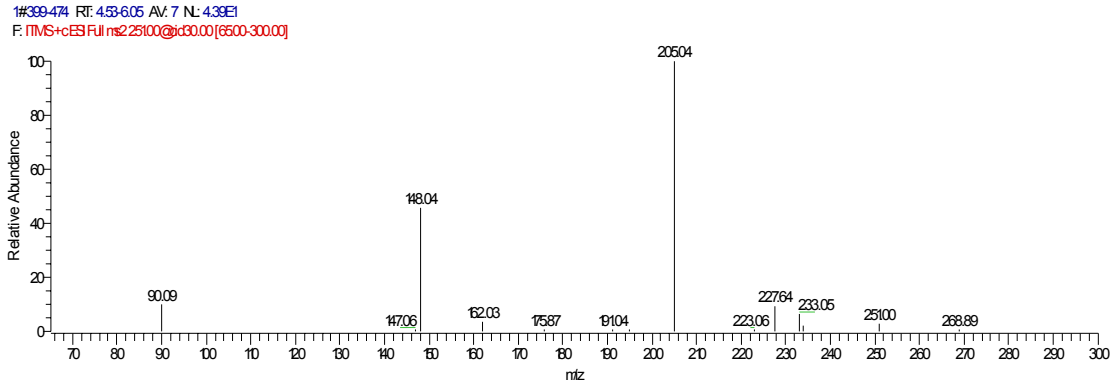
5.29
10.10
15.68
19.24
25.57
29.37
34.95
38.25
M+1 =251
NL: 4.49E1
Base Peak F:
ITMS+c ESI Full
ms2
251.00@id30.00
[65.00-300.00]
MS1
Relative Abundance
Time(min)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F: ITMS+c ESI Full ms2 251.00@id30.00 [65.00-300.00]
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148.04
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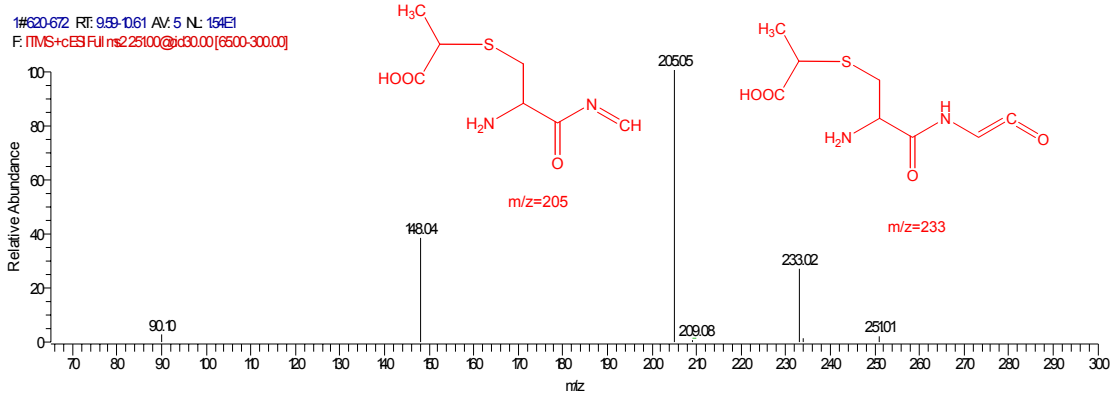
233.05
251.00
268.89
Relative Abundance
m/z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F: ITMS+c ESI Full ms2 251.00@id30.00 [65.00-300.00]
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B

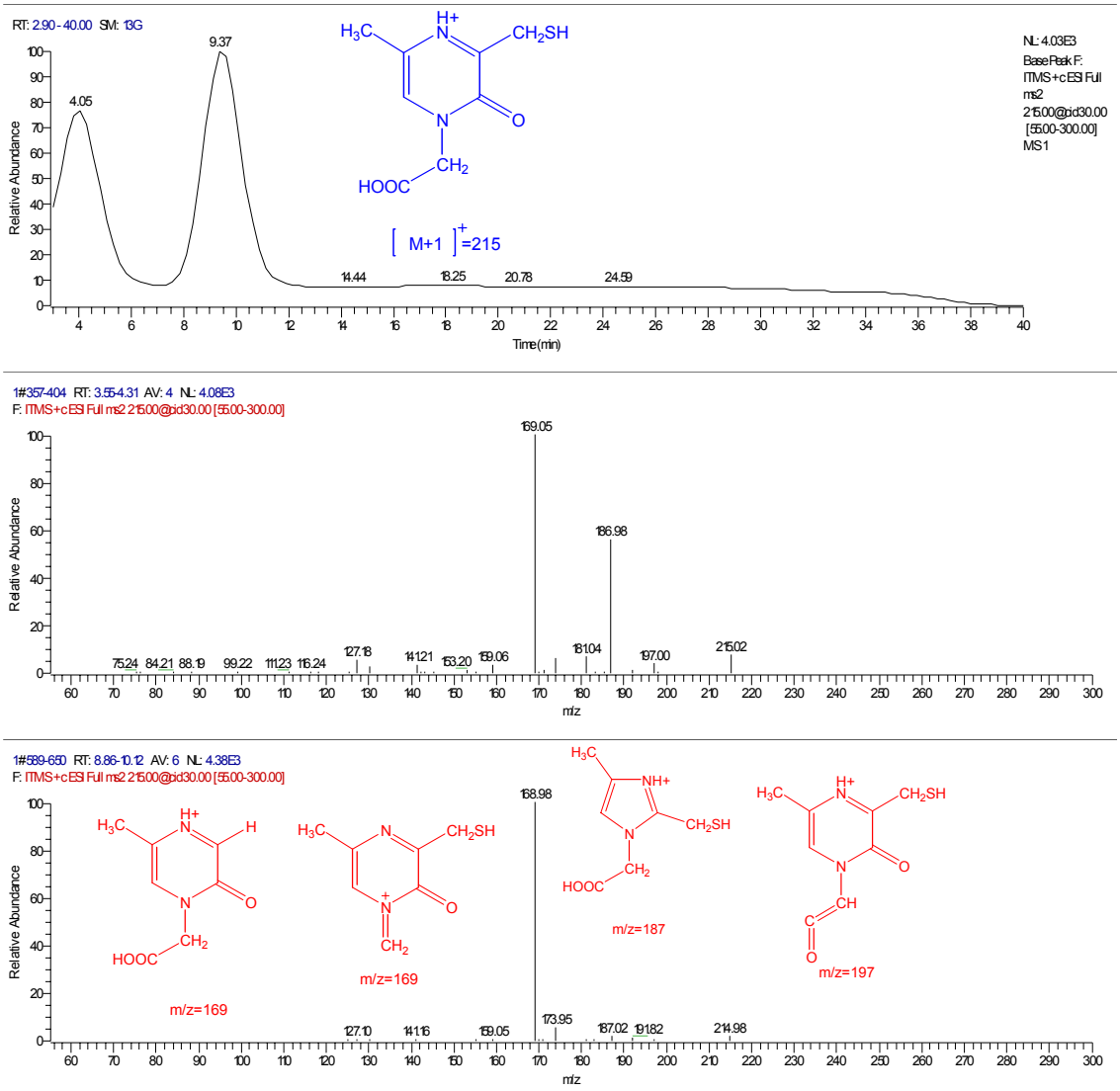

Figure 26. LC/MS Analysis of Cys-Gly. A mixture of MG and Cys-Gly in aqueous solution had been incubated at 37 ^{o}C for a week and then store at room temperature for 4 weeks before the LC-MS analysis.

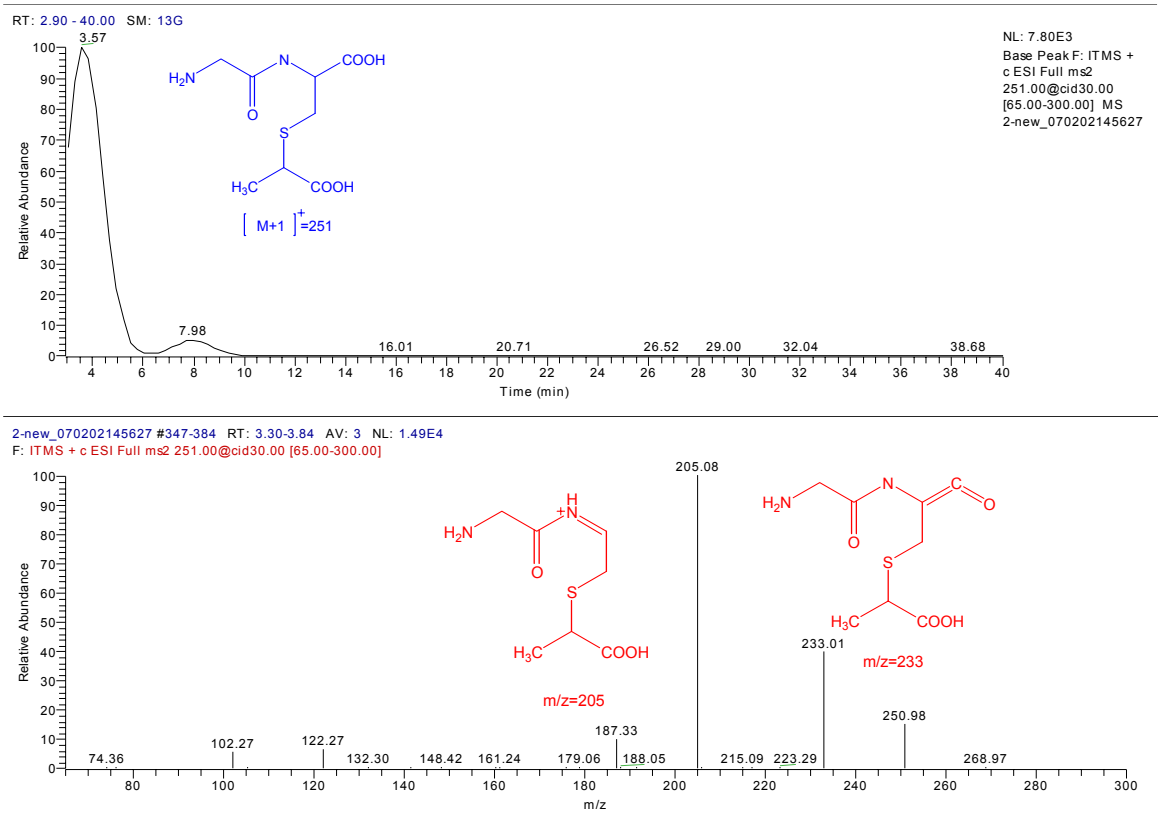

Figure 27. LC/MS Analysis of Gly-Cys. A mixture of MG and Gly-Cys in aqueous solution had been incubated at 37 ^{o}C for a week and then store at room temperature for 4 weeks before the LC-MS analysis.

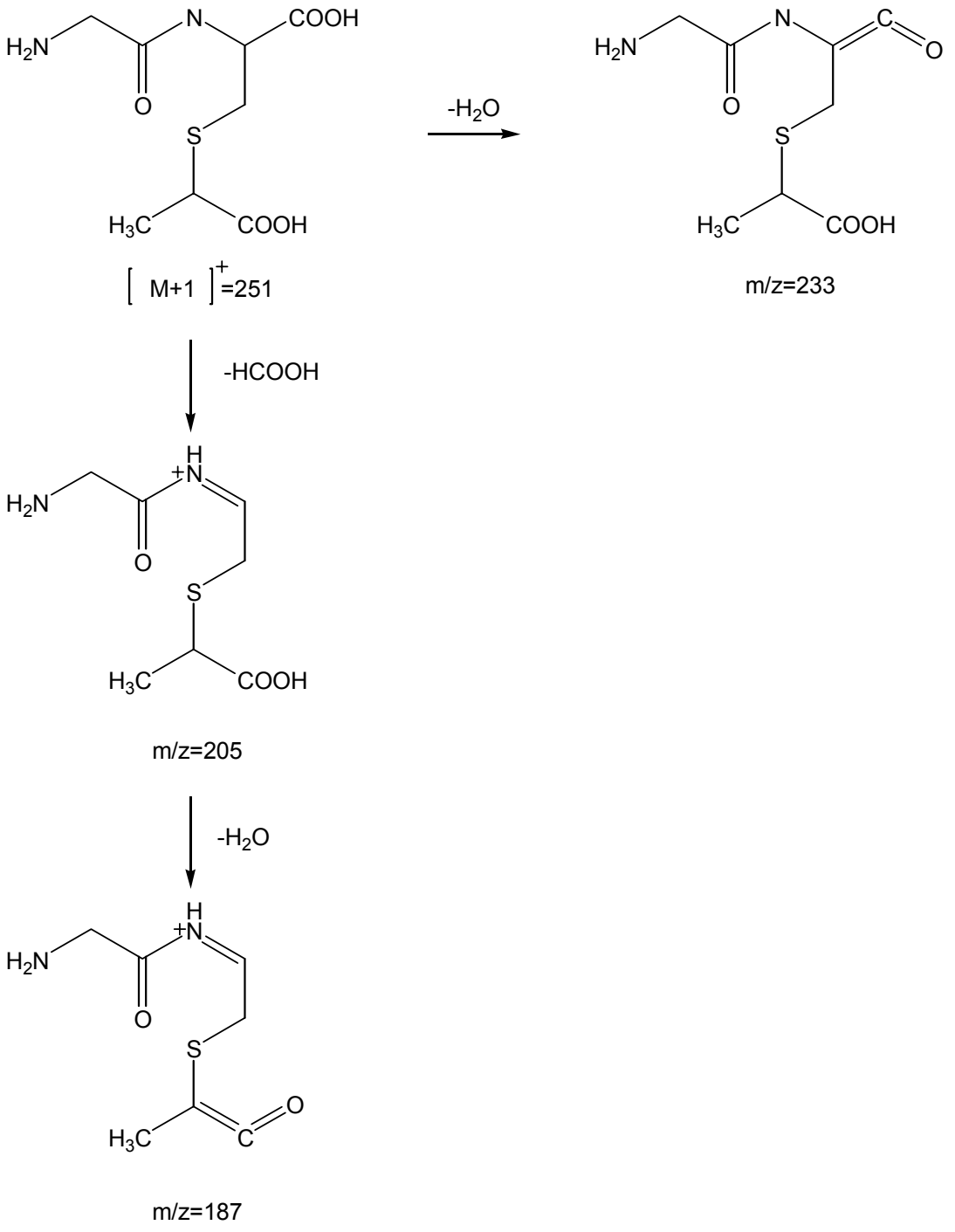

Figure 28. Possible mass fragmentation mechanism of the CEC derivative from MG and Gly-Cys.

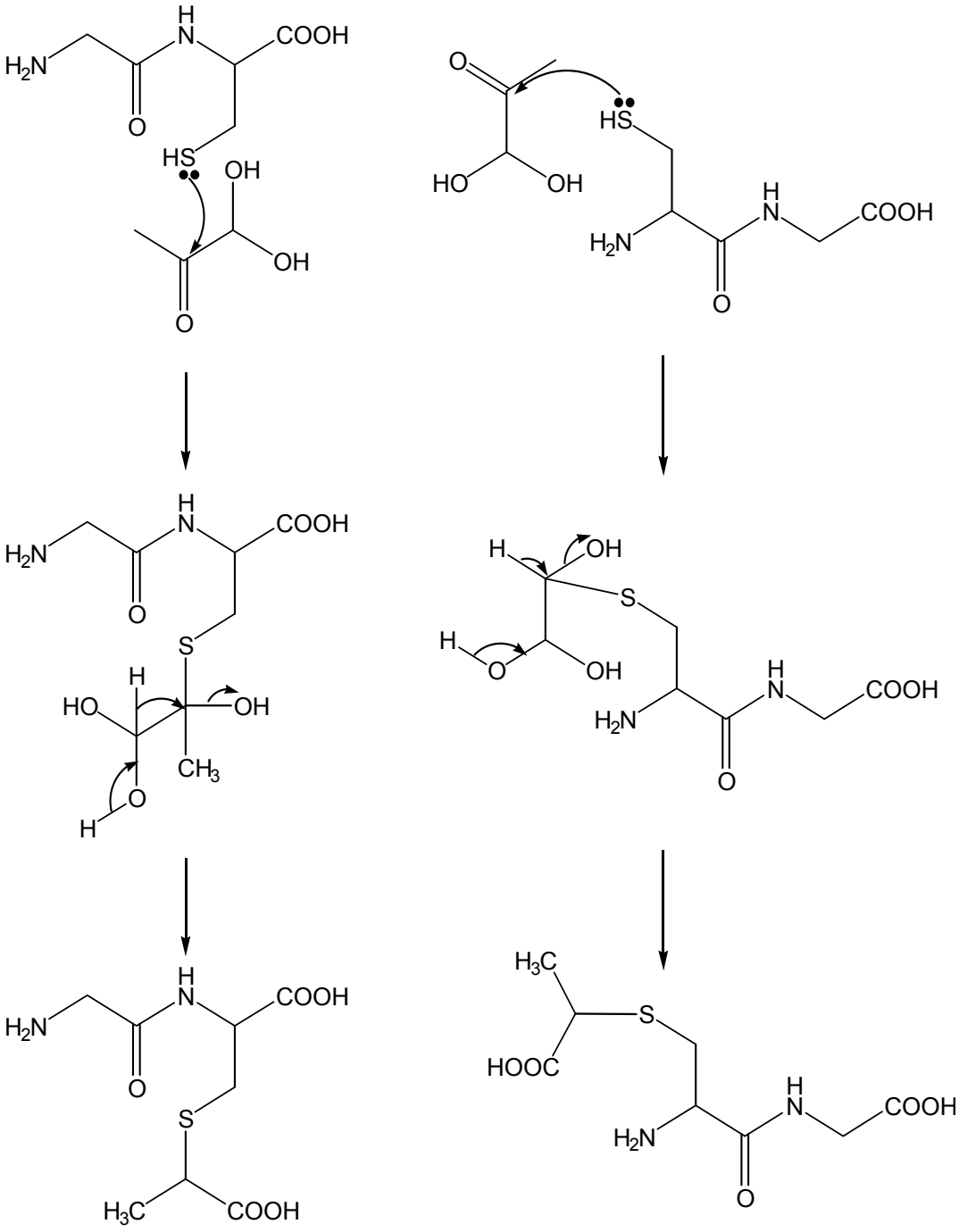

Figure 29. The possible mechanism for the formation of CEC derivative from the addition of Gly-Cys and Cys-Gly to MG

It is interesting to note that in the reaction mixture of Gly-Cys and MG, there is no [M+1] at 215 can be found (Fig. 27). The possible structures of these pyrazinone derivatives formed from Cys-Gly and MG are with molecular weight of 214. Figure 26B shows the mass chromatogram of [M+1] ion at m/z 215 which is the pyrazinone products between MG and Cys-Gly. There are two peaks having [M+1] ion of 215. From the mass spectral fragmentation pattern, it is believed that the peak at the retention of 9.37 min could be the expected pyrazinone derivative from Cys-Gly and MG. Figure 30 shows the possible fragmentation mechanism. The M+1 ion of 215 can loss a molecular of CH_2=S leading to major ion at m/z 169. The other peak at retention time of 4.05 min (Figure 26B) shows an unusual loss of CO from the [M+1] ion which is not consistent with the mass spectral fragmentation of other pyrazinones observed in this study.

As discussed before, γ-Glu-Cys was also observed to react with MG .The LC/MS Analysis of the product of MG and γ-Glu-Cys and the possible fragmentation was illustrated at figure 31 and figure 32.

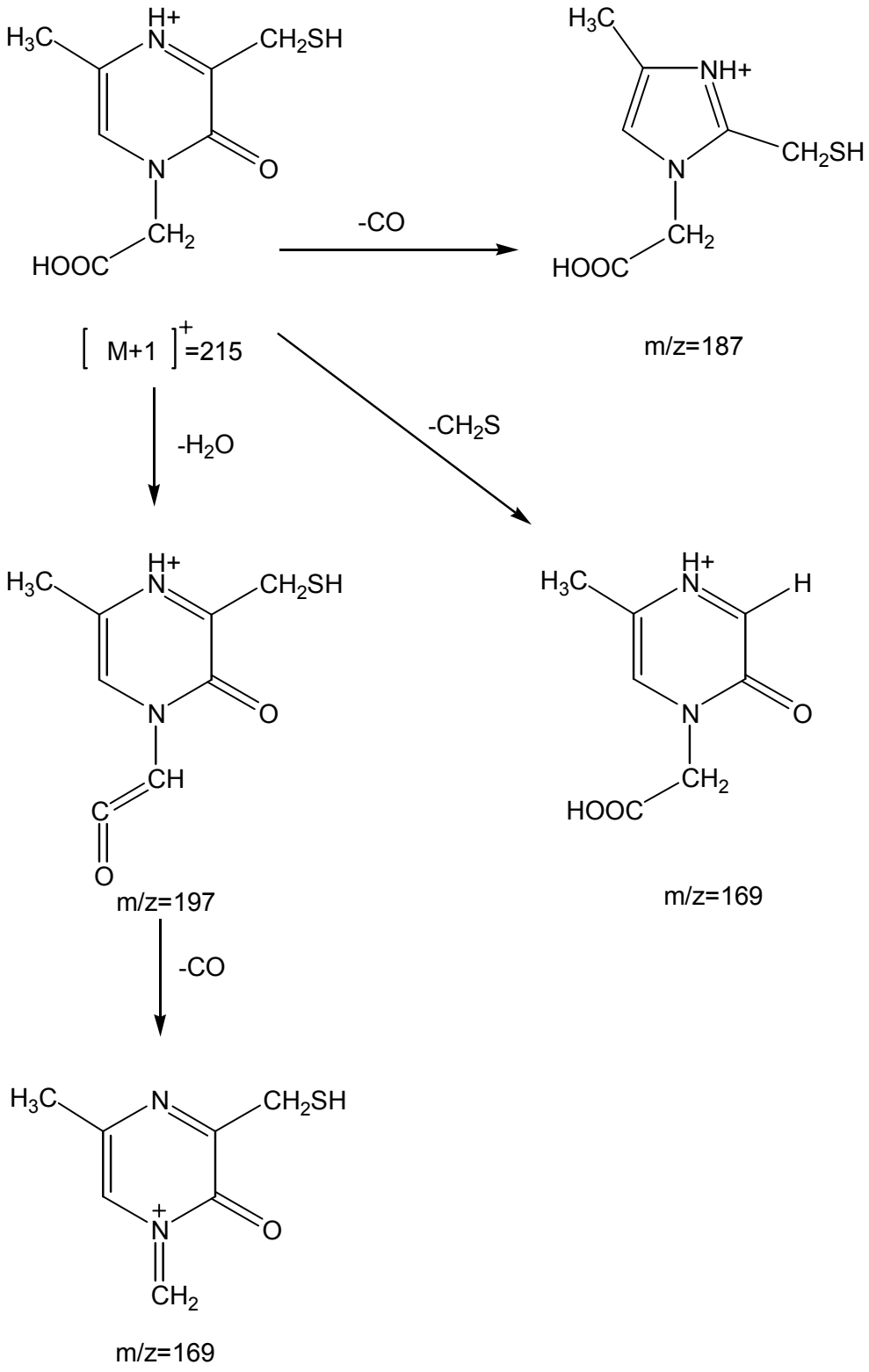

Figure 30. Possible mass fragmentation mechanism of the pyrazinone derivative from MG and Cys-Gly.

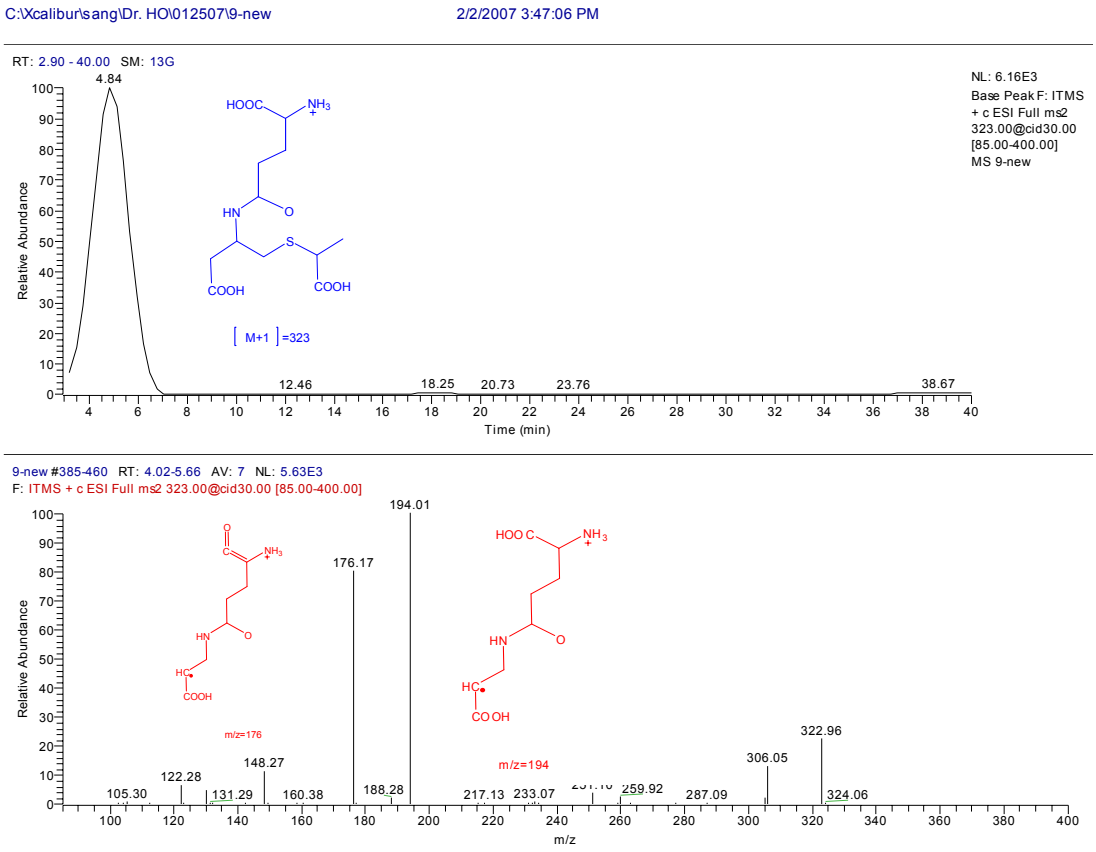

Figure 31. LC/MS Analysis of γ-Glu-Cys. A mixture of MG and γ-Glu-Cys in aqueous solution had been incubated at 37 ^{o}C for a week and then store at room temperature for 4 weeks before the LC-MS analysis.

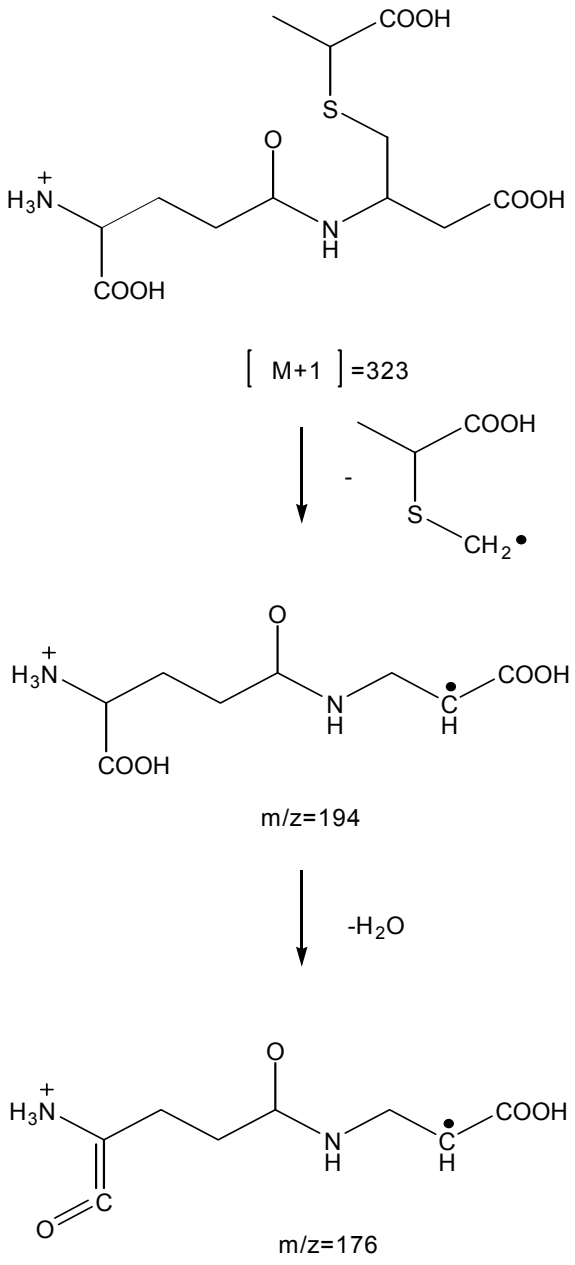

Figure 32. Possible mass fragmentation mechanism of the adduct from MG and γ-Glu-Cys.

4.3.3 Kinetic Study of Non-Cys Containing Peptides and MG

Different from Cys-containing peptides and based on the mechanism of pyrazinone formation reported at physiological conditions (Krause, *et al*., 2004), we believed that the differences of trapping efficiency among Gly-Phe, Gly-Gly, and Gly-Leu are due to the nucleophilic properties the side chain of the C-terminal amino acids provide. Phe with the electron pushing group provides the highest trapping efficiency while Leu with the steric hindrance effect showed the lowest efficiency (Fig. 23 and Fig 33). Based on the Krause's observation, 5-methyl-pyrazinone is the main product with high regioselectivity (Krause et al., 2004). The proposed formation mechanism was shown in Figure 30, however, the proposed structure needs to be confirmed by product separation and NMR spectral analysis.

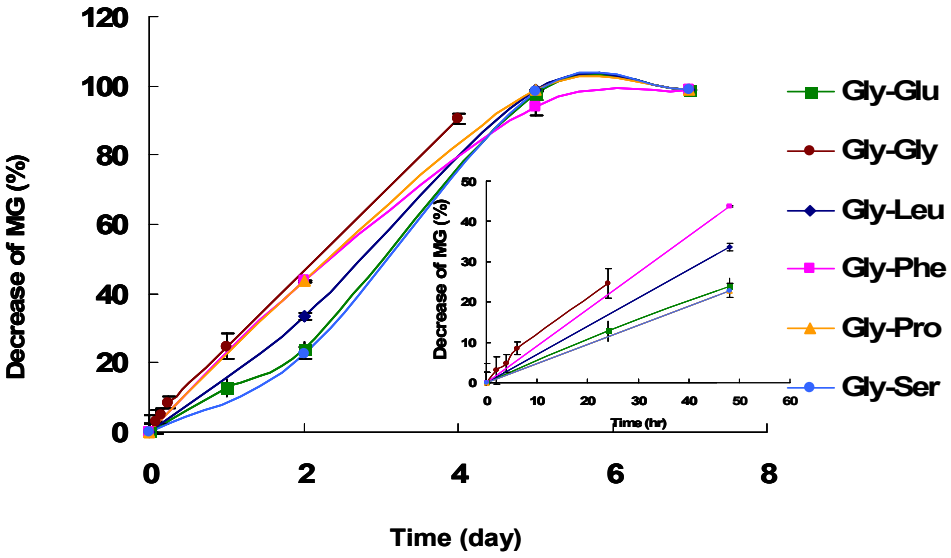

Figure 33. Kinetic study of trapping of MG among Non-Cys containing dipeptides. Gly-Glu, Gly-Gly, Gly-Leu, Gly-Phe, Gly-Pro, and Gly-Ser were included to reveal the kinetic trends of MG trapping ability.

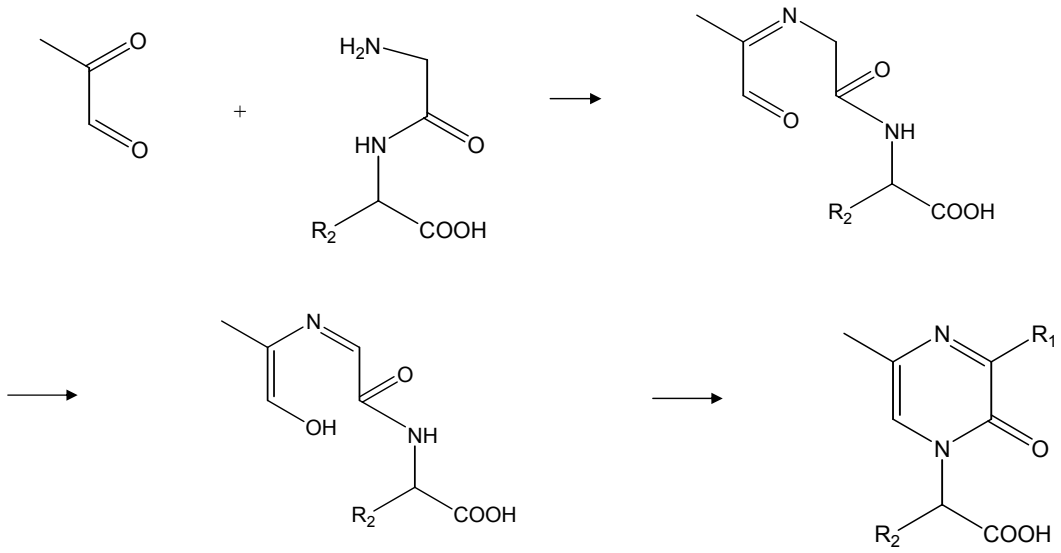

Figure 34. Proposed mechanism of 5-methyl-pyrazinone formation from MG and Gly-N-terminal dipeptides.

All the tested dipeptides depleted the MG completely at the end of the observation period (4 to 7 days) The decline of MG was in a linear trend with the R square equals to 0.9959 (Fig. 35) as that it is a time dependent degradation. The Equation of the trend line is y=0.5213x+100.51 while y represents the decrease of MG in percentage and x presents the time in day. The reaction between MG and dipeptides should be concentration-dependent. The degradation of MG should be concentration dependent as well. At day four, about 50% MG was degraded.

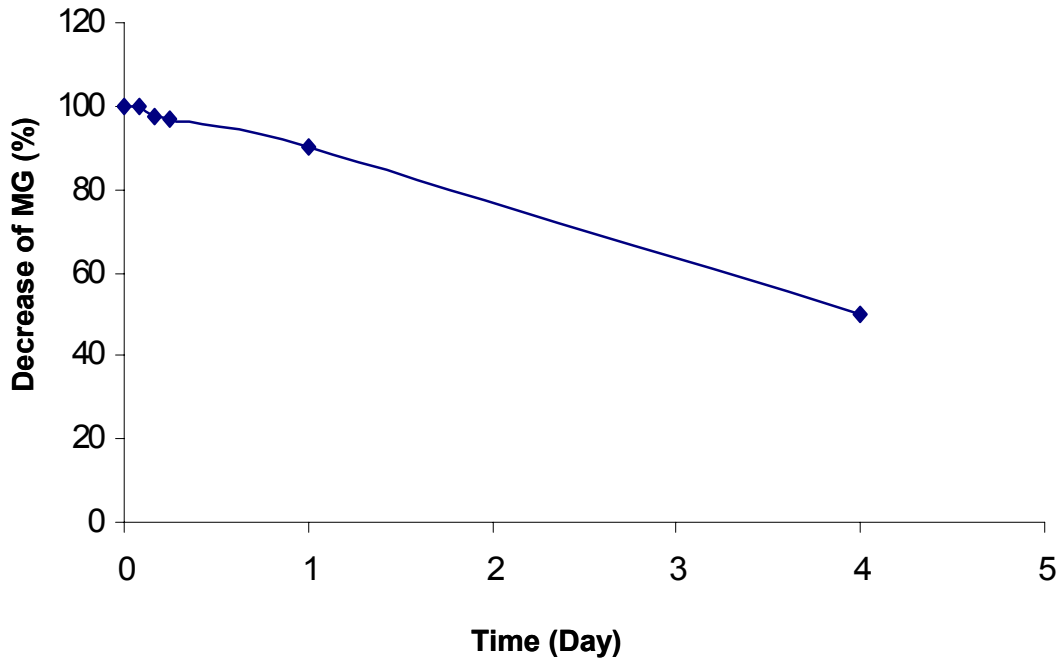

Figure 35. The degradation of MG under physiological condition. 1.15umole/2ml of MG in pH 7.4 PBS had been incubated at 37 ^{o}C to investigate its naturally degradation.

Since the peptide nitrogen in dipeptide, Gly-Pro, is a ternary amine, it is not possible to form pyrazinone adduct between Gly-Pro and methylglyoxal. It is expected that the product from the reaction of Gly-Pro and methylglyoxal will be a Schiff base or Amadori product. All adducts were identified by LC-MS and the possible fragmentations were described separately as follow.

4.3.4 Identification of Adducts from the Reaction of Methylglyoxal with Cys-containing Dipeptides

4.3.4.1 Identification of Adducts from the Reaction of Methylglyoxal with Gly-Glu:

The expected pyrazinone adduct, 4-carboxy-4-(5-methyl-2-oxo-2H-pyrazin-1-yl)-butanoic acid with molecular weight of 240. Figure 36 shows the mass chromatogram of [M+1] ion at m/z 241. The mass spectrum of peak at retention time of 10.18 min is shown in Figure 36. Figure 37 shows the proposed fragmentation mechanism of this molecule. It is consistent with the expected structure of adduct, 1-carboxymethyl-5-methyl-2-pyrazinone. The M+1 ion of 241 can loss a molecular of H_2O leading to ion at m/z 223. On the other hand, neutral loss of CO_2 from M+1 ion will lead to fragment ion at m/z 197.

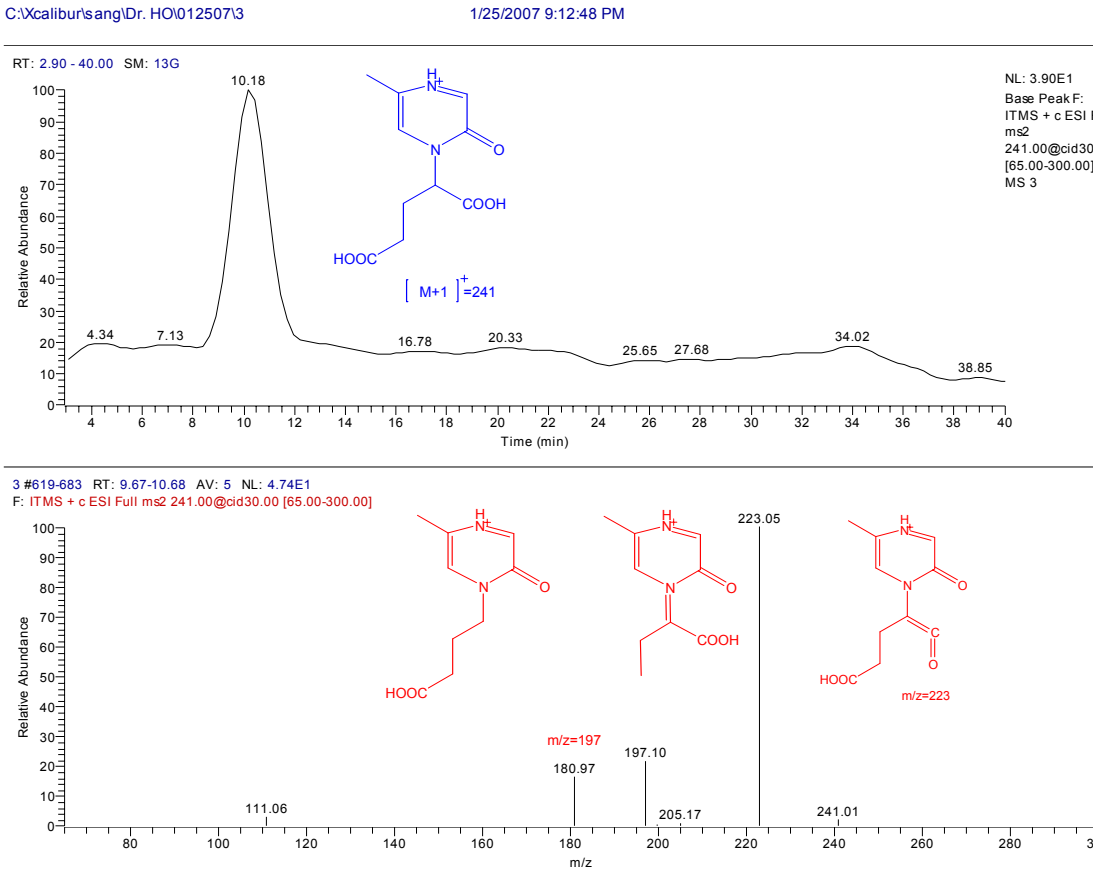

Figure 36. LC/MS Analysis of Gly-Glu. A mixture of MG and Gly-Glu in aqueous solution had been incubated at 37 ^{o}C for a week and then store at room temperature for 4 weeks before the LC-MS analysis.

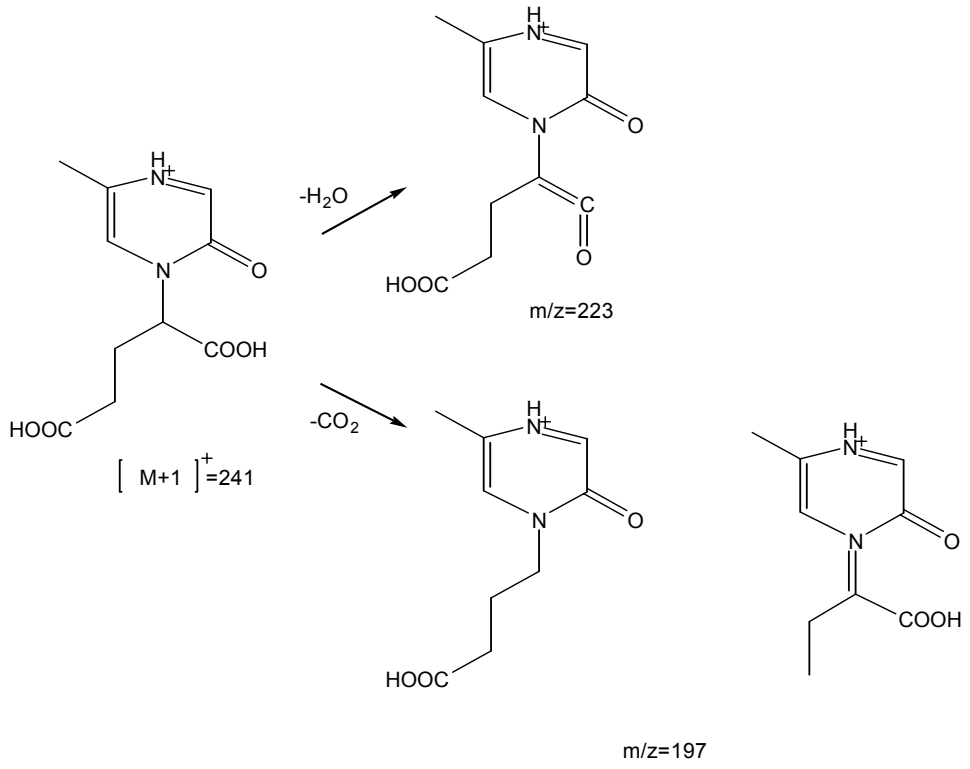

Figure 37. Possible mass fragmentation mechanism of the pyrazinone derivative from MG and Gly-Glu.

4.3.4.2 Identification of Adduct from the Reaction of Methylglyoxal with Gly-Leu

A mixture of methylglyoxal and diglycine in aqueous solution was prepared to look for the expected pyrazinone adduct, 1-[(α-isobutyl)-carboxymethyl]-5-methyl-2-pyrazinone with molecular weight of 224. Figure 38 shows the mass chromatogram of [M+1] ion at m/z 225. The mass spectrum of peak at retention time of 23.93 min is shown in Figure 38. Figure 39 shows the proposed fragmentation mechanism of this molecule. It is consistent with the expected structure of adduct, 1-carboxymethyl-5-methyl-2-pyrazinone. The M+1 ion of 225 can loss a molecular of H_2O leading to ion at

m/z 207. On the other hand, neutral loss of CO_2 from M+1 ion will lead to fragment ion at m/z 181 subsequent loss of a molecule of hydrogen will form ion at m/z 179.

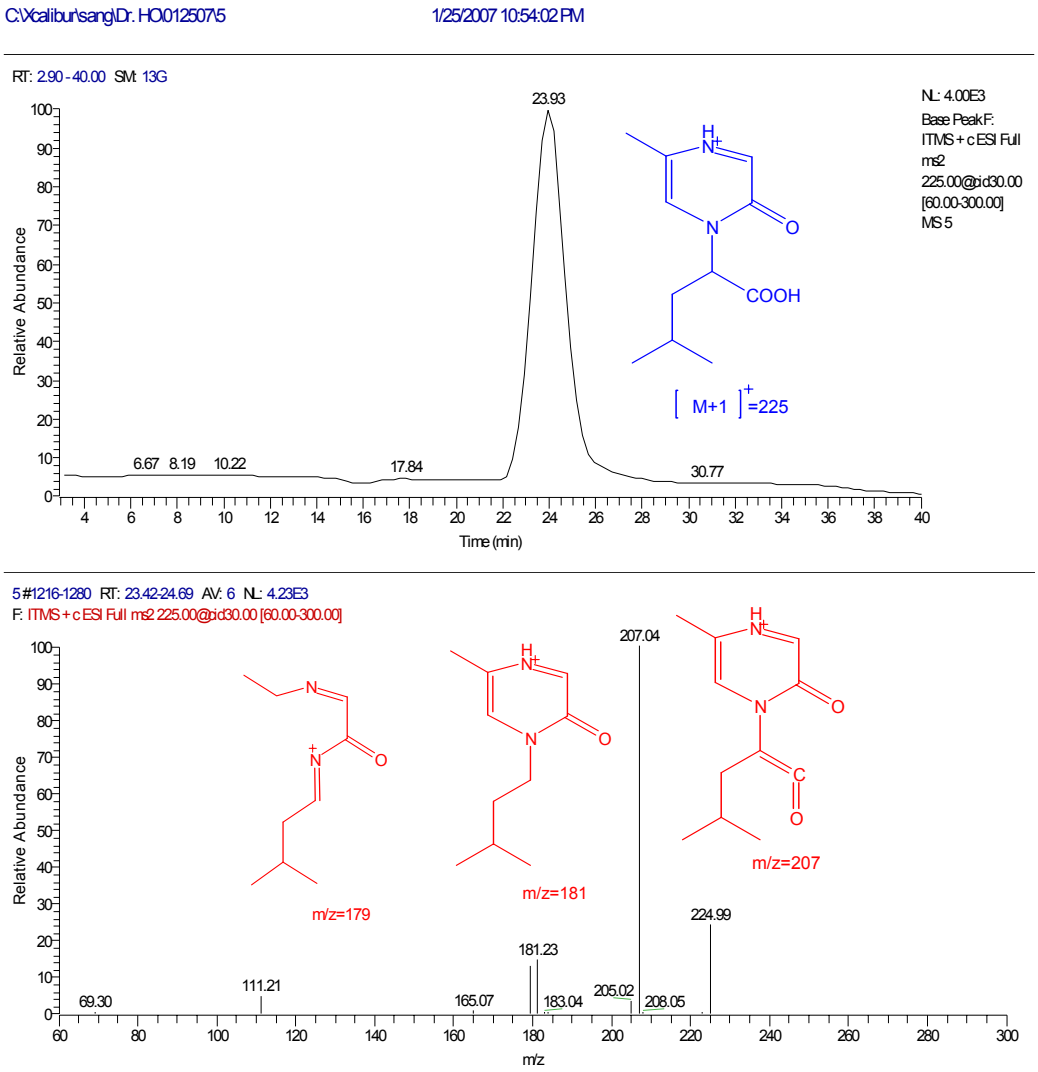

Figure 38. LC/MS Analysis of Gly-Leu. A mixture of MG and Gly-Leu in aqueous solution had been incubated at 37 ^{o}C for a week and then store at room temperature for 4 weeks before the LC-MS analysis.

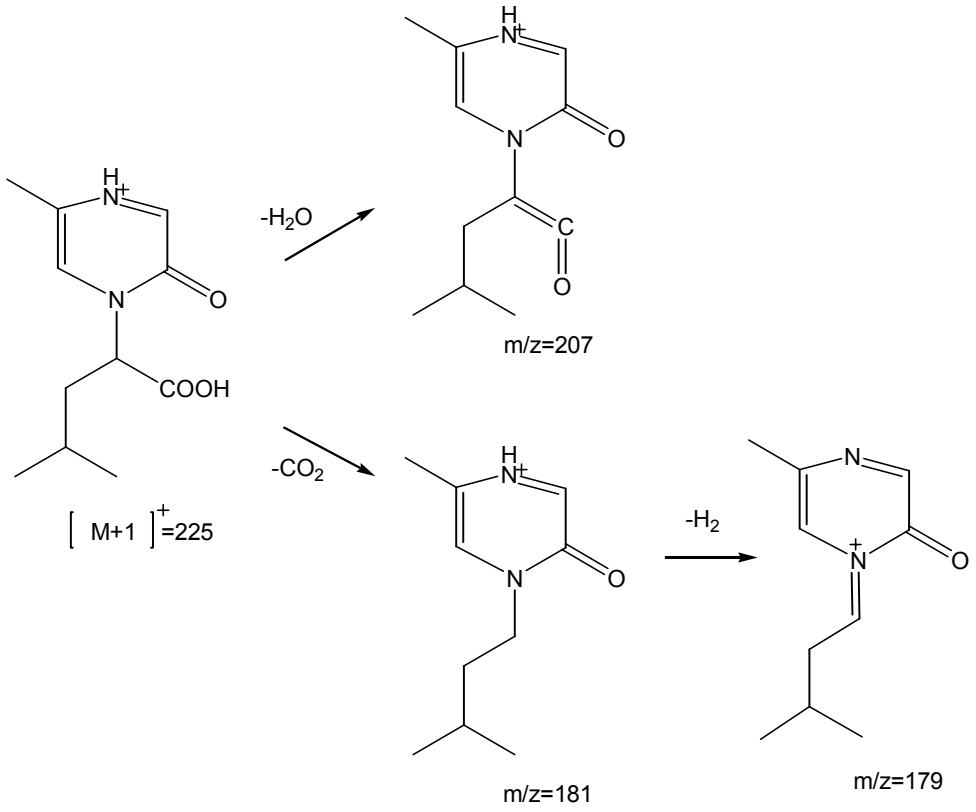

Figure 39. Possible mass fragmentation mechanism of the pyrazinone derivative from MG and Gly-Leu.

4.3.4.3 Identification of Adduct from the Reaction of Methylglyoxal with Gly-Phe

A mixture of MG and diglycine in aqueous solution was prepared to look for the expected pyrazinone adduct, 1-[(α-benzyl)-carboxymethyl]-5-methyl-2-pyrazinone with molecular weight of 258. Figure 40 shows the mass chromatogram of [M+1] ion at m/z 259. The mass spectrum of peak at retention time of 28.01 min is shown in Figure 40. Figure 41 shows the proposed fragmentation mechanism of this molecule. It is consistent with the expected structure of adduct, 1-carboxymethyl-5-methyl-2-pyrazinone. The M+1 ion of 259 can loss a molecular of H_2O leading to ion at m/z 241. On the other hand,

neutral loss of CO_2 from M+1 ion will lead to fragment ion at m/z 215 subsequent loss of a molecule of hydrogen will form ion at m/z 213.

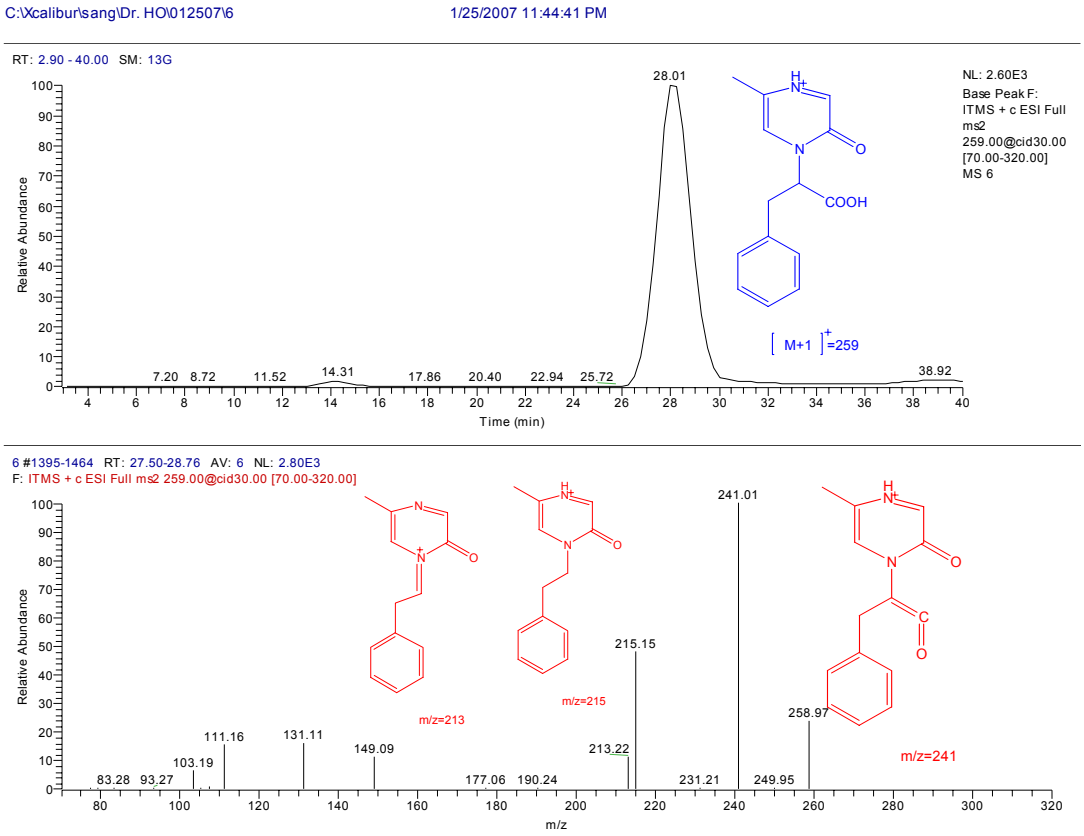

Figure 40. LC/MS Analysis of Gly-Phe. A mixture of MG and Gly-Phe in aqueous solution had been incubated at 37 ^{o}C for a week and then store at room temperature for 4 weeks before the LC-MS analysis.

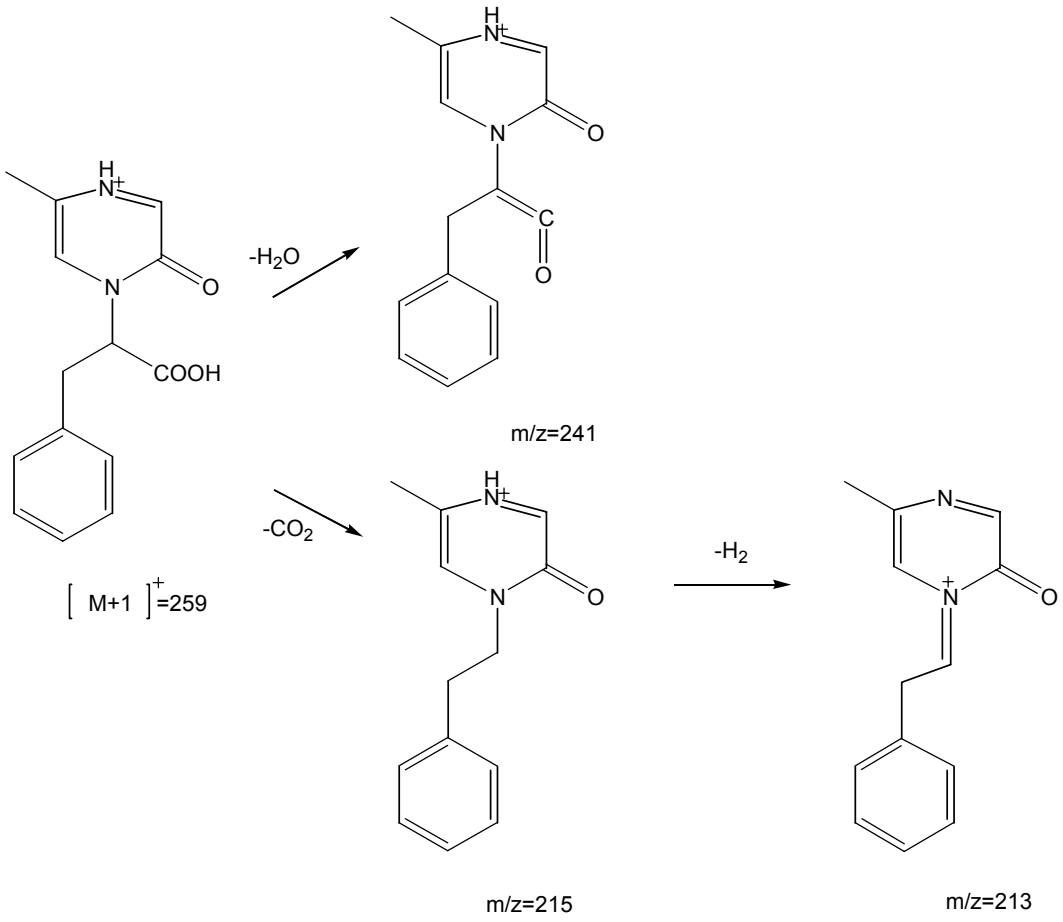

Figure 41. Possible mass fragmentation mechanism of the pyrazinone derivative from MG and Gly-Phe.

4.3.4.4 Identification of Adduct from the Reaction of Methylglyoxal with Gly-Pro

Because of the ring structure on the side chain of Gly-Pro, It is impossible for Gly-Pro to form the pyrazinone structure. A Schiff base adduct was proposed and examined when a mixture of MG and Gly-Pro in aqueous solution was incubated at 37 ^{o}C for a week and then store at room temperature for 4 weeks. The resulting reaction mixture was subjected to LC-MS to look for the expected Schiff base adduct with molecular

weight of 226. Figure 42 shows the mass chromatogram of [M+1] ion at m/z 227. The mass spectrum of peak at retention time of 3.81 min is shown in Figure 42. The mass fragmentation of this compound clearly suggests that the C=N bond formed between the primary NH_2 group of Gly-Pro and the ketonic carbonyl group of the MG. This is in agreement with the observation of Krause *et al* (2004) that the aldehydic carbonyl group of MG is mainly hydrated in the aqueous solution. Figure 43 shows the proposed fragmentation mechanism of this Schiff base molecule. The M+1 ion of 227 can loss a molecular of H_2O leading to ion at m/z 209. On the other hand, neutral loss of CO_2 from M+1 ion will lead to fragment ion at m/z 183. The loss of a molecule of formaldehyde from ion at m/z 183 will lead to ion at m/z 153, subsequent loss of a molecule of C_2H_4 from the pyrrolidine ring will form ion at m/z 125.

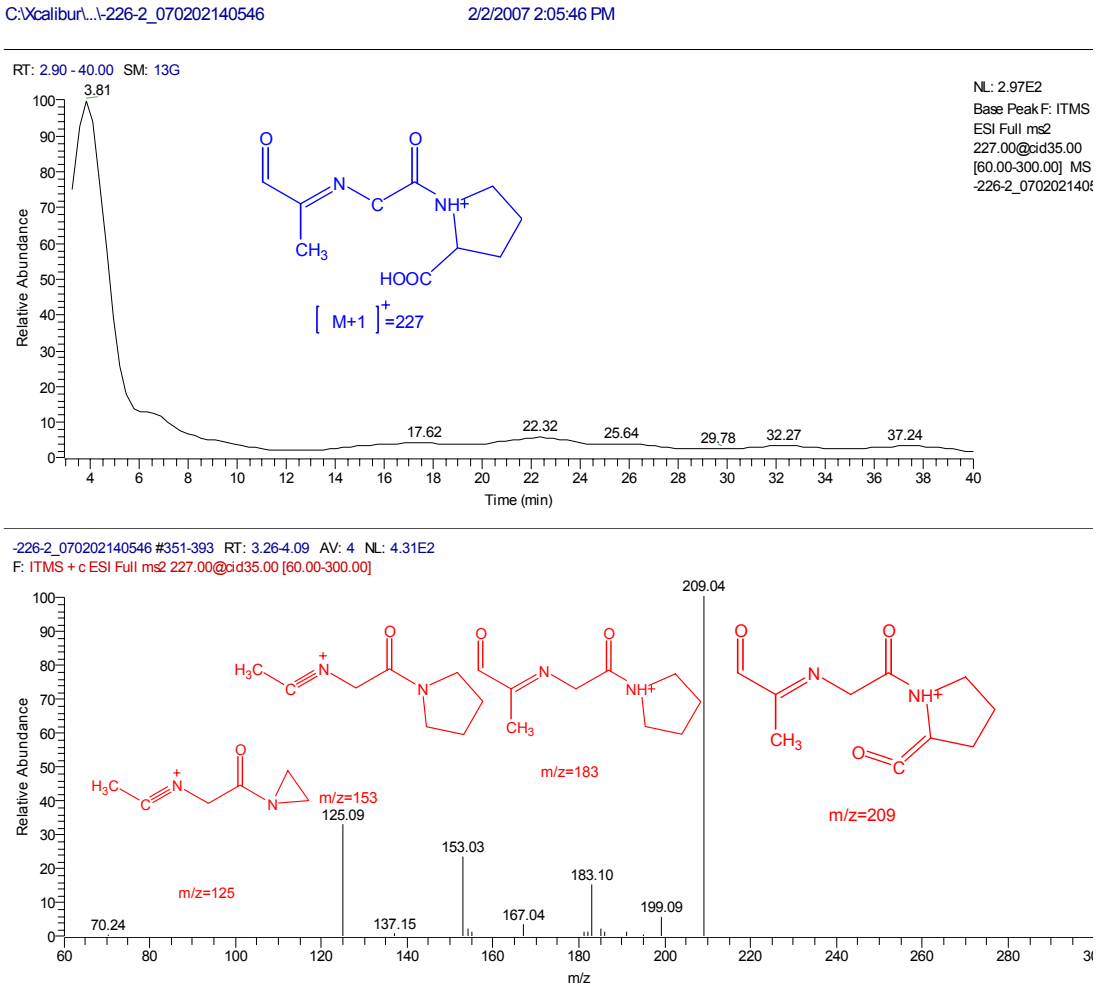

Figure 42. LC/MS Analysis of Gly-Pro. A mixture of MG and Gly-Pro in aqueous solution had been incubated at 37 ^{o}C for a week and then store at room temperature for 4 weeks before the LC-MS analysis.

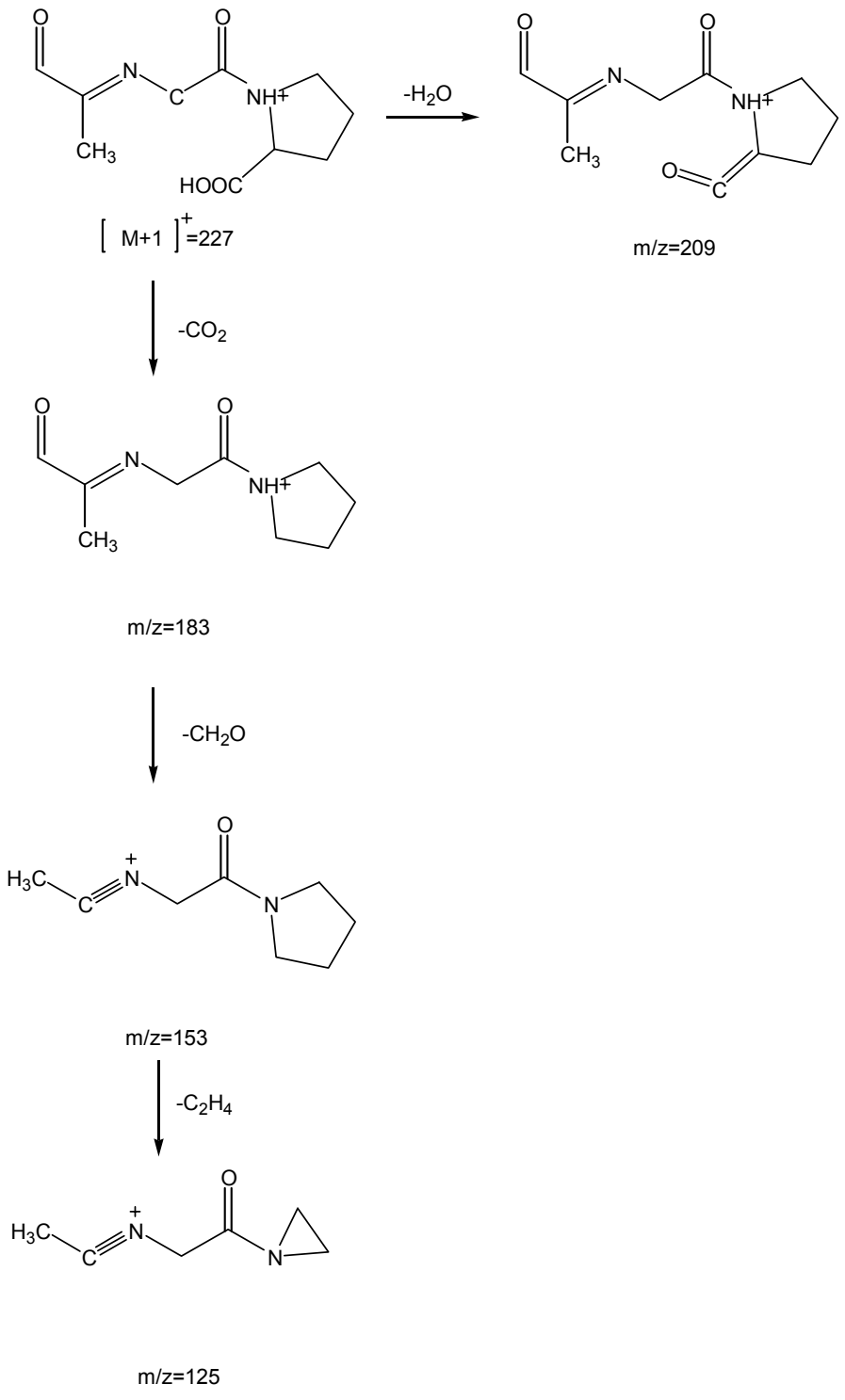

Figure 43. Possible mass fragmentation mechanism of the Schiff base from MG and Gly-Pro.

4.3.4.5 Identification of Adduct from the Reaction of Methylglyoxal with Gly-Ser

A mixture of MG and diglycine in aqueous solution was prepared to look for the expected pyrazinone adduct, 1-[(α-hydroxymethyl)-carboxymethyl]-5-methyl-2-pyrazinone with molecular weight of 198. Figure 44 shows the mass chromatogram of [M+1] ion at m/z 199. The mass spectrum of peak at retention time of 5.73 min is shown in Figure 44. Figure 45 shows the proposed fragmentation mechanism of this molecule. It is consistent with the expected structure of adduct, 1-carboxymethyl-5-methyl-2-pyrazinone. The M+1 ion of 199 can loss a molecular of H_2O leading to ion at m/z 181 subsequent loss of a molecule of water will from ion at m/z 163. On the other hand, neutral loss of CO_2 from M+1 ion will lead to fragment ion at m/z 155 subsequent loss of a molecule of hydrogen will form ion at m/z 137.

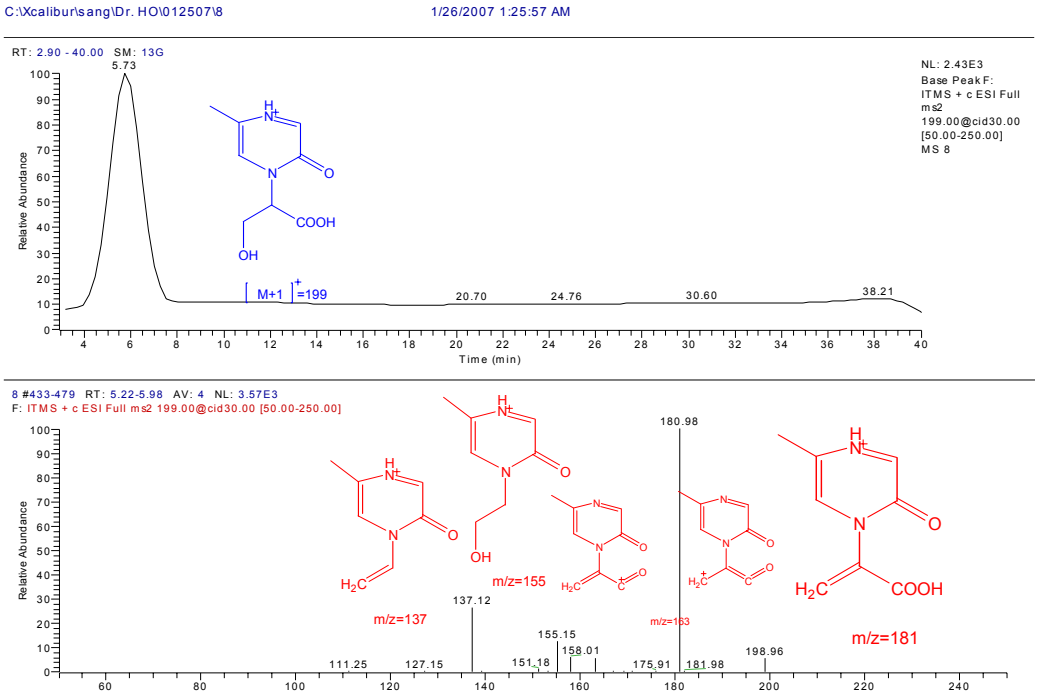

Figure 44. LC/MS Analysis of Gly-Ser. A mixture of MG and Gly-Ser in aqueous solution had been incubated at 37 ^{o}C for a week and then store at room temperature for 4 weeks before the LC-MS analysis.

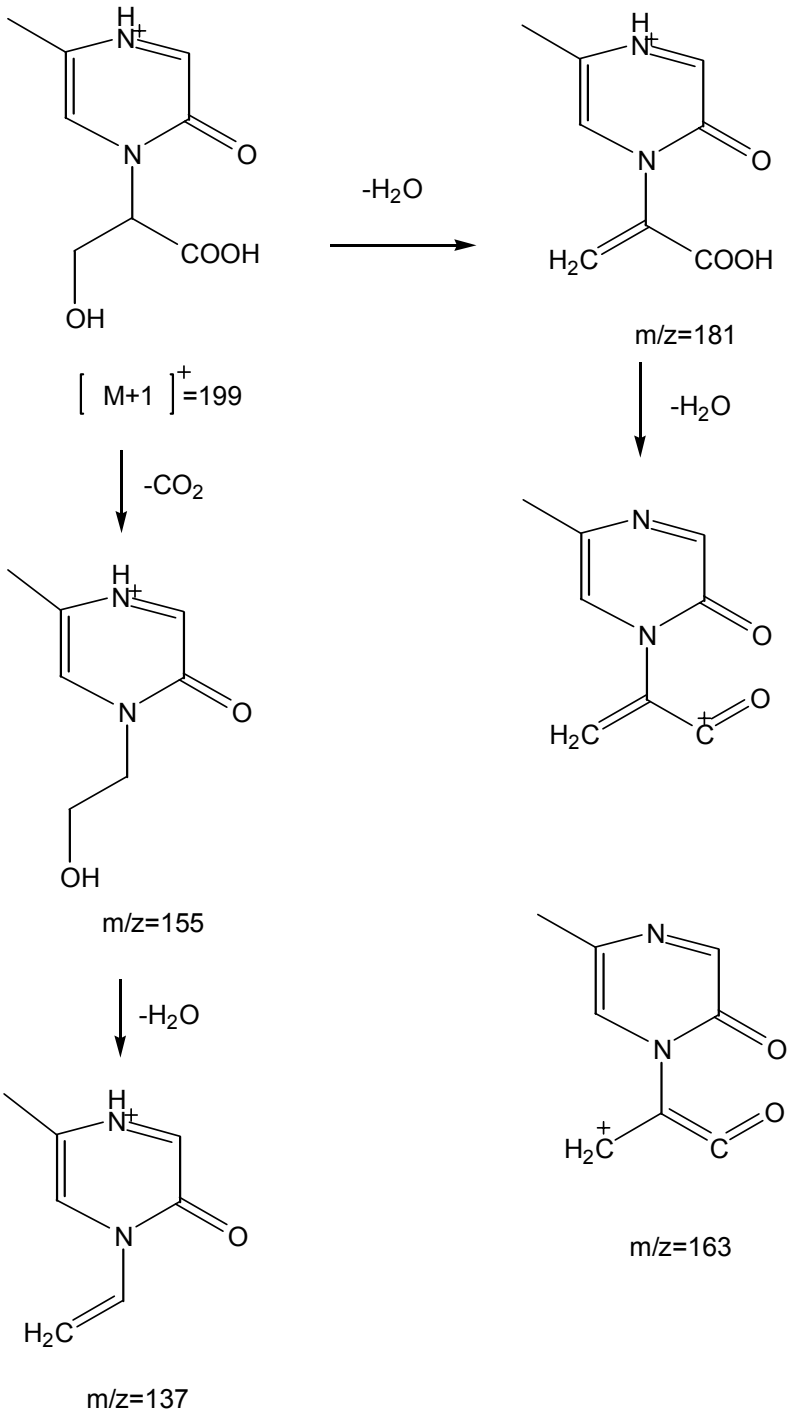

Figure 45. Possible mass fragmentation mechanism of the pyrazinone derivative from MG and Gly-Ser.

4.3.5 Comparison between Diglycine and Triglycine

To better explore the MG trapping mechanism, seven day time course studies have been conducted. Diglycine (DG) shows the steadily scavenging of MG in 7 day period while pyrazinone is generated accordingly (Fig. 46). The amount of pyrazinone was represented by the sum of AUC at the retention of 7 min from the HPLC chromatogram.

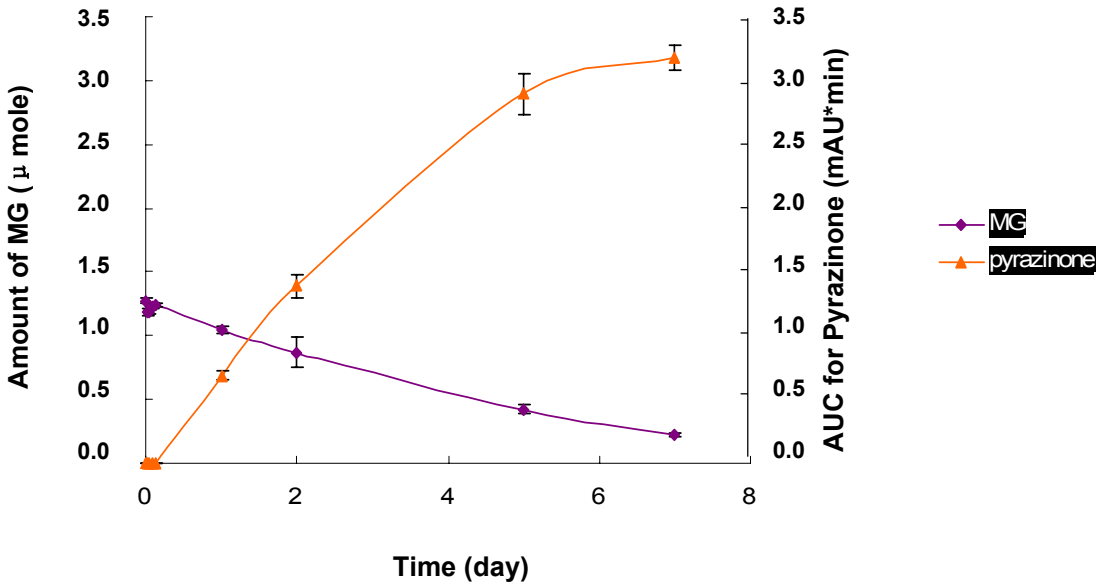

Figure 46. The kinetic study of reaction of diglycine and MG by monitoring the decline of MG and the generation of pyrazinone.

The peak of 11.50 was used to calculate the absolute amount of MG from the standard curve generated from the indicated range of MQ. The AUC of the peaks at retention time of 7 min was used to calculated the production of pyrazinone.

Comparing the decrease of MG by DG and TG provided the influence of the peptides length on MG trapping. As shown on Fig. 47, DG showed a time dependent consumption of MG with R square of 0.9991. TG, on the other hand, decreased MG rapidly from beginning and exceeded 50% decrease of MG in one day. As mentioned before, TG actually showed a much efficient trapping ability compared to all the tested dipeptides except Gly-Cys and Cys-Gly tested in the primary test. Cys-containing peptides as discussed before would trap MG by forming CEC derivatives and/or pyrazinone. TG seemed to have different mechanism to trap MG to show such a different reactivity as well. The LC/MS was applied as the preliminary study of the adduct products.

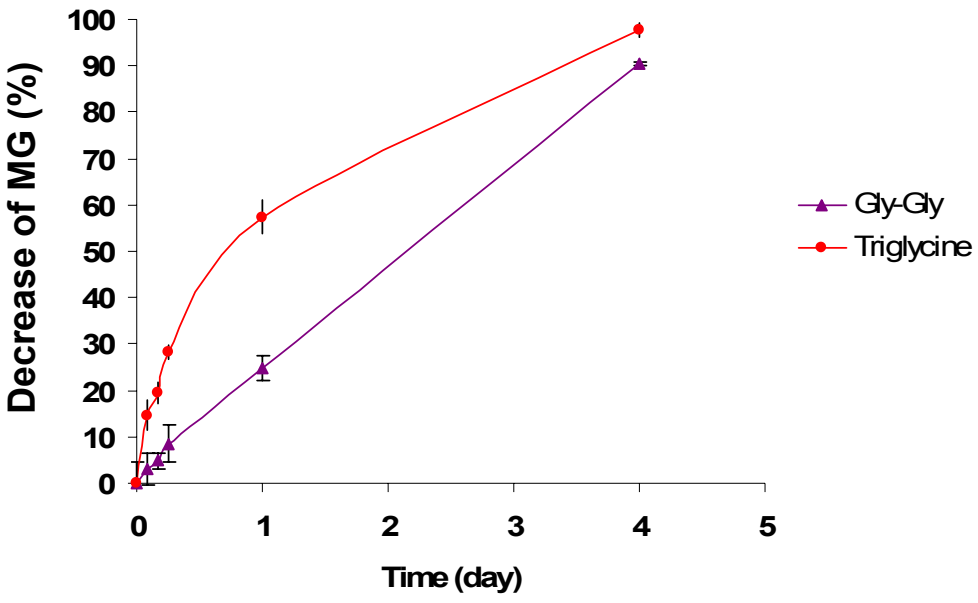

Figure 47. The kinetic study of reaction of diglycine and TG with MG.

4.3.6. Identification of Adduct from the Reaction of Methylglyoxal with Diglycineamd Triglycine

4.3.6.1 Identification of Adduct from the Reaction of Methylglyoxal with Diglycine

A mixture of MG and diglycine in aqueous solution was prepared to look for the expected pyrazinone adduct, 1-carboxymethyl-5-methyl-2-pyrazinone with molecular weight of 168. Figure 48 shows the mass chromatogram of [M+1] ion at m/z 169. The mass spectrum of peak at retention time of 40.21 min is shown in Figure 48. Figure 49 shows the proposed fragmentation mechanism of this molecule. It is consistent with the expected structure of adduct, 1-carboxymethyl-5-methyl-2-pyrazinone. The M+1 ion of 169 can loss a molecule of H_2O leading to ion at m/z 151. On the other hand, neutral loss of CO_2 from M+1 ion will lead to fragment ion at m/z 125, subsequent loss of a molecule of hydrogen will form ion at m/z 123.

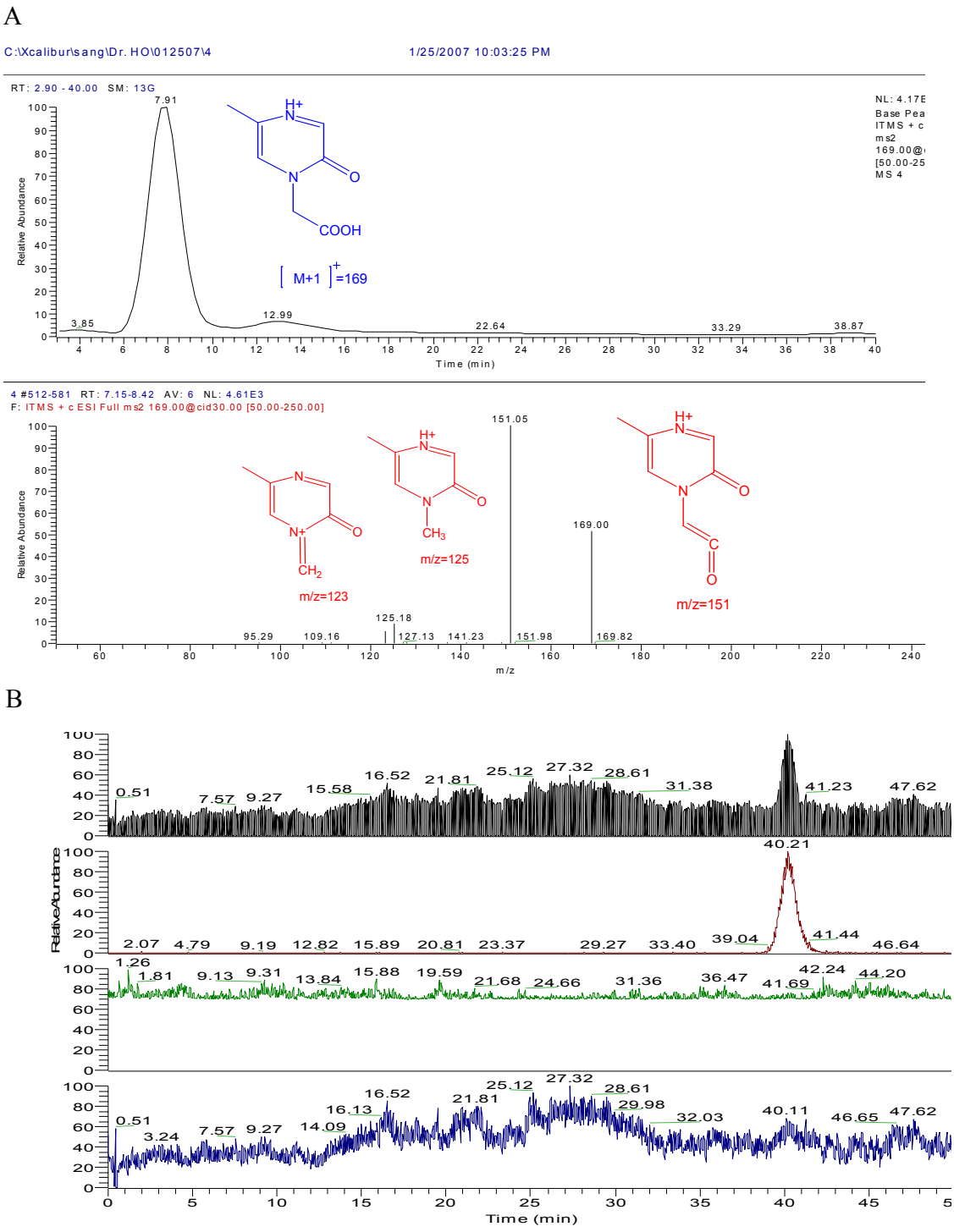

Figure 48. LC/MS Analysis of Gly-Gly. A mixture of MG and Gly-Gly in aqueous solution had been incubated at 37 ^{o}C for a week and then store at room temperature for 4 weeks before the LC-MS analysis.

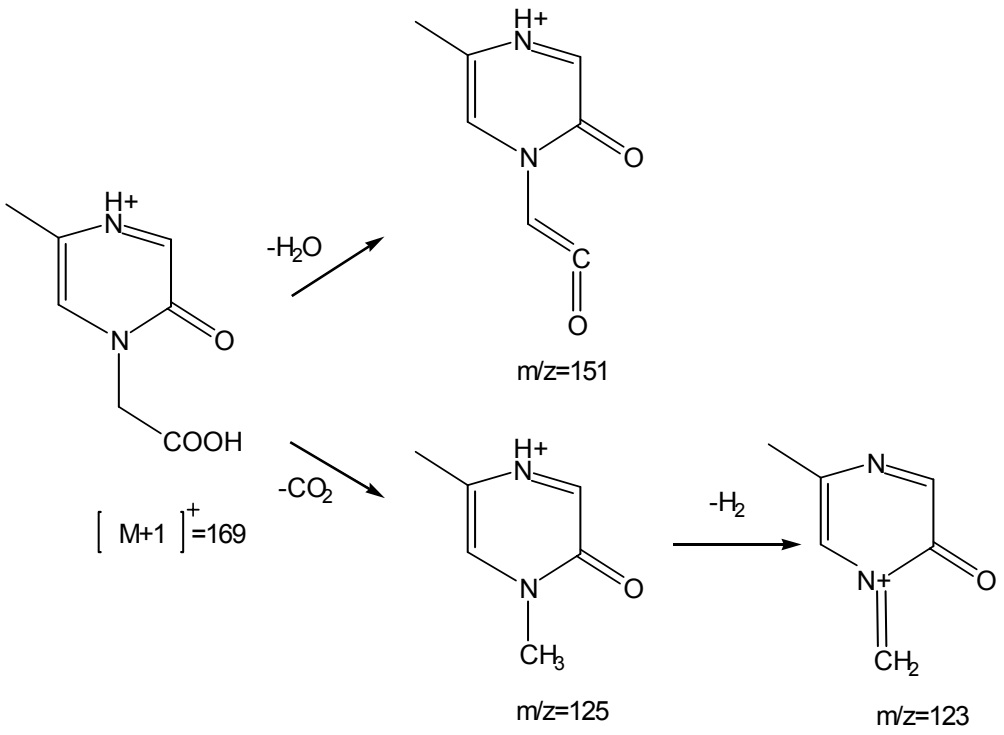

Figure 49. Possible mass fragmentation mechanism of the pyrazinone derivative from MG and Gly-Gly.

4.3.6.2 Identification of Adduct from the Reaction of Methylglyoxal with Triglycine

A mixture of MG and TG in aqueous solution was prepared to look for the expected pyrazinone adduct, *N*-[2-(5-methyl-2-oxo-2H-pyrazin-1-yl)-acetyl]-glycine with molecular weight of 198. Figure 50A shows the mass chromatogram of [M+1] ion at m/z 226. Figure 50B shows the mass chromatogram of [M+1] ion at m/z 226 at retention time of 7.94 min. Figure 51 shows the proposed fragmentation mechanism of this molecule. It is consistent with the expected structure of adduct, *N*-[2-(5-methyl-2-oxo-2H-pyrazin-1-yl)-acetyl]-glycine. The M+1 ion of 226 can glycine and form the major ion at m/z 151.

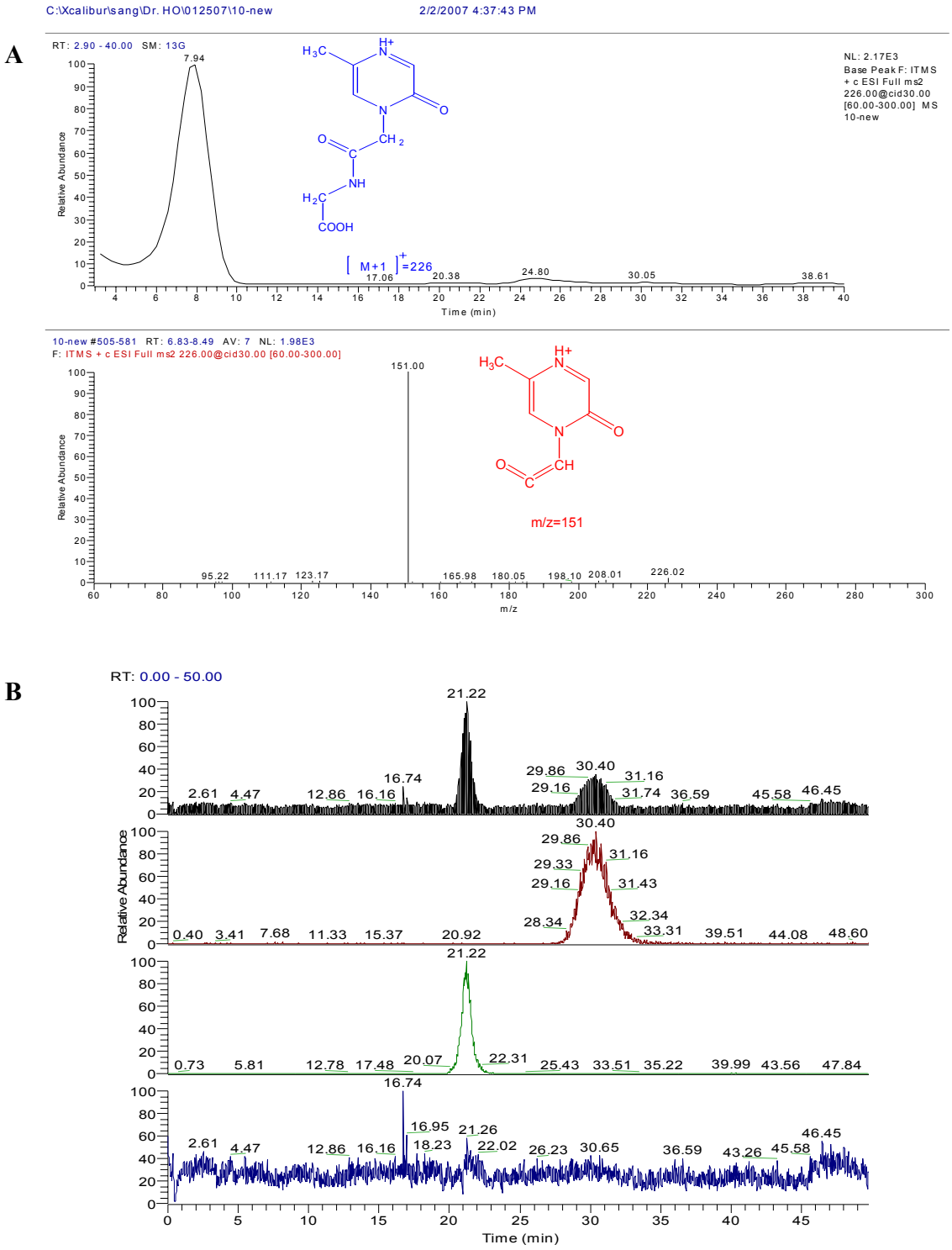

Figure 50. LC/MS Analysis of triglycine. A mixture of MG and triglycine in aqueous solution had been incubated at 37 ^{o}C for a week and then store at room temperature for 4 weeks before the LC-MS analysis.

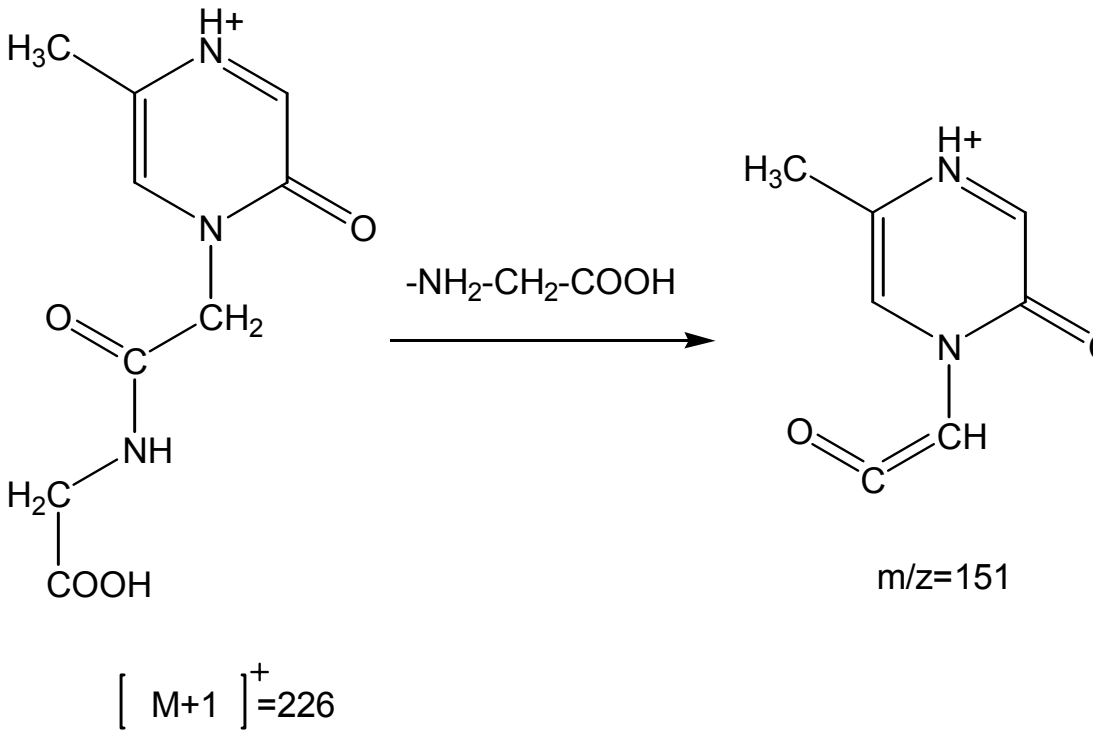

Figure 51. Possible mass fragmentation mechanism of the pyrazinone derivative from MG and triglycine.

The proposed mechanism for the reaction between TG and MG is shown in figure 52, which provided an explanation why TG traps MG more efficiently than DG. As all the experiments carried in our study were at pH 7.4 mimicking the physiological condition, the mild alkaline condition provided the hydroxide to stabilize TG structure by forming the negative charged at the middle peptide nitrogen atom. This negative charged atom will be the most nucleophilic one to most easily attack MG. As TG and MG forms the Schiff base, the amine group can then attack the keto group of MG breaking the peptide bond. It is then followed by the dehydration to form the Amadori product and the formation of pyrazinone finally.

It is interesting to compare the second panel of Figure 48B and the second panel of Figure 50B which are the negative LC/MS SRM at ion of m/z 167.. The chromatograms showed that the peak at 40 min at Fig. 48B is the same as the peak at 30 min at 50B due to the retention time shift. They are both the same negative ion species of 1-carboxymethyl-5-methyl-2-pyrazinone, the pyrazinone product from reaction of dipeptide and MG. It might indicate that either TG will degrade to DG, or the Schiff base or Amadori form between TG and MG will facilitate the cleavage of peptide bond, which was proposed above, to form 1-carboxymethyl-5-methyl-2-pyrazinone. The later one is more convincing since previous study from our lab showed that TG is quite stable even at higher temperatures (Lu, 2005). In addition, as mention before, TG forms the pyrazinone product much faster than DG, it is less possible for TG to degrade slowly to DG, a compound reacts with MG more slowly. However, glycine might need to be included to see if it provided a much efficient MG trapping ability to compensate the difference.

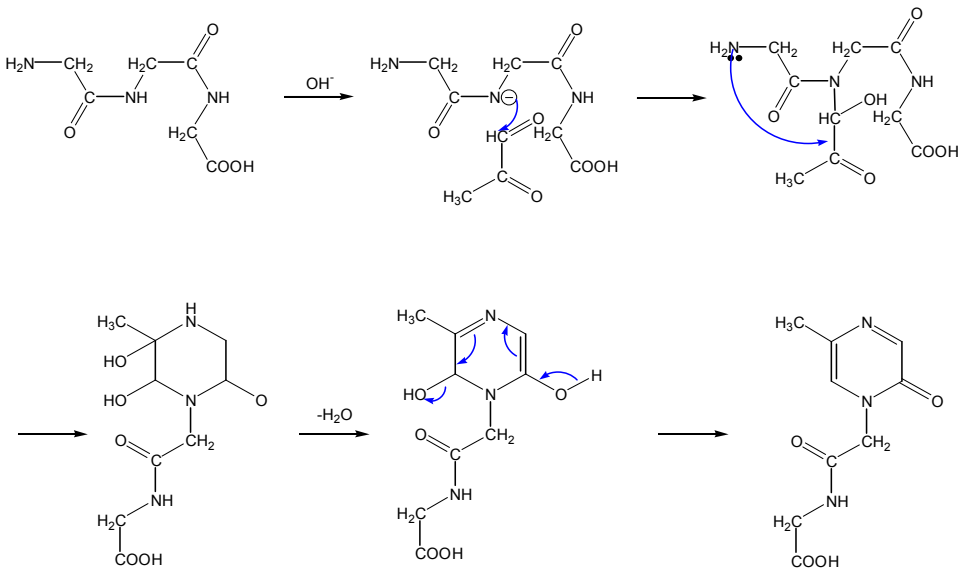

Figure 52. Proposed mechanism of 5-methyl-pyrazinone formation from MG and triglycine.

5. Conclusion

GC analysis with the PFBHA derivatization provided one way to quantify MG. The quantification of MG-*O*-PFB-oxime compounds was based on the sum of these signals in the gas chromatograms. There are six peaks for MG-O-PFB. Peaks 1 and 2 are the syn and anti O-PFB-oxime stereoisomer derivatives of IS (2-chlorobenzaldehyde) and peaks 3, 4, 5 and 6 are the syn+syn, syn+anti, anti+syn and anti+anti O-PFB-oxime stereoisomer derivatives of MG. The limit of quantification (LOQ) was determined as 5.1 μg/L. The internal standard, 3,4-hexanedione in our study, is required. In addition, the extraction process is a necessary step before the GC analysis.

Using the commercial available 2-methylquinoxaline to generate the standard curve, HPLC system provides a quantification method for MG determination in an absolute value. As that the internal standard compound is not needed in this method. We used 1,2-diaminobezene to derivatize MG which resulted in a single peak, 2-methylquinoxaline, in HPLC chromatogram. This excludes the bias as the need to sum up areas of many peaks as in the GC method. The range of 0.05 mM to 3 mM 2-methylquinoxaline was used in our study with the R square of 0.997. In addition, HPLC method eliminates the extraction process which may prevent the possible lost of MG or its derivatives during the procedure. HPLC with UV detector also detected TF-3 and pyrazinone generated from the reaction of diglycine and MG in our study.

In the present study, all the tea polyphenol compounds including pyrogallol, gallic acid, (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG), theaflavin, (TF1), theaflavin-3 or 3′-gallate (TF2) and theaflavin 3,3′-digallate (TF3) showed very good MG trapping abilities. Catechins seemed to trap MG at the ratio of 1 to 1. The primary adduct between EGCG and MG has

been identified (Lo, *et al.* 2006). It is concluded that the reaction between EGCG and MG dominantly occurs at the C8-position of the A ring of EGCG.

One molecule of theaflavins was observed to react with two molecules of MG in the present study. The time course study of TF3 and MG showed a rapid decline of MG in which 50% MG was trapped in ten minutes. In addition, TF3 was diminishing spontaneously at an even fast trend. This might indicate that the trapping of MG is stepwise, and the first addition was faster than the second addition of MG to TF3.

Several dipeptides and triglycine have been evaluated for their abilities to trap MG. Except Gly-Pro, all other non-Cys-containing dipeptides tested in our study may trap MG by the formation of pyrazinones. The pyrazinone structures were confirmed by LC/MS/MS. The trapping efficiency was investigated by time course study. It was shown that the reaction between MG and dipeptides was mostly completed at day five. Compared among Gly-Phe, Gly-Gly, and Gly-Leu, we believe the different trapping efficiency is mainly due to the nucleophilic properties the side chain of the C-terminal amino acids provide. Phenylalanine with the electron pushing group provides the highest trapping efficiency while Leu with the steric hindrance effect showed the lowest efficiency. In addition, 5-methylpyrazinone was proposed as the main product with high regioselectivity of the reaction based on the Krause's observation (Krause *et al*., 2004)

Gly-Pro, on the other hand, is a ternary amine not able to form pyrazionone. The major product between MG and Gly-Pro was proposed to be a Schiff base based on the data from LC-MS-MS.

The high nucleophile property of thiol group of Cys-containing dipeptides provides them other trapping mechanism than the formation of pyrazinone. Cys-dipeptide

may trap MG by forming CEC moiety. It is interesting that Cys-Gly trapped MG faster than Gly-Cys, 39.6% compared to 12.3% in 2-hr incubation and 82.5% compared to 61.9% in 1-day incubation. In addition, Gly-Cys cannot form pyrazinone in our observation, which might provide an explanation for the trapping activity since there are two ways for Cys-Gly to react with MG. Cysteine amino acid trapped the MG most rapidly among all the compounds we tested, more than 50% of MG was decreased by 2 hours. γ-Glu-Cys seemed to react with MG more efficiently than Gly-Cys as well, however, toward the end of the incubation (5-7 days), the MG was depleted by all Cys-containing compounds except *N*-acetylcysteine.

To investigate if the length of peptide influence trapping of MG, time course study was conducted with diglycine and triglycine. The results showed that TG trapped MG much more rapidly than DG. We proposed here that under slightly basic condition, triglycine may form a negative charged nitrogen atom, which might increase its nucleophile property and increase its reactivity to MG.

6. Suggested Future Studies

1. In the present study, the adducts between peptides and MG were tentatively identified by LC-MS-MS only. For the positive identification, those major products such as pyrazinones and CEC derivatives have to be isolated and purified by chromatographic techniques and properly identified by various NMR spectrometric techniques. This is particularly important for the positive assignment for the position of methyl group in the pyrazinone ring, and also for the unambiguous proof of the structure of CEC moiety.
2. The kinetics of the reactions between peptides and MG needs to be better understood. This requires monitoring not only the reactants but also the products using HPLC and HPLC-MS. The effects on time intervals and different concentration of reactants should be studied.
3. Based on the comparison of MG trapping efficiency between diglycine and triglycine, we need to further understand the structure and mechanism which cause this difference. It is recommended to design and synthesize more dipeptides and tripeptides for the study of structure-activity-relationship (SAR) of their MG trapping efficiency.
4. Polyphenols other than catechins are recommended to examine their abilities in trapping MG. It is then providing more desirable and effective use of a phytochemical to trap MG. The proper bioavailability of a phytochemical should be concerned the most to reach the expected MG trapping ability *in vivo*. From our study, green tea and black tea polyphenols do show the

excellent MG trapping ability, however, the lack of good bioavailability might hinder their effectiveness *in vivo*.

5. It is suggested to establish a cell culture system to evaluate the MG trapping ability of a dietary compound, to evaluate the difference between real and mimicking physiological environments. Current developed *in vitro* method for the evaluation of MG trapping efficiency of dietary compounds is simple and convenient, which is very useful for screen the possible MG trapping reagents. But the drawback is its ignorance of the bioavailability and potential metabolism of dietary compounds.

6. Current *in vitro* trapping of MG by dietary compounds has its importance in the application in decreasing the MG levels in processed foods and beverages. Other study in our laboratory has shown that carbonated beverages contain unusual high levels of reactive carbonyl species including MG, GO and 3-deoxyglucosone. Accordingly, the addition of green and black tea polyphenols has been shown to significantly reduce the level of MG in these carbonated beverages.

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