The modulatory effects of flavonoids on the enzymatic activities of transglutaminase2

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Declaration

I hereby certify that the work embodied in this thesis is the result of my own investigations except where reference has been made to published literature

Aknowledgment

First and primarily, all praise be to Allah, who gave me the strength to persevere and complete my thesis.

In addition, I would like to offer my huge thanks to my supervisor, Dr Phil Bonner, for his guidance, continuous support, eagerness, and great knowledge, as well as encouraging me to perform to the best of my abilities. I am also thankful for his assistance in writing this thesis. I am deeply proud to have been work under his supervision.

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Abstract

To date, a gluten-free diet is the only accepted form of therapy for coeliac disease (CD). Because of the important role of transglutaminase2 enzyme in the initiation of coeliac disease (CD).This study was aimed at the identification of TG2 inhibitors from natural sources, as a potential intervention in CD therapy. The natural products chosen for this study were dietary flavonoids. Kaempferol, morin, and quercetin displayed inhibition on hr TG2 (human recombinant transglutaminase 2) and in fewer extent gplTG2 (Guinea pig transglutaminase2) amine incorporation activity. However, the majority of flavonoids showed a trend of inhibitions (>50% of inhibition at 1.25, 12.5 and125µM) on the cross-linking activity of hrTG2.The flavonoids used did not show a significant inhibitory effect on the amount of ammonia released in the deamidating activity of hrTG2. A novel method for purification of flavonoids from food extracts was developed, using Immobilized Metal Ion Affinity Chromatography (IMAC). In addition, a BCA assay was optimised in a novel quantification method using quercetin as a standard flavonoid. In addition, to the pure flavonoids, flavonoids were extracted from different food samples. In the TG2 amine incorporation activity, all of food extracts display a significant inhibition effect towards the hrTG2 and gplTG2 (20-50% of inhibition). While in the TG2 cross-linking activity, the majority of food extracts did display an inhibition effect on the gpl TG2 cross-linking activity (50-70% of inhibition), but only the Strawberry and kale extract had an effect on hrTG2 activity (40-50% of inhibition). In the deamidation assay, no food extracts showed inhibition. A cell-based fluorescence assay confirmed the TG2 inhibitory characteristic of kaempferol on HT29 cell lines. This was among the first studies to examine the effects of flavonoids on the activity of TG2. The inhibition of transglutaminase 2 (TG2) deamidating activity can be considered as a potential therapeutic target in the treatment of coeliac disease (CD). All current TG2 assays measure amine incorporation, none can specifically measure TG2 deamidating activity because there would be little or no deamidation in the presence of an amine. A novel deamidating assay has been developed using o-pthaldehyde (OPA) to measure the ammonia released in deamidation. This assay demonstrated that the TG2 deamidation of *Vicia faba* storage proteins and gliadin peptides was enhanced by presence calcium and inhibited by EDTA.

Publications

Alshintari, M.A., Bonner, P. L., Hargreaves, A.J., and Dickenson, J. M. “The Effect of Dietary Flavonoids on Transglutaminase 2 Activity: A potential Role in Management of Coeliac Disease?”. School of Science and Technology 10th Annual Research Conference (STAR conference), Nottingham Trent University (May 2017). Poster and oral presentation abstract no (3).

Alshintari, M.A., Bonner, P. L., and Hargreaves, A.J. “The Effect of Dietary Flavonoids on Transglutaminase 2 Activity: A potential Role in Management of Coeliac Disease?”. Transglutaminases in Human Disease Processes (GRS) Gordon Research Conference and seminar June 16-22, 2018. Les Diablerets, Switzerland. Poster and abstract no (31).

Abbreviations

BCA: *Bicinchoninic* acid (Protein Assay)

BSA: Bovine serum albumin

BXC Biotin X cadaverine

CD: Coeliac disease

DMSO: Dimethyl sulphoxide

ECL: Enhanced chemiluminescence

ECM: Extracellular matrix

EDTA: Ethylene diaminetetraacetic acid

GDP guanosine 5'-diphosphate

Gpl TG2 Gunia Pig transglutaminase2

GTP guanosine 5'-triphosphate

hrTG2: Human Recombinant transglutaminase2

HPLC: High pressure liquid chromatography

HT29: Human colon adenocarcinoma cell line

MTT: 3-(4-5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

OPA: *o*-phthalaldehyde

PAGE: Polyacrylamide gel electrophoresis.

PBS: Phosphate buffer saline

R283: 1, 3, dimethyl-2-[(2-oxopropyl) thio] imidazolium chloride

ROS Reactive oxygen species

SDS: Sodium dodecyl sulphate

TEMED: Tetramethylethylene amine

TG: Transglutaminase

Z-DON:Benzyloxycarbonyl-(6-Diazo-5-oxonorleucinyl)-L-Valinyl-L-Prolinyl-L-Leucinmethylester

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Chapter 1 : General Introduction

1.1 Introduction

Transglutaminases (EC: 2.3.2.13; TGs), also known as protein-glutamine γ-glutamyl transferases, are calcium-dependent enzymes which catalyse posttranslational modifications to many proteins. This happens through inter- or intramolecular covalent cross-linking by an acyl-transfer between the γ-carboxyamide groups of glutamine residues and ε-amino groups of lysine residues or other primary amines leading to the formation of covalent isodipeptide cross-links. (Martins, et al. 2014). Transglutaminases are a 9- member family of multifunctional proteins. Eight TGs (TG1-7 and factor XIIIa (FXIIIa) possess transamidation/deamidation activity, while band 4.2 does not have a catalytic role but mainly acts as a structural protein of erythrocyte membranes (Kárpáti, et al., 2018).

1.2 History of the discovery of transglutaminases

In 1957, Sarkar and co-workers clarified homogenates of brain, kidney, and liver of guinea pig showing the transamidation activities catalysed by TGs for the first time (Sarkar, Clarke and Waelsch 1957). Clarke et al., (1959), also observed the transamidation activity of TG in the guinea-pig liver. Pisano et al., (1969) found that Factor XIIIa had the ability to cross-link and stabilise fibrin monomers as the final step in the blood coagulation cascade, a finding that has established the idea that these enzymes can modify proteins and act as biological glues(Griffin, Casadio and Bergamini 2002).

1.3 Biological activities of transglutaminases

Transglutaminase enzymes cross-link many different proteins containing glutamine and lysine residues through a transamidation reaction (see Figure 1-1). The isodipeptide bonds formed between cross-linked proteins are peptidase resistant (Kanchan, Fuxreiter and Fésüs 2015). Cross-linking of proteins helps in some cellular functions such as stabilising the extracellular matrix (ECM), blood clotting, wound healing, bone growth, apoptosis, cell adhesion, cell survival and cell signaling (Kanchan, et al. 2015). In addition, to protein: protein cross-linking, TGs mediate the incorporation of primary amines into

proteins and, in the absence of amine nucleophiles, the deamidation of protein/peptide glutamine residues to glutamate (see Figure 1-1; (Kárpáti, et al. 2018). Transglutaminases are considered as "housekeeping" enzymes; the biological functions attributed to TG2 include but are not limited to, tissue repair, inflammation, the development of some cancer, fibrosis and coeliac disease (Kobayashi, et al. 1998) and (Bergamini, et al. 1999).

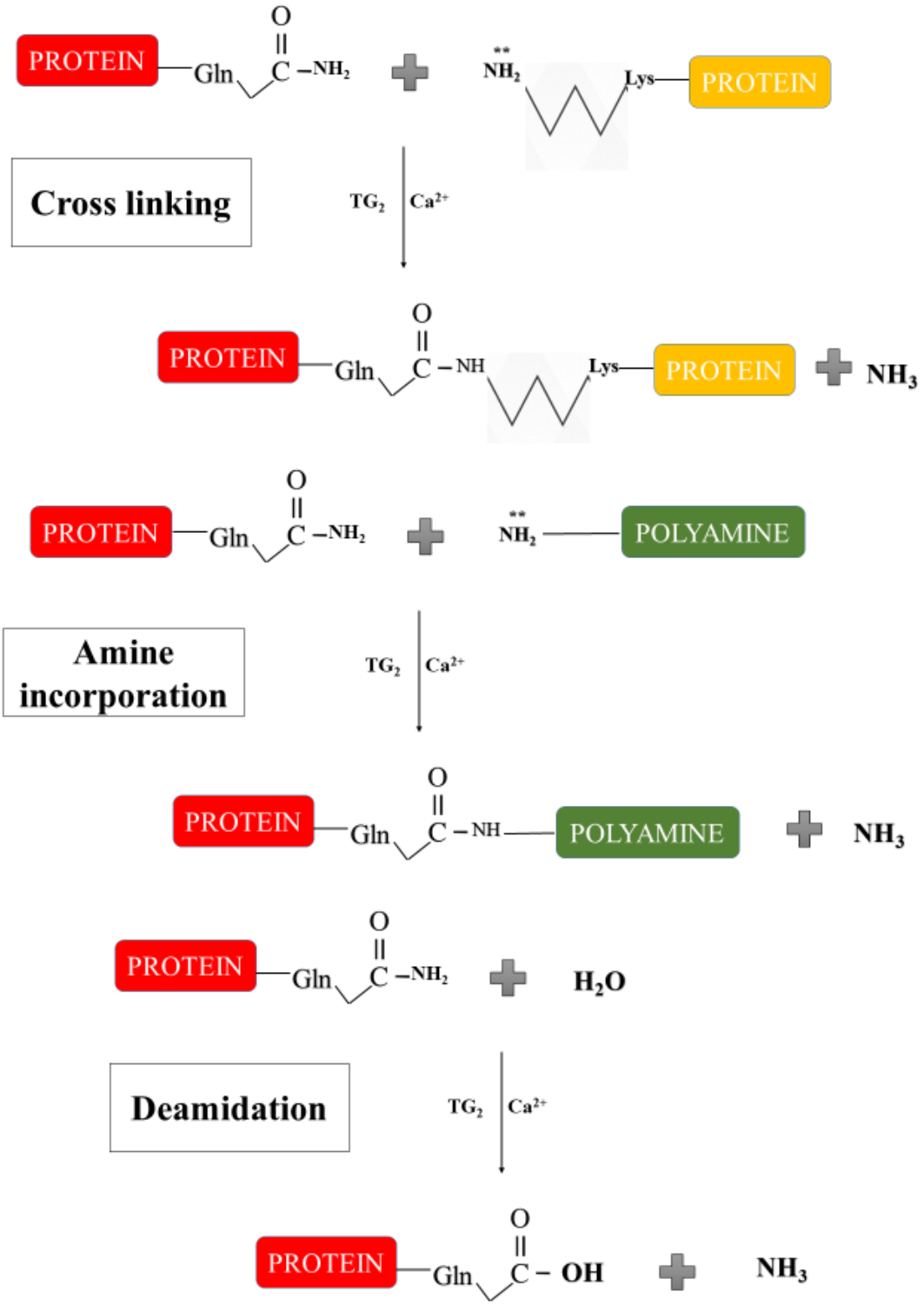


Figure 1-1: Schematic diagram for TGs activity

1.4 Isoforms of mammalian transglutaminases

The different isoforms of transglutaminases are distinguishable from each other due to their different physical properties and their distribution throughout the body (Lentini, et al. 2013). Although the catalytic activities of most TGs overlap, not all are homologous and sequence variations in substrate preference have been observed among TG enzymes (Makarova, Aravind and Koonin 1999). All of the mammalian isotypes of TGs are calcium-dependent except the erythrocyte band 4.2 protein, which is not catalytically active (Satchwell, et al. 2009). In addition, TG1, TG3, and Factor XIIIa are synthesised as zymogens, which are proteolytically cleaved to present the activated form of the enzyme (Pedersen, et al., 1994; Eckert, et al., 2005). The TG2 enzyme targets proteins which act as cellular scaffolds, controlling cell adhesion and regulating signal transduction (see Table 1-1) (Satchwell, et al. 2009).

Table 1-1: Human Transglutaminases

Gene	Protein and Other Names	Location	Size (kDa)	Principle Function	Tissue Distribution
TGM1	TG1, TGk, keratinocyte TG,	14q11.2	90	Cell envelope formation during keratinocyte differentiation	Membrane-bound keratinocytes
TGM2	TG2. Tissue TG, liver TG, endothelial TG, erythrocyte TG, Gh	20q11-12	80	Apoptosis, cell adhesion, matrix stabilization,signal transduction	Many tissues: cytosolic, nuclear, membrane, and extracellular
TGM3	TG3.TGE, callus TG, hair follicle TG, bovine snout TG	20q11-12	77	Cell envelope formation during keratinocyte differentiation	Hair follicle, epidermis, brain
TGM4	TG4. TGp androgen-regulated major secretory protein, vesiculase, dorsal prostate protein 1	3q21-22	77	Reproduction, especially in rodents as a result of semen coagulation	Prostate
TGM5	TG5. TGx	15q15.2	81	Cell envelope formation in keratinocytes	Foreskin keratinocytes, epithelial barrier lining, skeletal muscular striatum
TGM6	TG6. TGy	20q11	78	Not known	Testis and lung
TGM7	TG7. TGz	15q15.2	81	Not known	Ubiquitous but predominately in testis and lung
F13A1	FXIIIa. Fibrin-stabilizing factor, fibrinoligase,plasma TG, Laki-Lorand factor	6q24-25	83	Blood clotting, wound healing, bone synthesis	Platelets, placenta, synovial fluid, chondrocytes, astrocytes, macrophages, osteoclasts and osteoblasts

(Modified from Eckert et al., 2014)

1.4.1 Transglutaminase 1 (TGM1)

Transglutaminase 1 also known as keratinocyte TG resides in the stratified squamous epithelia of the skin, the lower female reproductive system, and the upper alimentary tract. As a result of proteolytic cleavage, a cellular Ca^{2+} level increase leads to the activation of TG1's catalytic activity (Sturniolo and Eckert 2005; Eckert, et al., 2014). Transglutaminase 1 protein was linked with the plasma membrane by a fatty acid acyl

linkage in an N-terminal cysteine residue. The enzyme was released from the membrane by proteolysis as 10-, 33-, and 66-kDa fragments (Kim, Chung and Steinert 1995). Impairment in the function of TG1 is responsible for the skin condition lamellar ichthyosis, occurring due to the inability of TG1 to form ester bonds (Nemes, et al., 1999); (Iismaa, et al., 2009). Transglutaminase1 is central for the cross-linking of substrate proteins essential for the complete development of the cornified envelope of the skin, a cross-linked scaffold located in the outermost layer of the epidermis (Yamane, et al., 2016).

1.4.2 Transglutaminase 2 (TGM2)

Tissue TG (TG2) is widely distributed in tissues and cell types; it is mainly a cytosolic protein but has been shown to be also present in the nucleus and on the plasma membrane of cells (Lorand and Graham 2003). It is encoded by the TGM2 gene on the human chromosome 20q11-12. Transglutaminase 2 shows GTPase, protein disulphide isomerase (PDI), ATPase and protein kinase activity, in addition to transamidation and deamidation reactions (Tatsukawa, et al. 2016). Both Ca^{2+} and GTP can regulate the enzyme's activity. The function of the TG2 isoform has been linked to coeliac disease, neurodegenerative disorders and cataract formation (Griffin, et al., 2002); (Tong, et al., 2013). This enzyme will be discussed in more detail in section 1.5.

1.4.3 Transglutaminase 3 (TGM3)

Transglutaminase 3 (TG3) is also known as epidermal TG exists in the follicles of hair, skin epidermis, and brain tissue. The TG3 gene (TGM3) promoter includes Sp1- and Ets-motifs (Lee, et al., 1996). Calcium enhances the expression of the pro-transglutaminase 3 mRNA and the final TG3 protein appears as two polypeptide chains formed from a single precursor protein after proteolysis. Transglutaminase 3, like TG2, binds to and hydrolyses GTP (Ahvazi, et al., 2003). Transglutaminase 3 is responsible for the hardening of the internal root sheath of the hair follicles via activation of the cross-linking of keratin and trichohyalin intermediate filaments, which is crucial for the morphology of the hair fibre (Hitomi, et al., 2001); (Hitomi, et al., 2003) and (John, et al., 2012). It also contributes to the formation of the keratinocyte cell envelope through the later stages of differentiation (John, et al., 2012). Dysfunction of TG3 causes impaired development of hair and reduction in skin barrier function (John, et al., 2012) and (Bognar, et al., 2014).

Moreover, it is supposed that TG3 is the dominant autoantigen in dermatitis herpetiformis in coeliac patients (Sárdy, et al., 2002).

1.4.4 Transglutaminase 4 (TGM4)

Transglutaminase 4 (TG4), also known as prostate TG, exists in the prostatic fluids of the prostate gland and seminal plasma of humans (Dubbink, et al., 1999); (Grenard, Bates and Aeschlimann 2001) and (W. Jiang and Ablin 2011). Transglutaminase 4 mRNA levels in human prostate cancer cells increase following androgen treatment (Cho, et al., 2010). The TG4 isoform is encoded by TGM4 gene on human chromosome 3p21-22. The role of TG4 in humans is at present unknown. Some studies suggest that there is a link between the promotion of an aggressive prostate cancer phenotype and increased expression of TG4 (Jiang and Ablin 2011). Overexpression of TG4 has a probable role in stimulation and adherence of endothelial cells in prostate cancer, as these effects were decreased when TG4 expression was knocked down (Jiang, et al., 2009). Other studies suggest that TG4 improves fertility in males (Iismaa 2016).

1.4.5 Transglutaminase 5 (TGM5)

Transglutaminase 5 (TG5) is mostly present in foreskin keratinocytes, skeletal muscle and the epithelial barrier (Cassidy, et al., 2005). Transglutaminase 5, like TG2, is a calcium-dependent enzyme, which is regulated via GTP and ATP levels. Transglutaminase 5 is encoded by the TGM5 gene, which is located on human chromosome 15q15.2 and has a molecular weight of ~ 81 kDa. The enzyme is responsible for cross-linking loricrin, involucrin, and SPR3 in the skin epidermis (Melino, et al. 2001) and has a role in hyperkeratosis in patients with ichthyosis and psoriasis. Overexpression of TG5 was detected in, and indirectly related with, numerous pathologies of human epidermis, including ichthyosis Vulgaris, psoriasis and Darier's disease (Candi, et al., 2002). Transglutaminase 5 mutations can lead to skin peeling syndrome (Cassidy, et al., 2005).

1.4.6 Transglutaminase 6 (TGM6)

Transglutaminase 6 (TG6) is expressed in human lungs and testes, brain and in human neuronal carcinoma cells (Thomas, et al., 2013). It includes two polypeptide chains that are cleaved from a single precursor. The proteolytic cleavage of the proenzyme leads to the activation of the catalytic function of TG6 (Stamnaes, et al., 2010; Thomas, et al.,

2013) revealed the participation of TG6 in coeliac disease autoantibody-mediated gluten ataxia (Mulder, Rouvroye and van Dam 2018).

1.4.7 Transglutaminase 7 (TGM7)

The TG7 protein is encoded by the TGM7 gene located on human chromosome 15q15.2 (Griffin et al., 2002). This enzyme is expressed in the skin epidermis (Kuramoto et al., 2013).

1.4.8 Factor XIIIa (F13A1)

Plasma TG (FXIIIa) is found in plasma, platelets, macrophages, astrocytes, placenta, heart, eyes, dermal dendritic cells, chondrocytes, and synovial fluid in cells of the osteoblast lineage. Factor XIIIa is an important component of the blood coagulation cascade involved in the terminal stage of blood coagulation processes (Lorand and Graham 2003b). The A subunit is the catalytic site of FXIII, whereas, the B subunit acts as a carrier protein. Factor XIIIa catalyses cross-linking of fibrin molecules to stabilize fibrin clots, which are resistant to proteolysis and fibrinolysis (Tone et al., 2016). This activity occurs in the presence of Ca^{2+} ions and ultimately contributes to hemostasis and wound healing (Włodarczyk et al., 2014). Deficiency in Factor XIIIa is characterized by prolonged bleeding time and impaired wound healing. (Koseki-Kuno et al., 2003; Dardik et al., 2006; Nahrendorf et al., 2006; Tsujimoto et al., 2011).

1.4.9 Erythrocyte membrane protein band 4.2 (EPB42)

Band 4.2, which is encoded by the EPB42 gene, is a unique member of the mammalian TG family, which lacks catalytic activity. The lack of enzymatic activity is the result of the substitution of the active site cysteine residue for an alanine residue. Band 4.2 protein is located predominantly in foetal liver, bone marrow, spleen and erythrocytes and plays an important role in the organisation of cell stability and maintenance the integrity of the cell membrane (Peters et al., 1999). Band 4.2 protein prefers to bind with ATP instead of GTP, in contrast to the other TG family members (Azim et al., 1996).

1.5 Transglutaminase 2

Transglutaminase 2 (EC: 2.3.2.13) exhibits a number of functions with different molecules acting as substrates for this enzyme, such as proteins, amines, and water. For these reasons and its ubiquitous distribution, it is considered the most interesting member

of the TG family (Lerner et al., 2015). The TG2 gene is localized to chromosome 20q11-12, the protein has 687 amino acids, and it is the most studied and abundant of the nine members of the TG enzyme family (Odii and Coussons 2014).

1.5.1 Structure and conformational changes of TG2

The X-ray α-crystal structure of TG2 has reported the nature of the enzyme's complex structure (Liu et al., 2002). Human TG2 is composed of four globular domains: an N-terminal β-sandwich domain, which contains the binding site for fibronectin. A catalytic domain that consists of the catalytic triad (cysteine-histidine-aspartic acid) plus two tryptophan residues (Yee et al., 1994; Liu et al., 2002). Two C-terminal β-barrel domains, which are implicated in the ability of TG2 to bind and hydrolyse GDP/GTP (Achyuthan and Greenberg, 1987; Liu et al., 2002a) (See Figure 1-2). Three crystal structures of TG2 have been elucidated, one complexed with ATP, one with GDP and another complexed to an irreversible inhibitor (Liu et al., 2002b, Pinkas et al., 2007). Upon the binding of irreversible inhibitor at the active site, TG2 structure undergoes a large conformational change. Inactivation of TG2 via GTP and low calcium levels promotes a compact conformation, whereas activation of TG2 by calcium ions leads to an open conformation (See Figure 1-2). It has been proposed that, because of GTP binding, intracellular TG2 will be sustained in a closed conformation. However, in different circumstances such as cell lysis and necrosis in which calcium levels are increased, there will be a rapid activation of TG2 into an open conformation (Jang et al., 2014). The conformations of TG2 could be essential for its role in cell survival/death pathways (Gundemir, et al., 2013).

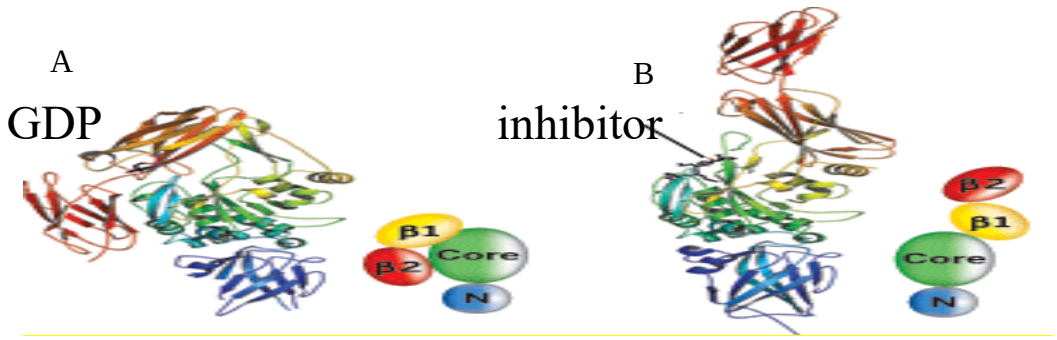


Figure 1-2: Schematic diagram for conformations of TG2.

Overall Structures of GDP-Bound and Inhibitor-Bound TG2. The enzyme consists of 4 domains; an N terminal β sandwich domain (blue), a catalytic core (green) and two C terminal domains, β-barrel 1 (yellow) and β barrel 2 (red). (A) GDP-bound TG2. (B) TG2 inhibited with the active-site inhibitor Ac-P(DON)LPF-NH 2

(Modified from Situ Boman., 2008)

1.5.2 Catalytic functions of TG2

Tranglutaminase2 catalytic functions are divided into two main reaction pathways with a 2-step "ping pong" mechanism (Palmer and Bonner, 2007). In the first step, the sulphur of the active site cysteine (cys277) in TG2 (acyl acceptor) interacts with the γ-carbon of the protein/peptide bound glutamine (acyl donor). This leads to the formation of a thioester bond with the release of ammonia as a by-product (see Figure 1-3). In the second step, the thioester bond is attacked by a primary amine either in transamidation or, in the absence of suitable amine, with water in deamidation. The transamidation reaction of TG2 leads to protein cross-linking which has a critical role in wound healing, blood clot formation, apoptosis and semen coagulation (Lorand and Graham 2003a), thus offering more protection for the organs or tissues. This alteration also comes under the category of a posttranslational modification. (Ju-Hong, et al., 2003). Alternatively, in the transamidation pathway, the nucleophile can be a polyamine (e.g. putrecine, spermidine or spermine). The addition of a polyamine results in an additional positive charge to the surface of the target protein, altering the protein's isoelectric point (pI). In the deamidation reaction, the thioester-glutamine at the active site is displaced by water acting as the nucleophile, which converts protein/peptide bound glutamine to glutamate (Fleckenstein et al., 2002; Sollid & Jabri, 2011). The additional negative charge on the protein/peptide will alter some of the protein's properties such as isoelectric point (pI), surface charge and interactions with other neighbouring amino acids on the target protein and its interactions with other proteins.

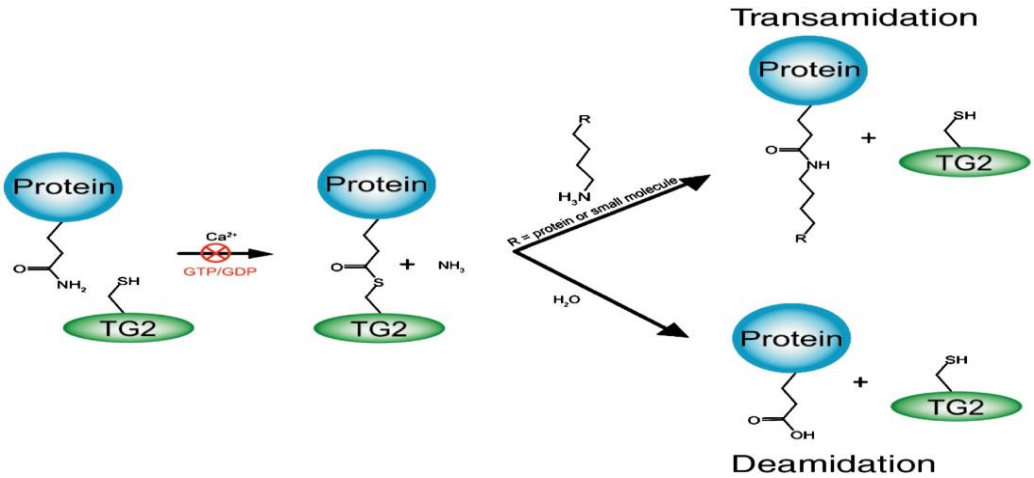


Figure 1-3: Catalytic activity of TG2

Digram shows that TG2 can catalyse the transamidation of glutamine to a suitable amine or the deamidation of glutamine to glutamic acid.

It is crucial to emphasise that water is an inferior nucleophile to amines so there would little or no deamidation in the presence of an amine (Folk, 1983; Fleckenstein et al., 2002). The deamidation activity of TG2 is central to the initiation of coeliac disease, by converting glutamine in a gliadin peptide to glutamate causing an immune response in CD patients, resulting in the typical symptoms of the disease (Sollid & Jabri, 2011). In the literature, the deamidation reaction is described to have a slower rate than the transamidation reaction (Fleckenstein et al., 2002).

Transglutaminase 2 has been reported to have a number of additional activities, one of which is related to its role as a cellular G protein (Gh). Hydrolysis of GTP to GDP can be achieved by TG2, which as a G protein in a combination with the plasma membrane-associated α1 adrenergic receptor via the transfer of signaling to the activation of phospholipase C (Nakaoka et al., 1994). Transglutaminase2 also exhibits phosphorylation and kinase activity, which are modulated through stimulation of A_1 adenosine receptor by protein kinase and extracellular Ca^{2+}-dependent pathways (Vyas et al., 2016). Transglutaminase 2 as a protein kinase has been shown to phosphorylate different proteins including histones in breast cancer cells retinoblastoma protein in fibroblasts and p53 (Mishra et al., 2007). One more activity of TG2 is its ability to act as protein disulphide isomerase (PDI) in the cytosol (Hasegawa, et al., 2003). Protein disulphide isomerase is a member of the thioredoxin family normally existing in the lumen of the endoplasmic reticulum (ER). Transglutaminase 2 may have a cytoplasmic or extracellular PDI role in the correction of disulphide bridges inside polypeptides, which may also correct the conformation of different proteins (Ferrari and Söling 1999). The PDI activity of TG2 is independent of Ca^{2+} ions and nucleotides, it active in cytosol where there are a high nucleotide concentration and low Ca^{2+} concentration (Hasegawa, et al., 2003).However, the majority of cytoplasmic proteins do not have disulphide bonds (Cumming et al., 2004).

1.5.3 Cellular role of Transglutaminase 2

Unlike other members of the transglutaminase family, TG2 can be found in both the intracellular and the extracellular spaces of different types of tissues (Giera et al., 2018). Transglutaminase 2 has been found to be involved in both enzymatic and non-enzymatic cellular and extracellular interactions. Enzymatic interactions are formed between TG2 and substrate proteins or polyamines in the presence of calcium ions (Kanchan et al.,

2015) (See Figure 1-4). During normal cellular activities, when calcium levels are low (<0.1mM) and GTP levels are high (>1mM), TG2 has been proposed to be inactive (Zemskov et al., 2006). Cell lysis and necrosis can damage calcium homeostasis, resulting in increased translocation of Ca^{2+} into the intracellular environment ultimately increasing the activity of TG2 (Griffin et al., 2002). The concentration of Ca^{2+} needed for activation of TG2 differs *in vitro* and *in vivo*. For instance, the Ca^{2+}concentration required for activating TG2*in vitro* (500µM) is far higher than net resting intracellular Ca^{2+}ion concentrations (about 100 nM) (Ahvaziet al., 2003).

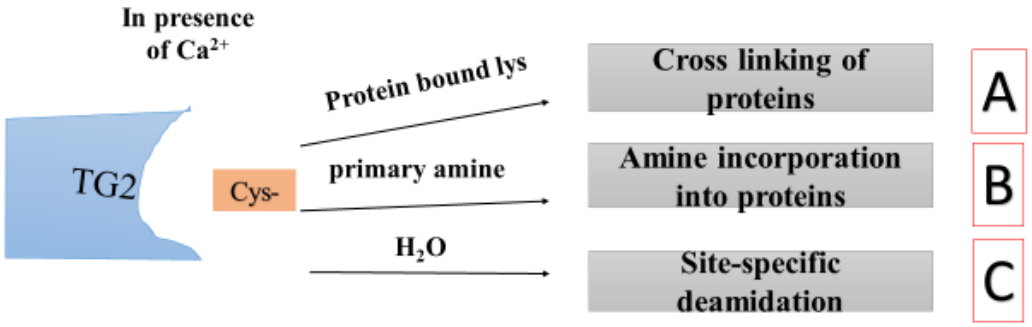


Figure 1-4: Biochemical activities of TG2.

TG2 catalyses Ca^{2+}-dependent acyl-transfer reaction between γ-carboxamide group of a specific protein-bound glutamine and either (A) the ε-amino group of a distinct protein-bound lysine residue (covalent protein cross-linking activity) or (B)primary amines such as polyamines(amine incorporation in proteins).(C)Water can replace amine donor substrates, leading to deamidation of the recognized glutamines.

Adapted from Primary and Piacentini (2002)

These TG2 activities facilitate it is a role in various cellular processes, such as interactions of cells with each other and with the extracellular matrix, apoptosis, gene transcription regulations and mitochondrial function (Yakubov et al., 2013).

1.5.4 TG2 Tissue Localization

Transglutaminase 2 is mainly cytosolic; nevertheless, the presence of TG2 has been demonstrated in the nucleus (Gundemir, et al., 2013). In addition, it has been shown to be present in other intracellular compartments and organelles such as endoplasmic reticulum, mitochondria, and cytoskeleton, as well as the extracellular environment and on the cell surface (Piacentini, et al., 2014). Transglutaminase2 is also expressed in many different cell types including; osteoblasts, fibroblasts, endothelial cells, smooth muscle cells, monocytes, neurons, chondrocyte, neutrophils, oligodendrocyte, epithelium, dendritic cells, stem cells and macrophages (Wang and Griffin,2012). The route by which TG2 is exported from the cell to undertake extracellular remodeling is not completely understood

(Scarpellini, et al., 2009; Iismaa, et al., 2016). A study by (Aeschlimann and Knäuper 2017) showed that TG2 externalization is mediated by the P2X7 receptors which are a member of the P2X family of nucleotide regulated ion channels that are activated by high concentrations of extracellular ATP (Hattori and Gouaux, 2012). Because of injury or inflammation, P2X7receptors will be activated, leading to the release of ATP from immune cells, serving as a danger indication amplification system. Transglutaminase 2 expression is highly up-regulated by acute phase injury cytokines. Consequently, P2X7 receptor-driven TG2 secretion will occur in such a situation (Aeschlimann and Knäuper 2017). It is also known that TG2 is exported from the intestinal epithelial cells into the intestinal *lamina propria* to operate in an extracellular form (Elli et al., 2009).

1.5.5 Transglutaminase 2 substrate properties

The identification of proteins and target amino acids, which act as substrates for TG2 activity, is important for discovering TG2's biological functions in diverse cell types and tissues. A considerable number of cytoplasmic TG2 protein substrates have been identified, such as enzymes, hormones, extracellular and intracellular structural proteins, and small heat shock proteins. Transglutaminase 2 appears to be more selective for glutamine residues on protein/peptides which is the first step in its “ping pong” kinetic pathway (Lorand and Graham, 2003a ; Esposito and Caputo, 2005).

1.5.6 Transglutaminase 2 in disease states

Transglutaminase 2 disruption has been implicated in many different diseases processes. For example, neurodegenerative disorders such as Huntington’s and Parkinson’s diseases (Hoffner and Djian, 2005), different cancers such as breast, ovarian and pancreatic cancers (Mangala and Mehta, 2005). In addition, during the diagnosis of type 1 diabetes, there is around 4% to 17% positive tests for TG2 antibodies (anti-tTG) (Bernassola et al., 2002; Parkkola et al., 2018). Coeliac disease is the one disease state that TG2 activity plays a crucial role, by mediating the deamidation of glutamine in a 33mer gliadin peptide initiating a chain of events leading to the destruction of the small intestinal villi (Molberg et al., 2000),

a) Transglutaminase 2 and neurodegenerative diseases

Transglutaminase 2 is involved in a number of neurodegenerative disorders including Huntington's, Alzheimer's, and Parkinson's diseases (Jeitner et al., 2009b; Min and Chung, 2018) due to several reasons. First, it is found in most regions of the brain, particularly in neurons (Appelt et al., 1996; Kim et al., 1999; Lesort et al., 1999). Second, the pathophysiology of all these diseases is characterised by the formation of insoluble protein aggregates (Mastroberardino and Piacentini, 2010). These aggregates are a result of the covalent cross-linking of pathogenic proteins by TG2, which is effectively irreversible. Thirdly, in neurodegenerative diseases where there are energy depletion and loss of calcium homeostasis, in these circumstances low GTP and high calcium levels may result in TG2 activation its intracellular cross-linking activity (see Section 1.3) (Lesort et al., 2000; Siegel & Khosla, 2007; Jeitner et al., 2009b; Grosso et al., 2012).

i. Transglutaminase 2 and Huntington's disease

Huntington's disease (HD) is a neurodegenerative disease that occurs because of the expansion of CAG trinucleotide repeats in the gene encoding huntingtin (htt). This expansion leads to a large number of contiguous glutamine residues in the htt protein. Aggregation of ubiquitinylated htt in the nucleus and the loss of neuronal cells is observed. One of the proposed mechanisms of htt aggregation is based on the action of TG2 because expanded polyglutamine repeats are excellent glutamine donor substrates for TG2-catalyzed cross-linking (Fesus and Piacentini 2002). In addition, TG2 mediates the cross-linking of mutation htt or its N-terminal fragment, generating an intra-nuclear inclusion with ε-(γ-glutamyl) lysine bonds in the frontal cortex of HD patient brains. There have been studies reporting that cystamine (a nonspecific TG2 inhibitor) treatment of HD patients extends survival, promoting the motor performance and minimising the number of cellular aggregates (Min and Chung, 2018).

ii. Transglutaminase 2 in Parkinson's disease and dementia with Lewy bodies

Parkinson's disease is a neurodegenerative disorder, which is characterised by the loss of dopaminergic neurones in the *substantia nigra pars compacta*, formation of aggregates α-synuclein and cytoplasmic proteins to form Lewy bodies (Junn et al., 2003). Transglutaminase2 has a pathogenic role in Parkinson's disease and dementia with Lewy bodies. These diseases are associated with the protein α synuclein, which is an excellent

substrate for TG2 (Junn et al., 2003). Transglutaminase 2 catalyses the formation of insoluble high molecular weight aggregates of α-synuclein in a calcium-dependent manner (Junn et al., 2003). These insoluble aggregates contain isodipeptide bonds, a process that is dependent on the concentration of TG2. The involvement of TG2 cross-linking activity in inducing the formation of these polymeric forms of α-synuclein is indicated by cystamine blocking α-synuclein aggregation (Junn et al., 2003; Grosso and Mouradian, 2012).

iii. Transglutaminase 2 and Alzheimer's disease

Alzheimer's disease is characterised by the formation of neurofibrillary tangles, extracellular senile plaques and neuronal loss of brain function (McKhann et al., 1984). Transglutaminase 2 has been found to be involved in the pathogenesis of Alzheimer's disease having been found to catalyse amyloid beta-protein oligomerisation and aggregation at a physiological level. Indeed, TG2 activity induces protofibril-like amyloid beta-protein assemblies that are peptidase-resistant and inhibit long-term potentiation (Buée et al., 2000; Lee et al., 2001). Therefore, TG2 activity could also contribute to Alzheimer's disease (AD) symptoms and progression. Work by Basso et al., (2012) demonstrates that, in addition to TG2, TG1 gene expression level is significantly increased following a stroke or due to oxidative stress *in vitro*. It has been further established that the protein cross-links formed by transglutaminases co-localise with pathological lesions in the Alzheimer's disease brain, thereby suggesting TG2 inhibitors may be appropriate for the treatment of Alzheimer's disease (Wilhelmus et al., 2009).

b) Transglutaminase 2 and Renal Fibrosis

Several studies have demonstrated the role of TG2 in the development of fibrotic conditions in humans (Shweke et al., 2008). The cross-linking actions of TG2 in ECM proteins leads to accumulation of proteolytic enzyme resistant cross-linked protein polymers in the kidney, thereby causing fibrotic lesions and renal failure (Johnson et al., 2003; Shweke et al., 2008). The TG2 mediated cross-linking of ECM proteins such as fibrinogen/fibrin, vitronectin, fibronectin, collagens I, III, IV, and V makes them resistant to degradation by matrix metalloproteinases (MMPs) (Aeschlimann & Thomazy, 2000; Fisher et al., 2009). Treatment with site-directed, irreversible inhibitors of TG activity led to a considerable improvement in both kidney function and prevention of renal remodeling, which highlights the importance of TG2 in both disease initiation and as a

potential therapeutic target for treatments (Johnson et al., 2007). Recently, a study by Furini et al., (2018) suggested that the therapeutic block of TG2 might potentially impede the ECM accumulation role of TG2 and the transfer of TG2 from cell to cell during fibrosis progression, and consequently diminish renal fibrosis (Furini et al., 2018).

c) Transglutaminase 2 and cardiovascular diseases

Cardiovascular diseases are one of the major sources of death worldwide (Yusuf et al., 2015). Cardiovascular diseases include various disorders involving the blood vessels – e.g. coronary heart disease, cerebrovascular and peripheral arterial diseases– and the heart organ, such as rheumatic and congenital heart disease. Studies have reported the important function of TG2 in the biology and pathophysiology of heart disease. For example, TG2has been implicated in promoting vasoconstriction of vascular smooth muscle cells, leading to raised blood pressure (hypertension) (Janiak et al., 2006). Through its cross-linking activity, TG2 caused a reduction in the lumen diameter of vessels of arteries. In addition, transglutaminase 2 has an effect on cardiac hypertrophy (heart enlargement) which is considered as a potential risk factor connected with heart failure and ischaemic heart disease (Shiojima et al., 2005). In atherosclerosis, TG2 is widely expressed in smooth muscle cells, macrophages, and endothelial cells and it was reported to accumulate in plaques to interact with atherosclerotic processes (Haroon et al., 2001; Szondy et al., 2017). Nitric oxide is known to inhibit TG2 protein cross-linking function through S-nitrosation (Lai et al., 2001). In addition, nitric oxide opposes TG2-dependent small-artery remodeling in response to vasoconstrictive stimuli (Bakker et al., 2008; Pistea et al. 2008). Reduced nitric oxide bioavailability in aging vasculature leads to diminished TG2 S-nitrosation, then increased cross-linking activity in the extracellular matrix (ECM). Therefore, nitric oxide can directly inhibit TG2 cross-linking function via S-nitrosation (Lai, et al. 2001) and regulate TG2 by constraining it to the cytosol.

d) Transglutaminase 2 and cancer

Transglutaminase 2 has a vital and physiological role in inflammation mainly via its modulation the structure and stability of ECM with its influence on gene expression. It also regulates tumour progression, via various intracellular and extracellular ways, depending on its pro-cross-link and signalling transduction mediation propensities, which attenuates cell adhesion and promotes ECM proteolysis (Huang and Liu, 2015). Transglutaminase 2 is overexpressed in breast, ovarian, brain, and pancreatic tumours;

however, several studies described TG2 as a pro-tumourigenic protein that enhances metastasis, cellular invasion, and chemoresistance. These actions have been attributed to stimulation of oncogenic signalling, controlled in part by the transcription factors nuclear factor-kappa B (NF-κB), cyclic adenosine monophosphate response element–binding protein (CREB) and by protein kinase B (Akt) (Yakubov, 2013). Generally, TG2 performs vital functions in inflammation mainly via its modification of the extracellular matrix (ECM) stability and structure (Huang, et al., 2015).

e) Transglutaminases and age-related cataract formation

Transglutaminase 2 has been implicated in the development of age-related cataract formation (Boros et al., 2008). The β-crystallin component is a major part of the lens proteins that are needed for the refractivity of the light onto the retina (Soderberg, 1990; Jeong and Kim, 2015). These proteins contain numerous glutamine residues, which can become sites of deamidation mediated by TG2 prior to the proteins being cross-linked (Takemoto and Boyle 1998; Lapko et al., 2002; Wilmarth et al., 2006; Boros et al., 2008). Boros and co-workers proposed that TG2 was able to mediate age-related cataract formation through disruption of β-crystallin structure (Boros et al., 2008).

f) Apoptotic and anti-apoptotic role of TG2

Apoptosis is a process of programmed cell death whereby the deletion of cells from a tissue occurs because of stimulation of specific death-signaling pathways (Tiwari et al., 2015). This stimulation of pathways is affected by different environmental factors such as redox potential and ultraviolet light. Apoptosis results in DNA fragmentation, cell volume reduction and phagocytosis of the apoptotic cell via nearby phagocytes. Dys-regulation or inhibition of apoptosis may lead to human disease (Fadok et al.,. 1998, Matute-Bello and Martin, 2003). Therefore, the management of apoptosis is central for cell survival, whereas upregulation of apoptosis can lead to a human disease condition. Transglutaminase 2 has been linked with pro-apoptotic and anti-apoptotic roles, depending on cell type, mode of death stimulation and the localization of the enzyme (Milakovic et al., 2004; Cao et al., 2008; Gundemir et al., 2013; Garabuczi et al., 2013). One probable anti-apoptotic role of TG2 is due to its ability to prevent the degradation of retinoblastoma protein, which is an important suppressor for apoptosis, consequently sustaining the anti-apoptotic role of retinoblastoma protein (Boehm et al., 2002; Milakovic et al., 2004). On the other hand, TG2 has been reported to encourage the release

of apoptosis regulators such as cytochromes C and B through conformational modifications by its BH3-like (Bcl-2 homology) domain (Rodolfo et al., 2004).

g) Transglutaminase 2 and coeliac disease

The TG2 posttranslational modifications of gliadin peptides that produced by the catalytic activity of dietary peptidases on gluten have been proven to cause the generation of autoantibodies in the gluten sensitivity disease or coeliac disease (CD) (Lionetti and Catassi, 2011). The deamidating action of TG2 is primarily responsible for the initiation of the coeliac disease (see section 1-8). Transglutaminase 2 deamidates an undigested gliadin (33mer) peptide found in the intestinal *lamina propria* (Dahlbom, et al., 2005; Lionetti and Catassi, 2011). Thereafter, the deamidated glutamine residue on the gliadin peptide becomes a significantly better binder to the HLA DQ2/DQ8 receptors. This leads to the activation of T cells, the production of cytokines and the production of autoantibodies against TG2 (Nadalutti et al., 2013). This autoimmune response causes damage to the small intestine occurs, which involves disruption of the villi, thus leading to the development of typical CD symptoms (Lionetti and Catassi, 2011). It is important to mention that CD occurs only in humans and does not have a good animal or cell culture model (Discussed in detail later in section 1.8).

1.6 Transglutaminase inhibitors

The implication of TG2 in the range of pathologies discussed above demonstrated the necessity for potent and specific inhibitors. Studies have shown that the pathogenic function of TG2 is mainly linked to its role in protein cross-linking and deamidation, with little evidence to suggest the involvement of its GTPase activity. (Keillor et al., 2015)

1.6.1 Types of Transglutaminase inhibitors

Transglutaminase 2 inhibitors are generally classified into reversible and irreversible inhibitors based on the transamidation activity of TG2. The effect of reversible inhibitors can be reversed after incubation of the enzyme with inhibitor followed by dialysis. On the other hand, the activity of irreversible inhibitors is not reversed after incubation of the enzyme with inhibitor followed by dialysis.

1.6.2 Reversible inhibitors

Reversible TG inhibitors act by blocking the binding of the substrate amine to the active site of the enzyme; however, they do not modify the enzyme covalently. This type of inhibitor acts by inhibiting the function of the enzyme by competing as a substrate in the cross-linking reaction (Keillor et al., 2015). The reversible TG inhibitors can be classified into (a) competitive and (b) non-competitive amine inhibitors (Siegel & Khosla, 2007; Badarau et al., 2013).

a) Competitive amine inhibitors

Competitive amine inhibitors are relatively non-toxic in living cells and chemically stable; these inhibitors, which include putrescine, cadaverine, and methylamine, are the most widely used TG inhibitors and are commercially available (Siegel & Khosla, 2007). Reversible TG2 inhibitors block the access of the substrate to the enzyme active site without covalently modifying the latter. These competitive amine inhibitors moderate TG2 activity in the transamidation reaction by competing with natural amine-containing substrates, such as protein-bound lysine residues. One such agent that is commonly used to inhibit TG2 activity is monodansylcadaverine (MDC). The similarity in structure of MDC with the lysine side chain permits MDC to be used as a competitive substrate to inhibit amine incorporation and cross-linking of natural protein substrates (See

Figure 1-5). In addition, it can be utilised as an amine donor substrate for the fluorescence incorporation assay of TG2 transamidating activity (Yuan et al., 2005, Budillon et al., 2013).

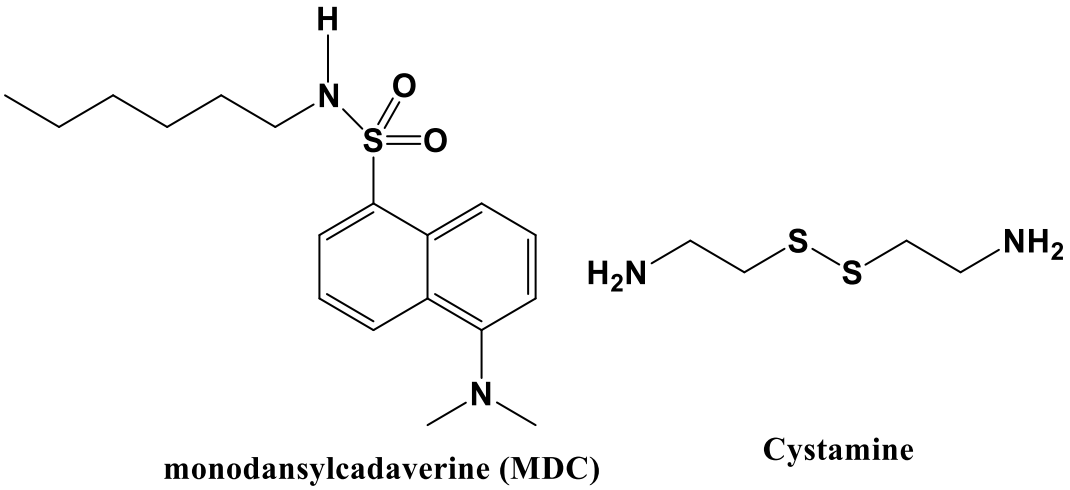


Figure 1-5: Structure of DMC and cystamine

Cystamine is a non-specific TG inhibitor that exerts its inhibition of TG2, through the formation of Nβ-(γ-L-glutamyl)-cystamine bonds at the active site of TG2 and possibly interacting with the sulphydryl residues on the surface of TG2. The formation of these bonds would compete with the generation of other TG-catalysed reactions such as polyamination, protein cross-linking, and the deamination of proteins (Jeitner et al., 2005; Okauchi, et al., 2009). The reduced form of cystamine, which is cysteamine or 2-mercaptoethylamine (MEA) has been shown to be a competitive amine inhibitor of TG2 in a time-dependent manner through an irreversible inhibition mechanism (Lorand & Conrad, 1984; Siegel & Khosla, 2007).

b) Non-competitive amine inhibitors

These inhibitors inhibit TG allosterically by competing for the co-factors of the enzyme, such as GTP and GDP (Badura et al., 2013). These inhibitors are able to bind to TG independent of GTP, but they may potentially compete with GTP for the same site of binding to TG2 (Duval et al., 2005; Case & Stein., 2007).

1.6.3 Irreversible Inhibitors

Irreversible inhibitors act by blocking the binding of the substrate to TG2 via covalently binding to the active site cysteine of the enzyme (Badarau et al., 2013). An example of an irreversible inhibitor of TG2 is iodoacetamide, which forms a thioester bond with the active site cysteine of TG2, leading to inhibiting of TG2 activity (Siegel & Khosla, 2007)(See Figure 1-6).

Iodoacetamide

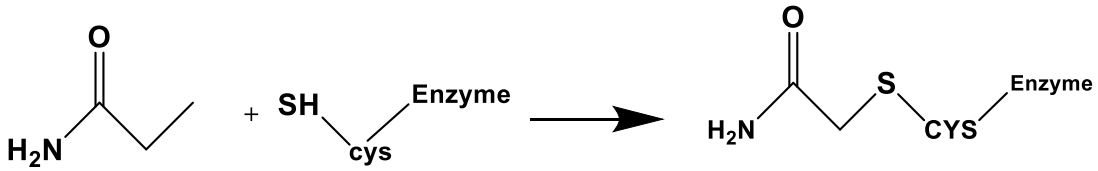


Figure 1-6: Mechanism of TG2 inhibition by iodoacetamide.

Another example of an irreversible inhibitor of TG is 3-halo-4, 5-dihydroisoxazole, which acts in a similar way to iodoacetamide by reacting with a thiol group on TG2 and forming a thioester bond at the active site, which cannot be broken (Keillor et al., 2015). Michael acceptors which are α and β-unsaturated carbonyl derivatives that have been used to inhibit many cysteine peptidases have been modified to become irreversible inhibitors of TG2 (Pardin et al., 2006). Pliura and coworkers (1992) prepared a group of inhibitors that

have a dimethyl sulfonium component and reported their ability to inhibit epidermal transglutaminase (TG3) and TG2 (Pliura et al., 1992). A reagent company Zedira (DE) established products that are used in scientific literature: These include Z-DON, which is a very potent and specific inhibitor of TG2 (IC_{50} about 0.02 μM) and is membrane permeable, in addition to TAMRA-DON which is a fluorescent blocker of TG2 with an IC_{50} of about 0.1 μM (https://zedira.com/Mechanism-of-TG-inhibitors/DON-compounds). Two irreversible TG inhibitors NTU R283 and carboxybenzoyl-glutamyl lysine (referred to as NTU-R281) have been synthesised. The inhibitor R283 was found to reduce the toxicity induced by gliadin in *ex vivo* and *in vitro* studies (Rauhavirta et al., 2013). While R283 does not enter the cell, R281 is cell-permeable.

1.6.4 Natural inhibitors

Inhibitors of transglutaminases have been discovered in extracts from different natural sources. A study was done by Jong et al., (2001) has reported that bovine milk contains an inhibitor of TG2 in several types of activity assay. This inhibitor was detected in a milk fraction without casein and whey proteins (De Jong et al., 2003). Another natural TG2 inhibitor was extracted from garlic, as mentioned in a study by Lee et al., (2007); this inhibitor was thermostable, had low molecular weight and it also reversibly inhibited microbial transglutaminase (Lee et al., 2007). Recently, a study by Madagi et al., (2018) reported that phytochemicals such as nobiletin and curcumin displayed a potential inhibition of TG2 and could be implicated for treatment of lung cancer. The isolation and development of new reliable natural TG inhibitors could be advantageous to help cure various pathological impacts of TGs. The major reasons for using these natural inhibitors include that they are easily available and, because they are ingested on a regular basis, they have potentially no adverse side effects. Natural inhibitors have thus played a critical role in drug discovery and have led to a revolution in pharmacology and medicine (Dash, 2018). Therefore, one of the main aims of this study was to screen some natural products for the presence of TG2 inhibitors, which could be used in the management of coeliac disease.

1.7 Assays for measuring TG2 activity

In order to screen modulators of TG2 activity, it is necessary to use enzyme assays to recognise inhibitors/activators of the enzyme (Pietsch et al., 2013). The classical assay methods, such as standard end-point, fluorimetric and colorimetric assays are still used,

although there is always the need to develop more sensitive assays (Song et al., 2016). Some methods that are used for measuring TG2 activity are explained below as representative examples.

1.7.1 Transamidation assay using radioisotopes.

Radioactivity assays are very sensitive methods. Lorand et al., (1972) developed a filter paper assay for measuring the activity of transamidating enzymes by the incorporation of radioactive amine substrates into proteins. The main advantages of this technique are the use of minimal amounts of reagents, as well as the simplicity of handling a large number of test samples simultaneously (Lorand et al., 1972). This technique was used to assess the TG2 activity of the brain, prefrontal cortex, and cerebellum samples of Alzheimer's disease subjects. In this assay, TG2 activity is measured directly by incorporation of the tritiated amine nucleophile putrescine into a glutamine-containing protein substrate N, N dimethyl casein (NMC) (Song et al., 2016).

1.7.2 Fluorescence Anisotropy Assay.

Detection of fluorescence anisotropy caused by the cross-linking of a fluorescein-labelled glutamine donor peptide to a larger protein acceptor (BSA) by transamidation activity of TG2 was achieved with the assay developed by Kenniston et al., (2013). Transamidation reactions between donor peptide and acceptor BSA resulting in a cross-linking product leads to bigger changes in the fluorescence anisotropy.

1.7.3 Fluorescence Transamidation Assay.

In this assay, the activity was determined by a fluorescent Lys substrate (KXD) as the amine nucleophile-labelled N, N-dimethylated casein (NMC) which is Gln-containing protein substrate, at the end of the transamidation reaction. The TG-catalysed covalent coupling of KXD and NMC causes a shift in strength and wavelength of fluorescence of the dansyl group (Case et al., 2005). The fluorescence transamidation assay could be applied for selectivity profiling across other transamidating enzymes (TG1, TG3, TG6 and factor XIIIa) and is widely used for screening to discover TG2 inhibitors (Schaertl et al., 2010).

1.7.4 Enzyme-Coupled Assay.

The enzyme-coupled assay was firstly developed by Day and Keillor (1999), based on the indirect measurement of TG2 activity through detecting the quantity of NADH by measuring absorbance at 340nm (Day and Keillor, 1999). The assay involves two steps; in first step ammonia is released as a result of TG2 action on its substrate (protein/peptide bound glutamine), while in the second step, reductive amination of α-ketoglutarate (α-KG) with consumption of NADH occurs to yield glutamate, this latter reaction being catalysed by glutamate dehydrogenase (GDH)(Oteng-Pabi et al., 2013; Michael et al., 2015) . In addition, this assay could be used to evaluate the activity and kinetic parameters of TG2 substrates (Song et al., 2016).

1.7.5 Absorbance Based Assay

A nonradioactive transglutaminase assay was developed by Slaughter and co-workers using 5-(biotinamido) - pentylamine and NMC (N, N-dimethylated casein) (Slaughter et al., 1992). In this assay, a microtiter plate is coated by NMC followed by incubation with 5-(biotinamido) pentylamine and TG. Then, by using streptavidin-alkaline phosphatase, the transamidation of 5-(biotinamido)-pentylamine and NMC was detected and measured by absorbance at 405 nm. Another absorbance based assay for transglutaminase activities has been developed by Seiving et al., (1980) and then optimised by Choi et al., (1992). This assay was established to monitor cross-linking activities between casein bound to microtiter plates and free biotinylated casein by the sample. Then quantitation of immobilized biotin-labeled casein, which formed as the result of the enzymatic reaction, was conducted by avidin or streptavidin conjugated enzymes. Trigwell and co-workers (2004) developed an assay by replacing the free biotinylated casein substrate with biotinylated hexapeptide (biotin-TVQQEL),which leads to reduction in the background signal and increases the assay sensitivity(Trigwell et al., 2004).

1.8 Coeliac disease

Coeliac disease (CD) is an autoimmune small bowel disease that can be triggered in genetically susceptible individuals by ingestion of gluten and similar proteins contained in wheat, rye, and barley (Wingren et al., 2012). Partially digested gluten can prompt an autoimmune response, which causes damage to intestinal villi, thereby reducing the absorptive surface of the small intestine (Assa, 2017). Patients with the early developing

coeliac disease still have a normal small-intestinal mucosal villus structure, but they may already have TG2-autoantibody in their circulation, leading to increasing in a number of inflammatory cells in their small-bowel mucosa. Moreover, these patients may suffer from various gastrointestinal symptoms, such as abdominal pain and diarrhoea, before development of villous atrophy (Kalliokoski et al., 2016).

1.8.1 Pathogenicity of coeliac disease

The major environmental factor responsible for the development of the coeliac disease is gluten, which is a collective term for the prolamin storage proteins of the cereal grains wheat, rye, and barley. Its partial digestion by gastric, pancreatic, and brush border peptidases leaves long peptides up to 33 amino acids in length containing glutamines and prolines (Shan et al., 2002). These peptides pass to the *lamina propria* of the small intestine through transcellular or paracellular routes (Matysiak et al., 2008; Visser et al., 2009; Schumann et al., 2017). Because of the accumulation of gliadin peptides, the permeability of the intestines will increase, which allows macromolecules to enter and exit through the gaps in the intestine. Intestinal permeability can be evaluated by numerous methods (Kim and Ko 2018), such as measuring the excretion of orally ingested sugars like sucrose, mannitol, cellulose and lactulose that are absorbed from different regions of the gastrointestinal tract in trace quantities and are excreted unchanged in the urine (Farhadi et al., 2003). The lactulose to mannitol (L/M) ratio is considered to be a sensitive and accurate marker of small bowel permeability and the urinary excretion of sucralose can be used to estimate whole gut (small bowel and colon) permeability (Bjarnason et al., 1994; Meddings and Gibbons, 1998). Urinary lactulose and mannitol have been quantitated by several methods including thin layer and paper chromatography, enzymatic assays, gas chromatography and HPLC (Farhadi et al., 2003). Furthermore, plasma haptoglobin-2 (zonulin) concentrations are used to assess intestinal permeability (Wang et al., 2000). However, this may be inaccurate as the haptoglobulin 2 protein has a completely different role to its precursor protein. Haptoglobin-2-precursor (pre-HP2) was identified as the precursor of haptoglobin-2 HP (Tripathi et al., 2009). The Haptoglobin protein (HP2) is a protein that circulates in the plasma and mops up free iron ion to avoid oxidative stress. Human haptoglobin (HP) occurs as two common alleles, HP1and HP2. The HPHP2 allele only exists in humans. The pro-protein of HPHP2 (pre-HP2) is active as zonulin (Sturgeon et al., 2017). Therefore, only patients who have an HP2 gene can produce pre HP2 (zonulin). This protein controls the permeability of the

intestine epithelial cells by controlling the binding between the epithelial cells of the intestinal mucosa (Kim and Ko, 2018). The pre-hp2 expression is upregulated in patients with celiac disease and type 1 diabetes (Wang et al., 2000; Drago et al., 2006). In affected individuals, an adaptive immune reaction occurs, which is dependent on the deamidation of a 33mer gliadin peptide (LQLQPFPQPELPYPQPELPYPQPELPYPQPQPF) by intestinal TG2 (Dieterich et al., 1997). Deamidation changes one glutamine to glutamate in the 33mer gliadin peptide (changes are underlined) and enhances the immunogenicity of the peptide, greatly enhancing its binding to HLA-DQ2 or HLA-DQ8 molecules and subsequently to antigen-presenting cells (APCs). The structure of DQ2 was found in complexes with an immunogenic epitope from gluten, namely QLQPFPQPELPY (Kim et al., 2004). During this process, autoantibodies against TG2, gliadin, and actin are formed. These antibodies are thought to contribute to extra-intestinal complications of coeliac disease, such as gluten ataxia and dermatitis herpetiformis (Lebwohl et al., 2018) (See Figure 1-7)

Coeliac disease has a genetic element in that patients have specific variants of the HLA class II genes HLA-DQA1 and HLA-DQB1 (Lundin and Wijmenga, 2015). More than 90% of CD patients are HLA-DQ2 positive and most of the others are HLA-DQ8 positive. A study by Liu, et al., (2017) in the United States reported that, by the age of 15 years, an estimated 3.1% of the population develops CD, with a risk of 14·2% among those homozygous for DQ2 and 1·5% through those who had one copy of DQ8. Nearly all CD patients possess HLA-DQ2, HLA-DQ8, or half HLA-DQ2, whereas, up to 40% of people in the Americas, Europe, and Southeast Asia also have these alleles. This means that these genes are necessary but not sufficient for CD to display, contributing to only about 40% of the genetic risk for the disease (Lebwohl et al., 2018). Moreover, an important role of other environmental factors in the pathogenesis of CD was suggested, including exposure to heavy metals, gastrointestinal infections, drugs and the intestinal microbiome (Elli et al., 2018).

1.8.2 Epidemiology

Coeliac disease is considered an infrequent or rare disorder; the diagnosis of CD was based on the detection of typical symptoms of the gastrointestinal tract and confirmed by the small intestinal biopsy. In recent times, an international study investigated wide-ranging population samples in four different European countries; on average, the total

prevalence of CD was 1%, with big variations between countries (2.0% in Finland, 1.2% in Italy, 0.9% in Northern Ireland, and 0.3% in Germany). This study has reported that many CD cases would remain undetected without serological screening (Catassi et al., 2014). Recently, a study to estimate the prevalence of CD in Australia showed that 1.2% of adult men and 1.9% of adult women proved positive (Walker et al., 2017). Recent studies for the epidemiology of CD performed in developing countries, show spread rates overlapping European Figures. For example, epidemiological studies performed in areas of the developing world show 0.79% in Libya, 0.6% in Tunisia, 0.53% in Egypt and 3% in Saudi Arabia (Costa et al., 2014). Coeliac disease is more frequent in women compared to men, with a female: male ratio of up to 2.8:1 (Gujral et al., 2012). For a long time, CD was believed to be a pediatric disease; however, a study in the UK reported that the highest rates of prevalence of CD were in people and aged less than 5 years and between 50 and 69 years. Moreover, this study reported a fourfold increase in the prevalence rate of CD in the United Kingdom over a period of 22 years (West et al., 2014). This may, in part, reflect the ease of correct diagnosis using the serological test over that time.

1.8.3 Symptoms of coeliac disease

Coeliac disease is commonly asymptomatic; indeed, the UK study commented that the mean duration of symptoms before an initial diagnosis of CD was 13 years. This because of, wide-ranging symptoms, with similarity to those in other conditions, and/or lack of attention and recognition of CD in the individuals and healthcare professionals (Gray et al., 2010). The classical symptoms in early childhood are diarrhea and failure to thrive after weaning (Lebwohl et al., 2015). Obviously, typical symptoms of CD comprise symptomatic malabsorption, abdominal pain and cramping, wind, nausea, diarrhoea with or without weight loss, iron deficiency and anaemia, increased levels of liver enzymes, irritable bowel syndrome, premature osteoporosis, discoloration of teeth, thyroid disease (Fasano and Catassi, 2001; Lionetti and Catass, 2011) .

1.8.4 Transglutaminase 2 role in coeliac disease

Transglutaminase 2 plays a vital role in coeliac disease by converting specific glutamine residues in gluten peptides into glutamic acid in deamidation reaction. This procedure raises the peptide binding (sequence) affinity of gluten peptides to the coeliac disease-associated HLA-molecules (du Pré and Sollid, 2015). The HLA-DQ2.5, HLA-DQ8, and HLA-DQ2.2 protein molecules all show a greater affinity for negatively charged anchor

residues, which are introduced into gluten peptides through deamidation by TG2 (du Pré and Sollid, 2015).

The HLA-DQ2/DQ8 bound glutamate-rich peptides present on the antigen-presenting cells to activate the $CD4^+$ T cells, which in turn activate the B cells in the *lamina propria* of the small intestine. The B cell clonal expansion results in the production of circulating anti-gliadin and anti-TG2 antibodies against self-TG2. The gluten-specific T cells have been proposed to cause the activation of TG2 specific B cells to produce anti-TG2 antibodies (see 1.8.1). All of these consecutive actions result in inflammation and intestinal epithelial layer destruction (See Figure 1-7) (Lionetti and Catass, 2011). The villi of the small intestine provide the absorptive surface for the nutrients into the blood. The destruction of the intestinal villi causes mal-absorption of nutrients into the blood, thereby causing typical symptoms of CD such as weight loss and anaemia (Lionetti & Catass, 2011).

(Adapted from Alaedini & Green (2005)

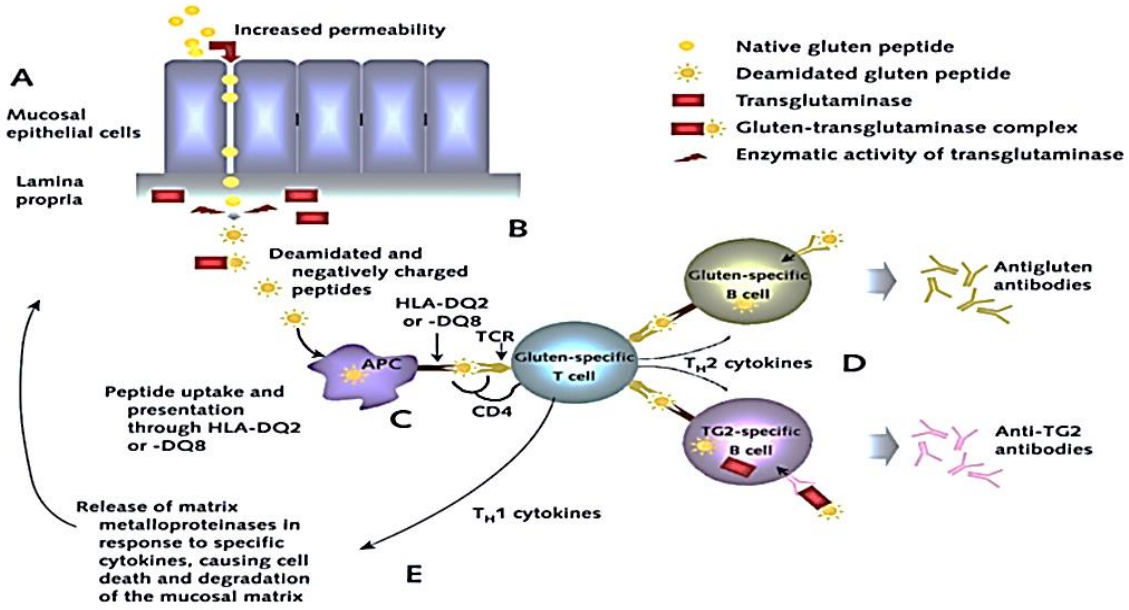


Figure 1-7: Schematic representation to show the development of CD after exposure to the gluten peptides

A; Because of increasing the intestinal permeability the gluten peptides cross the lamina propria. B; Deamidation of the glutamine residues in the gliadin peptides by intestinal TG2 leads to the formation of glutamate, creating epitopes with increased immunostimulatory potential. C; Deamidated gluten peptides have higher binding affinity for class I MHC HLA/DQ2/DQ8. These glutamate rich peptides on the HLA-DQ2/DQ8 activate the CD4+T cells which in turn activates the B cells. D; The clonal expansion of B cells results in the production of antibodies against gluten and TG2. E; In addition, the T cell activation produces pro-inflammatory cytokines that activate matrix metalloproteinases resulting inflammation and intestinal epithelial layer destruction.

1.8.5 Diagnosis of CD

The diagnosis of CD depends on a series of clinical signs, positive CD serological tests and histological findings of duodenal biopsies (Rubio et al., 2013). Serology and duodenal histology were undertaken after gluten-containing diet, to confirm mucosal healing in CD patients. Anti- TG2 IgA antibody (TG2-Ab) is the recommended serological test for CD screening in individuals < 2 years of age; this test has high sensitivity and specificity (> 95%) (Van Der Wind et al., 2010). Anti-endomysium antibody titre is another test with higher specificity (around 99%), but it is not used initially; is used as a confirmatory test because of technical difficulties and cost (Leffler, and Schuppan, 2010). Negative serological results do not mean the exclusion of the CD with 100% accuracy. Histological investigation of duodenal biopsy is still a crucial factor for the diagnosis of CD. The histological markers of mucosal damage of CD include villous atrophy (see Figure 1-8), increased intraepithelial lymphocytes (IELs), in combination with crypt hyperplasia (Marsh, 1992). The identification of HLA DQ2 and/or DQ8 through genetic testing can be used to rule out CD. This test could be used in certain clinical settings, in order to exclude the possibility of a likely future onset of CD in high-risk patients (Elli et al., 2018).

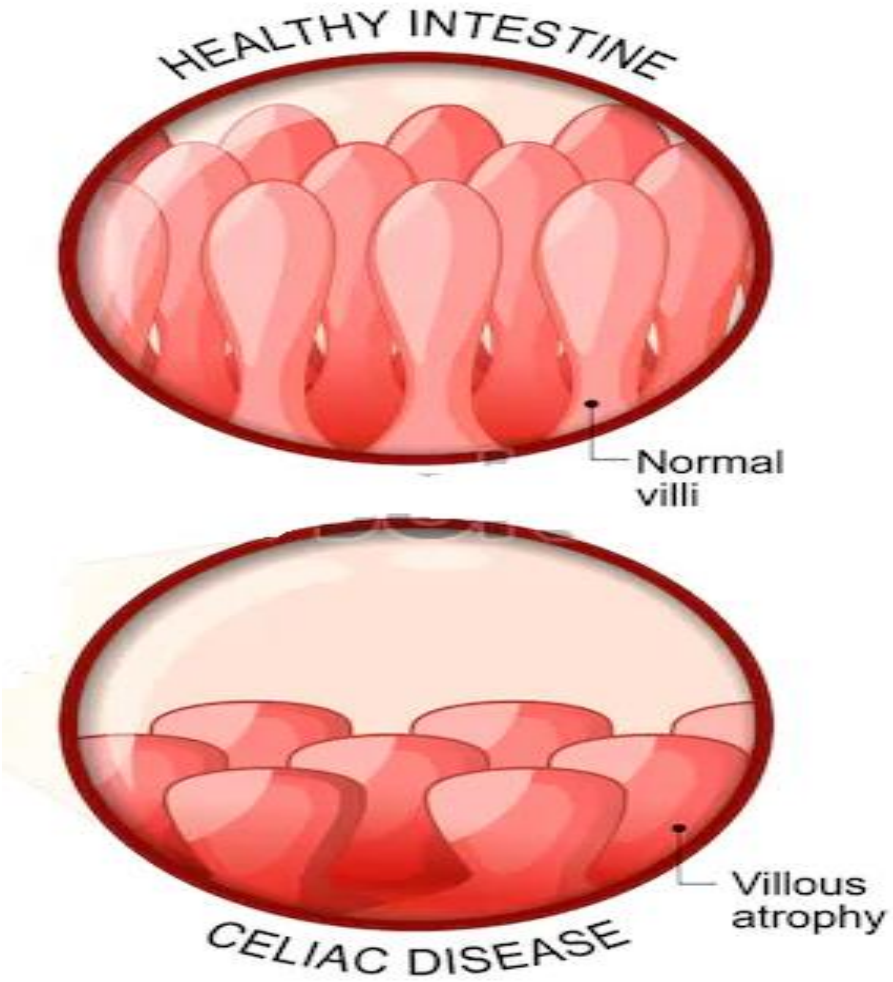


Figure 1-8: Changes in intestinal villi caused by CD.

Adopted from https://www.shutterstock.com/image-vector/celiac-disease-small-intestinal-normal-villi-794121334

1.8.6 CD complications

- Intestinal mucosal inflammation and calcium malabsorption can occur in CD patients, which leads to suffering from bone loss (Malamut and Cellier, 2015).
- There is an increased risk of diseases such as diabetes type I, autoimmune thyroiditis, autoimmune hepatitis, rheumatoid arthritis Addison's disease, antiphospholipid syndrome, myasthenia gravis and dermatitis herpetiformis (Cosnes et al., 2008; Iqbal et al., 20013).
- There is a higher risk of the development of obstetric complications in women with coeliac disease. This is because treatment with a gluten-free diet leads to a significant decrease in preterm birth (Saccone et al., 2016).

1.8.7 Therapeutic intervention for the treatment of CD

To date, the only accepted form of therapy for CD is the avoidance of gluten through dietary control (Pluges and Khosla, 2015). In recent years, the high quality of gluten-free products has improved; however, the adherence to these products depends on different individual and environmental factors (Hall et al., 2013). One study reported that in the UK, diet adherence in adult CD patients ranged from 36% to 96% (Ford et al., 2012). It has been established that the CD patients who keep to a gluten-free diet shown improvements in vitamin deficiencies and abnormalities linked with the disease (Cosnes et al., 2008). However, the majority of CD patients fail to adhere to a gluten-free diet. In some parts of the world, especially in developing or third world countries, gluten-free products are not readily available (Hauser et al., 2007). In addition, a gluten-free diet is usually more expensive. Therefore, it is challenging to adhere to a gluten-free diet for life and alternative therapeutic strategies are warranted for sufferers of CD (Hauser et al., 2007). Previous and current clinical trials for CD comprise treatment with glucocorticoids, zonulin (pre-HP2) antagonists, gluten-sequestering polymers, oral peptidases, gluten vaccines, probiotics and TG2 inhibitors (Pluges and Khosla, 2015).

a) Treatments with Glucocorticoids

Glucocorticoids are normally used to decrease the morbidity of immune-mediated diseases such as Crohn's disease and asthma. However, their significant side effects limit their uses in the treatment of lifelong diseases, such as CD. Budesonide, which is a glucocorticoid with poor oral bioavailability, is used in the treatment of Crohn's disease. Pilot studies reported that budesonide might provide clinical benefit to CD patients (Daum et al., 2006; Ciacci et al., 2009). Another pilot study has mentioned that using prednisolone, which is a glucocorticoid that has a higher oral bioavailability, induced a rapid reduction in epithelial apoptosis in celiac patients; however, it was accompanied with suppression of villous regeneration, suggesting that short courses of oral prednisolone could benefit specific patient groups (Das et al., 2012).

b) Oral peptidase treatments

The ability of several fungi, bacteria, and plants to degrade gluten has been used to develop oral peptidase therapies for CD. The high content of glutamine and proline in gliadin is the reason for gliadin resistance to breakdown by the intestinal brush border membrane peptidases, pepsin and pancreatic peptidases (Shan et al., 2002). Utilisation of

prolyl endopeptidases has been considered for detoxifying gluten peptides to give them the ability to cleave peptides at proline residues (Hausch et al., 2002). Prolyl endopeptidases are broadly expressed in both mammals and microbes; however, their levels in humans are insufficient for detoxifying gluten peptides. In contrast, recombinant prolyl endopeptidases from a diversity of bacteria and fungi have ability to proteolysis the gliadin peptides both *in vitro* and *in vivo* (Shan et al., 2004; Mitea et al., 2008).

c) Haptoglobin-2 (pre-HP2) antagonists

Haptoglobin-2 precursor protein (pre-HP2; previously known as zonulin) is an epithelial protein which has been shown to be involved in modulating barrier permeability. The intestinal barrier is normally impermeable to macromolecules but due to gluten exposure in coeliac patients, the permeability of this barrier will increase, leading to movement of gliadin peptides into the *lamina propria* (Fasano et al., 2001; Tripathi et al., 2009). Gluten peptides stimulate the overexpression of pre-HP2) in CD patients' intestinal tissue, consequently increasing intestinal permeability. As a result, the blocking of pre-HP2 has been proposed as a possible therapeutic strategy for the treatment of CD. Larazotide acetate inhibits the opening of tight junctions in epithelial cells in the small intestine (Di Pierro et al.,. 2001). This compound probably inhibits the pre-HP2 interaction and is thus thought to prevent gliadin-induced permeability both *ex vivo* and *in vivo (*Clemente et al., 2003; Drago, et al., 2006).

d) Treatments with probiotics

The ability of microorganisms to regulate the function of the intestinal barrier has motivated research towards using intestinal bacteria as probiotics for the treatment CD. The *in vitro* studies by De Angelis et al., (2006) demonstrated that several bifidobacteria species have the ability to hydrolyse gliadin peptides into less immunogenic peptides.

e) Treatments with therapeutic vaccines

The production of peptide-based immunotherapy that can specifically stop the adverse inflammatory cascade activated by gluten proteins is currently one of the most promising therapies for CD (Di Sabatino et al., 2018). This type of immunotherapy is targeted to the complete recovery of immune tolerance toward ingested gluten by targeting intestinal CD4+ T-cells, which have a crucial pathogenic role in CD, without affecting the systemic immune responses. Recently, the characterisation of the collection of gluten epitopes that is responsible for mucosal inflammation in CD has permitted the design of the Nexvax2

drug. Further studies are still needed to demonstrate the efficacy of this drug to protect CD patients from the damaging effects of gluten ingestion (Goel et al., 2017; Di Sabatino et al., 2018).

f) Treatments with TG2 inhibitors

The critical role of TG2 in CD makes inhibition of TG2 activity a potential therapeutic target. A number of TG2 inhibitors have been developed, including reversible inhibitors such as cinnamoyl compounds, thienopyrimidines, and acylidineoxindoles and irreversible inhibitors such as thiadiazoles, epoxides, and dihydroisoxazoles (Plugis and Khosla 2015). However, as TG2 is present in all cells, chemical inhibitors of the enzyme may have a detrimental effect on processes other than CD. Natural products such as garlic and milk present less of a toxicity problem, as they are ingested by many people, without adverse side effects. Milk and garlic have been shown to contain TG2-mediated transamidation inhibitors, (Williams et al., 2005; Lee et al., 2007). It is, therefore, important to **a)** develop an assay, which can measure the deamidating activity of TG2, and **b)** to screen natural products with known medicinal attributes in order to assess them as potential deamidation inhibitors and moderators of CD. Natural products chosen for this study were dietary flavonoids, due to their promising biological activity.

1.9 Flavonoids

The flavonoids, one of the most frequent and common groups of natural constituents, are important to man because many members are physiologically active. Nearly two thousand substances have been described and as a group they are generally distributed among vascular plants. (Harborne, J.B., 2013.) Flavonoids consist of a large group of polyphenolic compounds having benzo--pyrone structure, which is ubiquitously present in plants and in the human diet with numerous potentially health-beneficial biological activities. The natural sources of flavonoids are fruits and vegetables (Kumar and Pandey, 2013). Flavonoids have different biological activities, such as antioxidant, anti-inflammatory, cholesterol-lowering, antibacterial, hepatoprotective, anticancer, antiviral and many other biological functions reported in the literature (Kumar and Pandey, 2013; Huang, 2016; Mohana, 2016; Packzoski et al., 2017). Flavonoids are also known to be potent inhibitors of numerous enzymes, such as cyclo-oxygenase (COX), lipoxygenasexanthine oxidase (XO) and phosphoinositide 3-kinase (Panche et al., 2016) (More explanation in chapter 3).

1.10 Framework and aims of this study

As mentioned previously, the TG2 enzyme has an important role in the initiation of coeliac disease (CD) due to its ability to convert specific glutamine residues in gluten peptides into glutamic acid in a reaction termed as deamidation. However, to date, a gluten-free diet is the only accepted form of therapy for CD. Though safe and effective, this not an ideal lifestyle due to cost reasons and it has an impact upon the quality of life of the sufferer. Therefore, research for developing novel therapeutic strategies appears to be a reasonable approach to find alternative treatment options. The hypothesis that inhibition of the TG2 activity will be considered as a viable strategy for the treatment of CD. The side effects of using chemical TG2 inhibitors motivated the research towards the screening of TG2 inhibitors from natural sources. Natural products chosen for this study were dietary flavonoids, due to their promising biological activity. However, to recognise inhibitors/activators of TG2, numerous assays to measure and quantify transamidating TG2 activity have been established (Pietsch et al., 2013). The classical transamidation assay methods are standard end, fluorimetric and colorimetric assays, which are still used, although there is a need to develop an easy and more sensitive assay relevant to CD (Song, Minsoo, et al., 2016). While mass spectrometry methods have been successfully utilised to characterize deamidated peptides in complex mixtures, the results so far have largely been qualitative (Ong and Mann, 2005) with associated high costs. Therefore, an effort was made to develop and optimise a low-cost TG2 deamidation assay that could accurately quantify TG2-mediated deamidation and would be amenable to high throughput screening.

1.10.1 Aims of the thesis:

The main aims of the work presented in this thesis were-

- To screen the effects of different pure flavonoids and dietary flavonoids on the hrTG2 and gplTG2 transamidation and deamidation activity.
- To develop a novel method of collecting flavonoids from plant extracts using immobilised metal ion affinity chromatography (IMAC).
- To develop a novel colourimetric assay for flavonoids using bicinchoninic acid (BCA)
- To test the *in situ* activity of TG2 in HT29 cells and the effects of flavonoids on this cellular activity using, SDS-PAGE, Western blotting and fluorescence microscopy.
- To develop an assay to quantify the TG2-mediated deamidation activity using different substrates, such as plant storage protein gliadin peptide mixtures, the 33-mer gliadin peptide containing the HLADRQ2 binding sequence.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 General laboratory reagents

All laboratory reagents were of the highest grade and purchased from Sigma-Aldrich (UK), Lonza (UK) and Zedira (Germany) unless otherwise specified in the text. Pure flavonoids (kaempferol, luteolin, epicatechin, apigenin, myricetin, cyanidin, morin, naringin, hesperetin, hesperidin, quercetin, catechin, taxifolin) are obtained from sigma-Aldrich (UK). Casein,N',N'-dimethylcasein, MTT (3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), paraformaldehyde, Triton™ X-100, Phosphatase Inhibitor Cocktails 2 (sodium orthovanadate, sodium molybdate, sodium tartrate and imidiazole) and 3 (cantharidin, (–)-p-bromolevamisole oxalate, calyculin A), Ammonium persulphate (APS), ExtrAvidin-horseradish Peroxidase (HRP) and ExtrAvidin-FITC were obtained from Sigma- Aldrich Co. Ltd. (Gillingham, UK). The TG2 inhibitors Z-DON (Z-DON-Val-Pro-Leu-OMe) and purified human recombinant transglutaminase2 with purified guinea-pig liver TG2 were obtained from Zedira GmbH (Darmstadt, Germany). VectaShield® containing 4, 6-diamidino- 2-phenylinole (DAPI) was from Vector Laboratories Inc (Peterborough, UK). Biotin-TVQQEL was purchased from Pepceuticals (Enderby, UK). Biotin cadaverine (N-(5 aminopentyl) biotinamide) and biotin-X-cadaverine (5-([(N (biotinoyl) amino) hexanoyl] amino) pentylamine) were purchased from Invitrogen, UK (Loughborough, UK). N, N, N', N'- tetramethylethylenediamine (TEMED) was from (National Diagnostics, USA), 33-mer gliadin peptide((LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF)and pepceutical, UK****).

2.1.2 Food samples:

Apples (*Malus domestica)* Green tea (*Camellia sinensis*), Strawberry (*Fragaria vesca*), Kale (*Brassica oleracea*), Capers (*Capparis spinose*), Garlic (*Allium sativum*) and Onions (*Allium cepa*), were purchased from a local market.

2.1.3 Cell culture reagents

McCoys 5A medium, foetal bovine serum (FBS), penicillin (10,000 U/ml)/streptomycin (10,000 μg/ml) and trypsin (10 X) were purchased from BioWhittaker Lonza Group Ltd., UK. Phosphate buffered saline (PBS) was obtained from Life Technologies (Invitrogen, UK).

2.1.4 Cell line

Human Colonic adenocarcinoma cells (HT-29) from American Type (ATCC, USA).

2.2 Methods

2.2.1 Extraction methods

a) Extraction of proteins from cell lines

The HT29 cells were cultured (section 2.2.9a) and passaged (section2.2.9b). Once the cells were confluent, they were washed twice with ice-cold phosphate buffered saline (PBS) to remove the spent medium. Ice cold lysate buffer (50mM Tris/HCl pH 8.5 containing 0.5% (w/v) sodium deoxycholate, tissue culture 10mg ml^{-1} protease or 10 mg ml^{-1} phosphatase inhibitor cocktail 2 and 3 (Sigma, UK)) were added to the adherent cells while maintaining constant agitation for 30min at 4°C. The cells were scraped off the flask using plastic cell scraper. The cell suspension was gently transferred into a pre-cooled micro centrifuge tube. The cell suspension was homogenized and centrifuged (Eppendorf 5702 R) for 5.0min at 22^{o}C at 14,000 *x g.* The supernatant was used for measurement of protein concentration (section 2.2.6a), analysis by electrophoresis (section 2.2.7) and Western blotting (section 2.2.8).

b) Extraction of flavonoids:

All food samples were cleaned and freeze-dried (Telstar, LyoQuest, UK). Two hundred and fifty milligrams of freeze-dried materials were ground to powder in a mortar and pestle. The material was mixed with 6.0ml of 62% (v/v) methanol and 6.0ml of 6.0M HCl refluxed for 90min at 90^{O}C in the presence of 15.0mg of an anti-oxidant tert-Butylhydroquinone. Each sample was extracted in triplicate. After the refluxing the materials were cooled and centrifuged (5,000 *x g* for 15min), the volume was made up to 10.0ml with methanol and the extracts were stored at -20^{o}C in dark bottles until required. The extraction conditions (solvent polarity, the addition of acid, extraction time,

extraction temperature) were chosen from previously published literature (Vatai, et al., 2006; Spigno et al/. 2007; Putnik et al., 2016).

c) Extraction of *Vicia faba* storage proteins

Fifty grams of *Vicia faba* seeds (v *Aquadulce Claudia*) were soaked in water overnight. The cotyledons containing the storage proteins were homogenized in 200.0ml of 50.0mM Tris pH 8.0 buffer containing 0.5M NaCl. The homogenate was strained through muslin and centrifuged at 20.000 *x g* for 30.0minutes. The supernatant was collected and dialysed against water at 4^{o}C until the precipitated globulin storage proteins appeared in the sample. The dialysate was centrifuged at 20.000 *x g* for 30.0minutes and the precipitate was freeze-dried (Telstar, LyoQuest, UK) then stored at -20^{o}C.

2.2.2 Purification Methods:

a) Solid Phase Extraction (SPE)

The C_{18} Varian Bond Elute SPE cartridge (2.0ml) was washed with 2.0ml of methanol followed by 2.0ml of 5.0% (v/v) methanol. One millilitre of flavonoids extracts was loaded onto the washed C_{18} column and 1.0ml fractions collected from this point in glass sample vials (2.0ml Chromacol vials). The bound material was eluted with 1.0ml 80.0 % (v/v) methanol followed by 1.0ml methanol (100%)(Figure 2-1). The eluted samples were dried using a centrifugal concentrator (GeneVac, UK). The extracts were dispersed in 0.5 % (w/v) sodium deoxycholate using an ultrasonic bath (Fisherbrand, UK) before adding to an assay to measure TGase activity (See Section 0and (2.2.6b)

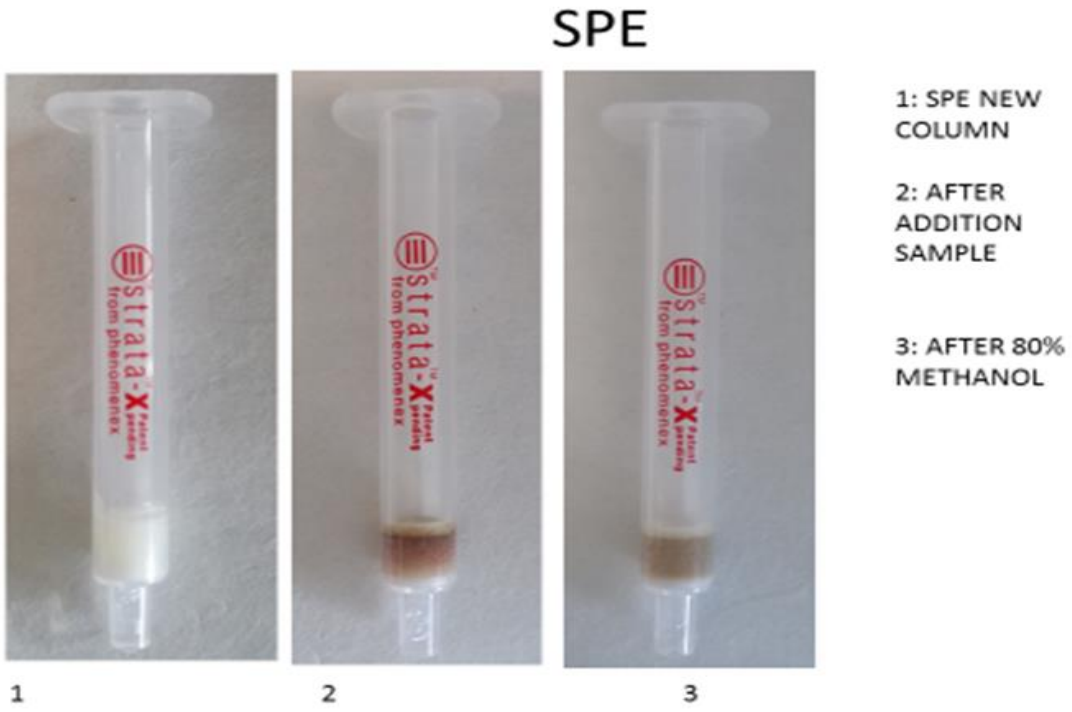


Figure 2-1: Illustration of the main steps of the SPE technique

1. Column after washing with 2.0 ml of 10% methanol. 2. Loading the column with the mixture made by 1.0 ml of sample and 9.0 ml of 100% methanol. 3. Analytes elution with 80% methanol.

b) HiTrap Chelating HP

All solutions were filtered through a 0.25µm syringe filter (Minisart syringe filter, Germany) prior to their application onto the (1.0ml) HiTrap Chelating HP (GE Healthcare, UK) column. The column was washed with 5.0ml of distilled and deionised water (DDW) before the application of 1.0ml of 15.0mM $CuSO_4$ to provide 75-80% column metal loading. After which the columns were washed with 5.0ml DDW followed by 10.0ml of methanol containing 0.2% (v/v) 5.0 M NaOH. The food extracts were removed from the freezer and allowed to equilibrate to room temperature. One ml of the extract was added to 3.0ml of methanol containing 0.2% (v/v) 5.0 M NaOH, the pH was brought to pH 8.0 by the addition of 1.0ml 20%(v/v) diethanolamine and the extract was applied to the copper chelating resin in a syringe at a flow rate of approximately 1.0ml min^{-1}. Unbound material was eluted with 10.0ml of methanol containing 0.2% (v/v) 5.0 M NaOH. The bound flavonoids were eluted with 5.0ml methanol containing 0.15% (v/v) TFA (prepared immediately before use) (See Figure 2-2). Fractions were dried using the centrifugal concentrator and dissolved in either sodium deoxycholate or DMSO before being included in biotin incorporation, cross-linking and deamidation assay to measure TG2 activity (See Section 2.2.6b).

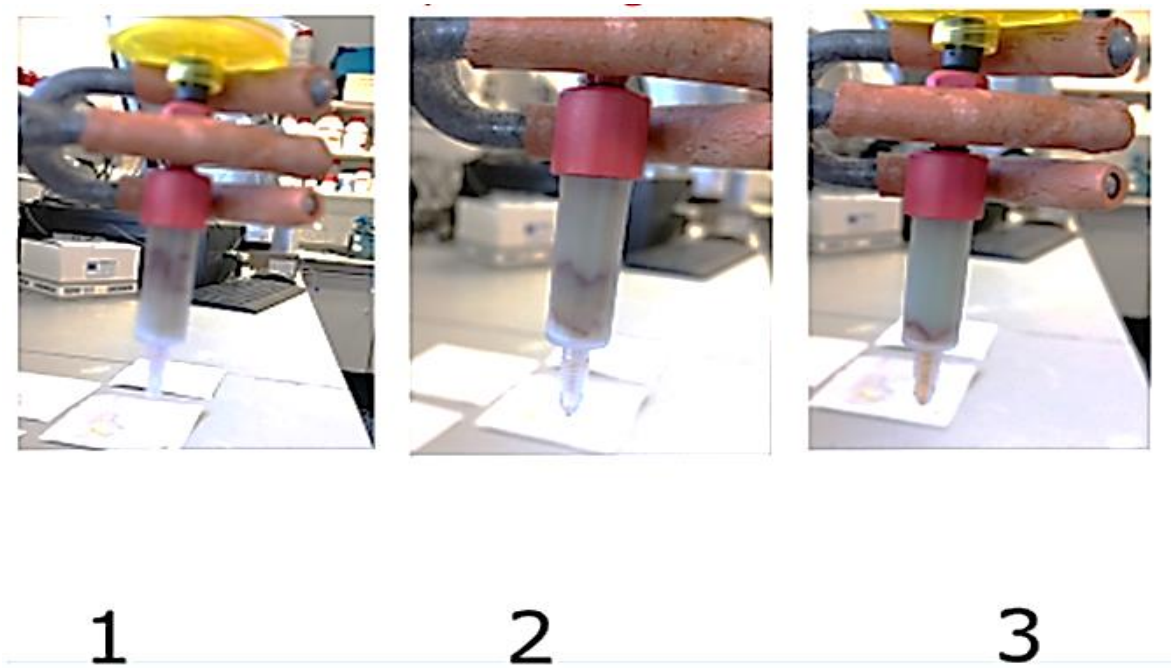


Figure 2-2: Illustration of the main step of the IMAC technique:

1, loading the column of With the mixture made by 1.0 ml of sample, 3.0 ml of 100% methanol with 0.2% (v/v) of 5M sodium hydroxide and 1.0 of 20% (v/v) diethanolamine. **2,** washing the column with 10.0 ml 100% methanol with 0.2% (v/v) of sodium hydroxide 5M to eliminate analytes that didn't bind the column. **3,** Elution of flavonoids by addition of 5.0 ml methanol with 0.15% (v/v) of TFA.

2.2.3 Qualitative analysis using High-Performance Liquid Chromatography (HPLC)

Chromatographic condition:

The mobile phase used**:** Distilled and deionised water + 0.02 % (v/v) of trifluoroacetic acid (Solvent A). Acetonitrile + 0.02 % (v/v) of trifluoroacetic acid (Solvent B); filtered through 0.2µm membrane. The column used C_{12} with TMS end-capping Synergi™ 4 µm Max-RP 80 Å, LC Column 250 x 4.6mm. The detector used LC 200a Series PDA Detector, Photo Diode Array Detector model, was provided by PerkinElmer; the UV wavelength used was 410nm(See Table 2-1). (Ganzera et al,. 2005)

Table 2-1: The gradient used in HPLC for flavonoids

Step	Time (min)	Solvent(A)	Solvent(B)
0	1	100	0
1	2	90	10
2	5	67	33
3	13	67	33
4	2	65	35
5	8	65	35
6	1	100	0
7	4	100	0
8	1	90	10
9	8	90	10

Pure flavonoids were prepared (1.0mM in acetonitrile) and filtered through 0.25µm syringe filter (Minisart syringe filter, Germany) before the injection to the HPLC. Samples obtained by SPE and IMAC were diluted 1:10 in acetonitrile and filtered before 0.25µm syringe filter (Minisart syringe filter, Germany) the injection on to the HPLC.

2.2.4 Digestion of gel bands for mass spectrometry

The biotinylated proteins were separated by 1D PAGE in 5-15% gradient gels (Bio-Rad, UK). The biotinylated bands were excised from the gel using a sterile scalpel, cut into smaller pieces and transferred slices to a microfuge tube. The gel pieces were covered in equal volumes (50.0mM ammonium bicarbonate, water, ACN) and then incubated at 37^{o}C for 5.0min. The supernatant surrounding the gel pieces was removed and replaced with equal volumes of 50.0mM ammonium bicarbonate, water and ACN followed by incubation at room temperature for 1.0min. The gel pieces were then mixed and dissolved in equal volumes of ACN in order to dehydrate them. The supernatant was removed and covered with 50.0mM ammonium bicarbonate for 5.0min until the gel is rehydrated. Acetonitrile was then added into the gel and incubated for 15min. The supernatant was removed and the gel covered with 100 % (v/v) ACN until the gel shrank. The supernatant

was removed, covered with water for 5.0 min until the gel dehydrated and the excess supernatant was removed. The gel pieces were then digested by (1.0µl Promega gold trypsin as prepared from 0.55µg μl^{-1}, 7.6 µl water and 16.6 µl of 100 mM ammonium bicarbonate pH 10.0) and then incubated for overnight at 37^{o}C. The trypsin reaction was terminated by adding 1.0µl volume of 1.0 % (v/v) TFA into the samples.

2.2.5 Mass spectrometry:

a) Flavonoids

Food extract samples were analysed by LC-MSMS. Briefly, 1.0uL of each sample was injected by Eksigent 425 LC system onto a trap column (Mobile Phase A; 0.1% formic acid, B; Acetonitrile with 0.1% formic acid). YMC Triart C18 guard column 0.3 x 5.0 mm, 300µm ID) at 10.0µL/min. Mobile phase A for 2.0min before gradient elution onto the analytical column (YMC Triart C18 150 x 0.3mm ID, 3.0 µm) in line to a Sciex TripleTOF 5600+ Duospray Source using a 50.0µm electrode, positive mode +5500V. The method was run as an MRM-HR (multiple reaction monitoring-high resolution) with a TOFMS survey scan 250ms accumulation time, m/z range 5.0 to 620.0, followed by 9.0 product ion scans for each known flavonoid as follows: Kaempferol 287.06, Myrecetin 319.05, Quercetin 303.05, Naringin 273.15, Hespiridin 611.19, morin 303.05, catechin 291.09, taxifolin 305.06, Hesperitin 303.09 (Figure 3-2). Declustering potential and collision energy were optimised by infusion for each flavonoid. Food extract samples were compared to known standards by LCMS and infusion.

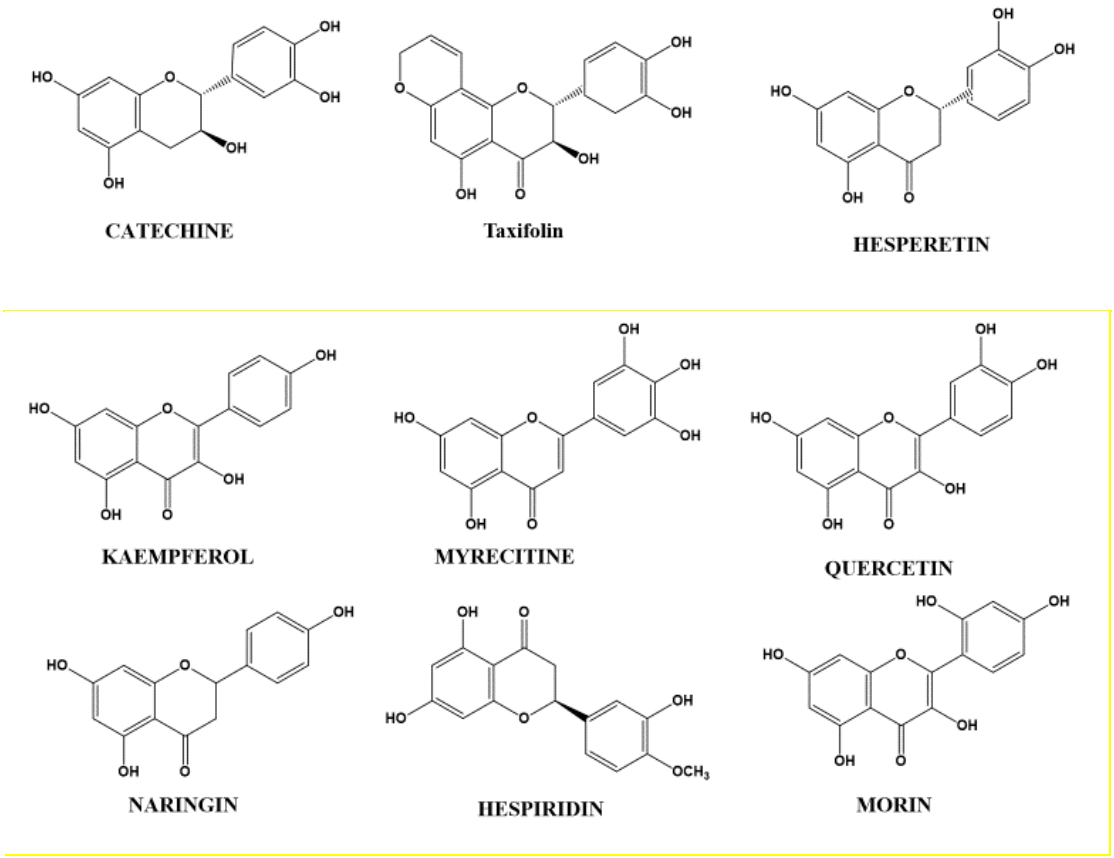


Figure 2-3:Flavonoids sturctures

b) Gliadin 33mer peptides

Samples (3.0 µL) were injected by Eksigent 425 LC system onto a trap column. (Mobile Phase A; 0.1% formic acid, B; Acetonitrile with 0.1% formic acid;). YMC Triart C18 guard column 0.3 x 5mm, 300 µm ID) at 10.0 µL/min mobile phase A for 2.0 min before gradient elution onto the analytical column (YMC Triart C18 150 x 0.3mm ID, 3.0µm) in line to a Sciex TripleTOF 6600 Duospray Source using a 50.0 µm electrode, positive mode +5500V. The method was set to IDA mode (Information dependent acquisition) with the following parameters: TOFMS survey scan m/z 900-2250 range, 250ms accumulation time, Top 15 ions fragmented 100ms accumulation time (m/z range 100-4000). The following linear gradients were used: mobile phase B increasing from 3% to 30% over 38.0 min; 40% B at 43.0min followed by column wash at 80% B and re-equilibration (57.0 min total run time). MS data were searched using PEAKS Studio 8.5 (Bioinformatics solutions inc. Canada) with the following settings; precursor mass 25.0 ppm, fragment ion 0.1 Da against custom Wheat database containing the gluten peptide.

Maximum missed cleavages 2, digest mode – none. PTM variable modification oxidation [M] and deamidation (NQ).

c) Gel Bands

Samples (4.0 µL) were injected by Eksigent 425 LC system onto a trap column (Mobile Phase A; 0.1% formic acid, B; Acetonitrile with 0.1% formic acid; YMC Triart C18 guard column 0.3 x 5.0mm, 300µm ID) at 10.0 µL/min mobile phase A for 2.0min before gradient elution onto the analytical column (YMC Triart C18 150 x 0.3mm ID, 3.0 µm) in line to a Sciex TripleTOF 6600 Duospray Source using a 50.0µm electrode, positive mode +5500V. The method was set to IDA mode (Information dependent acquisition) with the following parameters: TOFMS survey scan m/z 400-1250 range, 250ms accumulation time, Top 30 ions fragmented 50ms accumulation time (m/z range 100-1500). The following linear gradients were used: mobile phase B increasing from 2% to 30% over 20.0min; 40% B at 22.0min followed by column wash at 80% B and re-equilibration (32.0min total run time). MS data were searched using PEAKS Studio X (Bioinformatics solutions inc. Canada) with the following settings; precursor mass 25ppm, fragment ion 0.1 Da against the SwissProt human database (April 2019). Maximum missed cleavages 2, digest mode – semispecific. PTM fixed modification Carbamidomethylation, variable modification oxidation [M] and deamidation (NQ).

2.2.6 Assays

a) Bicinchoninic acid protein assay (BCA) assay

The principle of the BCA assay based on the formation of Cu^{2+} protein complex that then followed by reduction of Cu^{2+} to Cu^{1+} in alkaline conditions. The extent of reduction depends on the quantity of protein present, the more the protein higher the amount of reduction. The amount of reduction of alkaline Cu^{2+} was observed by the formation of purple-blue complex in alkaline environments. BCA works with all reagents that have a reducing centre including the peptide bond (Smith et al., 1985) reducing sugars (Anthon et al., 2002) and flavonoids. The BCA can be used for the measurement of protein concentrations in the range of 0.2 to 1.0mg ml^{-1} (Smith et al. 1985). Protein concentration was measured using a commercially available kit (Sigma-Aldrich Co Ltd, UK). Bovine serum albumin (BSA) was used as the protein standard. However, quercetin was used as a standard flavonoid for flavonoid quantification. In brief, protein standards were prepared using dilutions of 0-2.0mg ml^{-1} BSA to produce a linear standard curve,

quercetin was prepared using concentration from 40 to 400µM. Standards and samples (20.0µl) were added to a 96-well flat-bottomed plate in triplicate. A volume of 200.0µl of working BCA reagent (50 parts of reagent A to 1 part of reagent B) was added to each of the wells and the plate incubated at 37oC for 30.0min. Absorbance was read at 570 nm using a standard 96-well plate reader. The protein content of samples was obtained by measuring them against the BSA standard curve.

b) Transglutaminase deamidation activity assay

Optimization of ammonium chloride as standard for the deamidation assay was based on the Poulin et al., method (2007), with some modifications. Ammonium chloride in ultrapure water used to prepare different concentrations from stock of ammonium chloride (10.0mM, 1.0mM, and 0.1mM). A volume of 25.0µl of each dilutions of ammonium chloride were added to a black fluorescence 96 well microplates (Greiner), and supplemented with 200.0µl of working reagent containing; 500.0ml of sodium borate buffer solution, 2.5ml of sodium sulphite solution and 25.0ml of ultra-pure ethanol containing of 1.0g orthophthadialdehyde (OPA). Each microplate corresponding to different dilution of ammonium chloride was left for incubation at 20min incubation time at 60^0C. An intense fluorescent product is formed because of the OPA-amine reaction in the presence of 2-mercaptoethanol (See Figure 2-4). Release of ammonia readings in CLARIOstar Microplate Reader (BMG-LABTECH), relative fluorescence unit (RFU) was obtained at λex = 360nm and λem = 430nm.

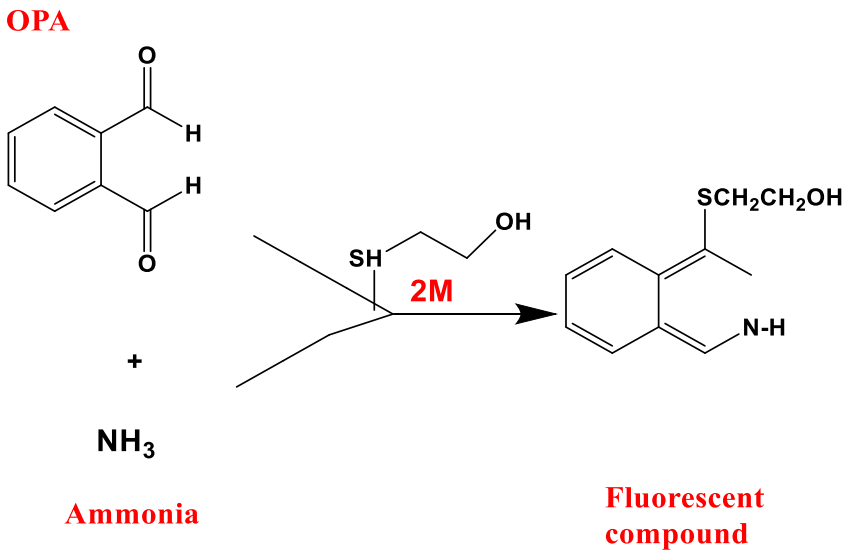


Figure 2-4: The orthophthadialdehyde (OPA) derivatization reaction

i. Transglutaminase 2 deamidation activity assay using *Vicia faba* storage protein as substrates

To a set of glass sample vials (2.0ml Chromacol vials, Phenomenex, UK), 100.0µl of storage proteins (10.0 mg $ml^{-1)}$,10.0 mM EDTA was added to three of the tubes while 10.0mM $CaCl_2$ was added to another three tubes, 100.0µl ultrapure water was added to three of glass tubes that serve as blank. Five µg ml^{-t} hrTG2 or 10.0µg ml^{-1} of gplTG2 (Zedira) was added to all of these tubes (blank no EDTA or $CaCl_2$, EDTA and $CaCl_2$). Hundredµl tris/HCl buffer (pH 8.0) was added to all tubes (blank no EDTA or $CaCl_2$, EDTA and $CaCl_2$), 100.0µl of storage proteins (See section 2.2.1c) and 400.0µl of ultrapure water was added to three of tubes as a control. The glass tubes were gas sealed and incubated overnight at 37^oC then 25.0µl was added from each sample to the wells of a black fluorescence microplate (Greiner) containing 1.0µl 2-mercaptoethanol. Then 200.0µl of working reagents containing; 500.0ml of sodium borate buffer solution, 2.5 ml of sodium sulphite solution and 25.0ml of ultra-pure ethanol containing 1.0g orthophthadialdehyde (OPA). Release of ammonia readings inCLARIOstar Microplate Reader (BMG-LABTECH), relative fluorescence unit (RFU) was obtained at λex = 360 nm and λem = 430 nm. The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one hour (1nmol hr^{-1}).

ii. Transglutaminase 2 deamidation activity assay using the gliadin protein as substrate.

Crude gliadin digest (G3375Sigma) 2.0mg was dissolved in isopropanol, evaporated by nitrogen gas and re-suspended in 1.0ml of 0.5% (w/v) sodium deoxycholate by sonication in an ultrasonic bath for 10min at room temperature. Gliadin protein samples were resuspended in deoxycholate (100.0µl). And used as substrate instead of storage proteins and applied to the assay for ammonia release that was described in section 2.2.6. The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one min (1nmol min^{-1}).

iii. Transglutaminase 2 deamidation activity assay using the 33-mer gliadin peptide as substrates.

The 33-mer gliadin peptide((LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF)and pepceutical, UK****) was prepared in DMSO (10.0mM)as stock then 1mM solution was incubated with 5.0 µg ml^{-t} hrTG2 in presence of $CaCl_2$ (10.0mM), EDTA(10.0mM) for 10 min at RT. Then applied to the assay for ammonia release that described in section 2.2.6.b. Release of ammonia readings in CLARIO star Microplate Reader (BMG-LABTECH), relative fluorescence unit (RFU) was obtained at λex = 360nm and λem = 430nm. The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one min (1nmol min^{-1}).

c) Transglutaminase transamidation activity assays

All the enzymatic assays for TGases have been carried out under optimal conditions of pH, Ca^{2+} and with N, N-dimethylcasein (acyl donor) or casein (acyl acceptor) as acyl donor substrates and biotin cadaverine (acyl acceptor) or TVQQEL as (acyl donor) substrates. Transglutaminase activity was measured by two different microplate assays.

i. Biotin-labeled cadaverine incorporation assay

The amine incorporating activity of TG2 was measured by biotin-cadaverine incorporation into N, N'-dimethylcasein. The assay was performed as described by Slaughter et al., (1992) with the modifications of Lilley et al.,(1998). Briefly, 96-well microtitre plates were coated overnight at 4°C with 250µl of N′, N′-dimethylcasein (10 mg ml^{-1} in 100mM Tris-HCl, pH 8.0). The plate was washed twice with distilled water and blocked with 250.0 µl of 3% (w/v) BSA in 100.0 mM Tris-HCl, pH 8.0 and incubated for 1h at room temperature with gentle agitation. The plate was washed twice with distilled water before the application of 150.0µl of either 6.67mM calcium chloride (required for enzyme activity) or 13.3 mM EDTA (used to detect background TG activity) assay buffer containing 225µM biotin cadaverine (a widely used substrate to monitor TG amine incorporating activity) and 2 mM 2 mercaptoethanol. The reaction was started by the addition of 50.0µl of samples or positive control (50 ng/well of human recombinant TG2) and negative control (100.0 mM Tris-HCl, pH 8.0). After incubation for 1 h at 37°C, plates were washed as before. Then, 200.0µl of 100.0mM Tris-HCl pH 8.0 containing 1% (w/v) BSA and ExtrAvidin®-HRP (1:5000 dilution) were added to each well and the plate incubated at 37°C for 45min then washed as before. The plate was developed with 200.0µl

of freshly made developing buffer (7.5 µg ml^{-1} 3, 3', 5, 5'-tetramethylbenzidine (TMB) and 0.0005% (v/v) H_2O_2 in 100mM sodium acetate buffer, pH 6.0) and incubated at room temperature for 15min. The reaction was terminated by adding 50.0µl of 5.0M sulphuric acid and the absorbance read at 450 nm. One unit of TG2 activity was defined as a change in absorbance at 450nm min^{-1}

ii. Biotin-labeled peptide cross-linking assay

The cross-linking activity of TG2 was measured via biotin-TVQQEL peptide incorporation into casein as described by Trigwell et al., (2004) with minor modifications. The 96-well microtitre plates were coated and incubated overnight at 4°C with casein at 1.0 mg ml^{-1} in 100.0 mM Tris-HCl pH 8.0 (250.0µl per well). The wells were washed twice with distilled water, before being blocked with 250.0µl of 3% (w/v) BSA in 100.0 mM Tris-HCl, pH 8.0 and incubated for 1 h at room temperature with gentle agitation. The plate was washed twice with distilled water before the application of 150.0µl of either 6.67 mM calcium chloride and or 13.3 mM EDTA assay buffer containing 5.0µM biotin-TVQQEL and 2.0 mM 2-mercaptoethanol. The reaction was started by the addition of 50.0 µl of samples or positive control (50.0 ng/well of human recombinant or guinea-pig liver TG2) and negative control (100.0 mM Tris-HCl, pH 8.0) and allowed to proceed for 1 h at 37°C. Reaction development and termination were performed as described for biotin-cadaverine assays. One unit of TG2 activity was defined as a change in absorbance 450nm of 0.01 at min^{-1}.

iii. Visualisation of *in situ* TG2 transamidase activity

The effect of the significant flavonoids on the TG2 activity was observed using immunofluorescence microscopy.HT29 (15,000 cells/well) cells were cultured for 24h on 8-well chamber slides (ibidi). The medium was then removed and slides incubated for 4 h with 1.0mM biotin-X-cadaverine (a cell-permeable TG2 substrate; Perry et al., 1995) in the culture medium before experimentation. Cells were then treated for 1h with the samples (kaempferol, morin, quercetin and Z-DON), washed three times (five min per wash) with PBS, fixed with 3.7 % (w/v) paraformaldehyde and permeabilised with 0.1% (v/v) Triton-X100, both in PBS for 15 min at room temperature. After washing, cells were blocked with 3% (w/v) BSA in PBS for 1 h at room temperature. The transglutaminase mediated biotin-X-cadaverine labelled protein substrates were detected by FITC-conjugated ExtrAvidin® (Sigma-Aldrich) (1:200 v/v) in blocking buffer. The slides were

incubated overnight at 4°C. The next day cells were washed as before and nuclei counter-stained using DAPI in Vectashield mounting medium. The slides were sealed with coverslips and stored at 4°C until required. Images were viewed using EVOS FL imaging system (ThermoFisher).

2.2.7 Electrophoresis:

To observe the activation or the expression of the proteins of interest, SDS- PAGE and Western blotting were employed.

a) Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The principle of the SDS-PAGE is built on denaturing of proteins and treatment with sodium dodecyl sulphate, which is anionic detergent. This process leads to the destruction of the secondary and tertiary structure of the protein. Moreover, the charge of the proteins when covered with SDS results in an equal mass to charge ratio on all the negative charged polypeptides. Therefore, the separation in SDS occurs solely by the size of the polypeptide chains. SDS-PAGE was carried out by the method described by (Laemmli et al.,1970)(See Table 2-2). Once the protein concentration of the samples was determined, (see section2.2.6a). The desired amount of protein was diluted in a 3:1 ratio with 4x reducing Laemmli buffer (8% w/v SDS, 40% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.01 % (w/v) bromophenol blue, 250.0 mM Tris/HCl pH 6.8; and boiled for 10min at 95°C to denature the proteins. Based on the molecular weight of the investigated proteins, acrylamide resolving gels were prepared (at different % acrylamide) for all experiments. Two glass plates with combs and spacers of 1.5 or 0.75 mm thickness were used. The 10% (w/v) acrylamide resolving gel mixture was prepared according to the required number of gels, as indicated in Table 2.2. Acrylamide polymerisation was initiated by the addition of volumes indicated of 10 % (w/v) ammonium persulfate and TEMED (N,N,N',N'- tetramethylethylenediamine), after which the mixture was swirled gently.

Table 2-2: Preparation of SDS-PAGE gel

Reagent	10% (w/v) acrylamide resolving gel	4% (w/v) acrylamide stacking gel
40% (w/v) 29:1 Acrylamide Bis Acrylamide solution (Molecular Biology Grade, AppliChem, USA)	3.3 ml	1.0 ml
Protogel® Resolving buffer (1.5 M Tris-HCl buffer pH 8.8, 0.4% (w/v) SDS)	2.5 ml	-
Protogel® stacking buffer (0.5 M Tris-HCl buffer pH 6.8, 0.4% (w/v) SDS)	-	2.0 ml
Distilled water	4.0 ml	6.1 ml
10 %(w/v) ammonium persulphate (APS)	100.0 µl	50.0 µl
TEMED (Melford, UK)	20.0 µl	10.0 µl

For each gel, about 8ml of resolving gel mixture was transferred into the glass cast to allow space for a stacking gel to be added later. Distilled water was carefully overlaid on the top layer of the freshly poured gel mixture to create a smooth interface and prevent any gel shrinkage. The gel mix was allowed to polymerise at room temperature for approximately 30 to 40min. Once the resolving gel had polymerised, water was removed, the required amount of 4% (w/v) polyacrylamide stacking gel was prepared and polymerised by the adding of 10% (w/v) APS and TEMED as indicated in Table 2-2. Immediately after the stacking gel was poured, a plastic comb was placed to form the sample wells. Following polymerisation, the gels were placed into a Biorad protean 3 electrophoretic tank to which electrophoresis buffer (0.01 % (w/v) SDS, 2.5mM Tris, 19.2 mM glycine, pH 8.3) was added. The protein samples (15-20 µg/well) were loaded along with 2.0 µg of protein ladder (BioRad, Hemel Hempstead, UK, 1610374). Electrophoresis was conducted at 120 V through the stacking gel and then at 160 V through the resolving gel.

b) Non denaturing PAGE (native gel)

The non-denaturing PAGE used for the separation of the native, unfolded and non-denatured proteins. Unlike SDS-PAGE which can separate only individual and denatured protein, non-denaturing PAGE was used to confirm the relevant native conformations of the proteins. The separation of the proteins by native PAGE relies on certain factors such as charge, size and 3D structure of the protein. To maintain the 3D structure of the protein, a suitable buffer was used depending on the charge and isoelectric point of the protein.

The non-denaturing PAGE gel contained 5% resolving gel with a pH 8.8 with larger pores, was prepared using the reagents (See Table 2-3Table 2-3). Storage protein samples were mixed with 5 x sample loading buffer containing; 2.5ml of 100 mM Tris/HCl pH (6.7), 1.0ml of glycerol, 6.4ml of ultrapure water and 1.0 % (v/v) bromophenol blue and centrifuged at 10,000 *x g* for 10.0 min. Then 10.0µl of the supernatant protein samples in presence of EDTA or $CaCl_2$ were loaded into 5.0 % polyacrylamide gel in a Bio-Rad mini gel system using a running buffer containing; 3.0 g of Tris/HCl pH 8.3, 14.4 g of glycine in water a final volume of 1.0ml. The gel was run at 170 V until the dye reached the bottom of the gel.

Table 2-3: Preparation and reagents for native gel

Reagent	5% (w/v) acrylamide resolving gel	4% (w/v) acrylamide stacking gel
40% (w/v) 29:1 Acrylamide Bis Acrylamide solution (Molecular Biology Grade, AppliChem, USA)	3.5 ml	1.0 ml
Protogel® Resolving buffer (1.5 M Tris-HCl buffer pH 8.8, 0.4% (w/v) SDS)	5.0 ml	-
Protogel® stacking buffer (0.5 M Tris-HCl buffer pH 6.8,)	-	2.0 ml
Distilled water	11.4 ml	6.1 ml
10 %(w/v) ammonium persulphate (APS)	60.0 µl	50.0 µl
TEMED (Melford, UK)	40.0 µl	10.0 µl

2.2.8 Western blotting

After the mixture of proteins is separated by SDS-PAGE, the separated polypeptides are then transferred to a membrane carrier. The membrane (normally nitrocellulose or polyvinylidene fluoride) is attached to the gel-forming a sandwich-like structure, which is then transferred to the electrophoresis chamber. Once the electric charge is applied, the protein bands travel in the vertical direction from the gel to the membrane and therefore are attached to the membrane. The membrane-bound proteins are termed as blotted proteins, which can then be further analysed according to the experimental need. Western blotting was based on a method by Towbin et al., (1979) with the modifications. Proteins were transferred to nitrocellulose membranes in a Bio-Rad Trans-Blot system. A set up of a wet transfer was performed, with the blotting cassettes set up as follows; pre-wet sponge - filter paper - gel- nitrocellulose membrane filter - filter paper – pre-wet sponge. The layers were then placed into a Western blotting cassette (Biorad) and closed gently to avoid air bubbles and placed in the transfer tank that contained chilled transfer buffer (25.0mM Tris, 192.0mM glycine pH 8.3 and 20% (v/v) methanol). Proteins were transferred at 90 V for 1h. After electrotransfer of the proteins, the membranes were washed with Tris-buffered saline (TBS), then blocked for 1 h at room temperature with 3% (w/v) BSA in TBS containing 0.1% (v/v) Tween-20 with mild agitation. Blocking is crucial to prevent non-specific binding of the antibodies to the membrane in subsequent steps. Blots were then probed with the one of the following primary antibodies β- actin or extravidin horse (HRP) peroxidase primary that is diluted in blocking buffer (1:1000) overnight with gentle agitation at 4°C. After incubation, the primary antibodies were removed, and blots washed three times for 5 min in TBS/Tween 20. After washing, blots were probed with the appropriate (1:1000) horseradish peroxidase (HRP)-conjugated secondary antibodies (New England Biolabs Ltd; Hitchin, UK) for 2h at room temperature in blocking buffer. Following removal of the secondary antibody, blots were extensively washed as above and developed using the Enhanced Chemiluminescence Detection System (Uptima, Interchim, France). Images of the blots captured and the bands were quantified by the iBright FL1000 imaging system. All band densities were measured and normalised to band densities for internal control. To confirm uniform protein loading in the gel primary antibodies specific to β actin were used.

2.2.9 Cell culture

a) Maintenance of cells

Human colonic adenocarcinoma cells (HT-29) was grown and maintained as a monolayer in the culture medium. Cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO2.

b) Sub-culture of cells

Cells were passaged or sub-cultured when growth reached 70-80% confluence (i.e. every 3-4 days). On reaching 80% confluence, the cell cultures were either used to seed monolayers on cell culture plates for assays, (for example; T75 flasks for Western blot analysis, and chamber slides for immunofluorescence staining) or passaged to maintain the cell line. Cells were passaged by removing the growth medium and washing cells with sterile phosphate buffered saline (PBS), then detached using trypsin (0.05 % w/v)/EDTA (0.02 % w/v) in PBS. Following trypsinisation, 10ml of fully supplemented McCoys 5A medium were added to the trypsinised cells. Then, the Ht29 suspended cells were harvested by centrifugation at 300-x *g* at room temperature for 5min. The supernatant was discarded, and the cell pellet resuspended in 1.0ml of growth medium and further sub-cultured (1:5 split ratio).

c) Counting and plating cells for experimental analysis

Cells were subjected to experimental analysis when they reached 70-80% confluence in the maintenance flasks. Initially, cells were passaged as described in the previous section and, after resuspending in 1.0 ml of the growth medium, automated cell counting carried on using a TC20 automated cell counter (BioRad, Hemel Hempstead, UK). The device utilised prepared TC20 Trypan blue dye (0.4% (w/v) Trypan blue in 0.81% (w/v) sodium chloride and 0.06% (w/v) potassium phosphate dibasic solution). Trypan blue is a vital stain that distinguishes between live and dead cells (Takahashi et al., 2006). The role of this dye is based on the blue acid dye chromophores which react and are taken up by the internal region of dead (non-viable) cells through a damaged membrane, whereas live (viable) cells do not take up this dye. A volume of 10.0µl of cell suspension was added to 10.0µl of Trypan blue solution and left for 4 min to allow cells to be exposed to the stain. A volume of 10.0µl of this mixture was then loaded into a chamber of the counting slide. The slide was inserted into the slide slot of the TC20 cell counter and cell counting was automatically initiated as soon as the cell counter detected the presence of the slide and

Trypan blue dye. Viable cell counts per ml were used to determine the volume necessary to seed the cells at a required cell density in the growth medium. The cell density of 50,000 cells/ml was used to plate out the cells. Flasks were then incubated at 37^{o}C.

d) *In situ* biotinylation of proteins in HT29 cell lines

Biotinylation refers to the binding of biotin amine conjugate with the biological proteins by cellular TG2. Identification of acyl-donor substrates of TG2 in human intestinal epithelial cells, based on the method by Orru et al., (2003). A cell-penetrating substrate of TG2; 5-(((N(Biotinoyl)amino)hexanoyl)amino) pentylamine, trifluoroacetic acid (biotin-X cadaverine, Invitrogen, UK) was diluted in dimethyl sulphoxide (DMSO) to achieve 180 mM stock solution. This substrate then diluted to 1.4 mM in growth medium and used to treat the cell lines, followed by incubation at 37^{o}C in presence of 5% CO_2 for 4.0 h. The cells were treated with flavonoids TG2 inhibitors for 1.0h at 37^{o}C. The cells were lysed with buffer (50 mM Tris/HCl pH 8.5 + 0.5%w/v sodium deoxycholate) and the lysates stored at -20^{o}C.

e) Cell viability assessment

i. MTT reduction assay

Cell viability was determined by measuring the activity of cellular dehydrogenases by MTT (3- (4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) reduction assay (Mosmann, 1983). The MTT tetrazolium dye was converted to water-insoluble purple formazan in the reductive cleavage of its tetrazolium ring by the respiratory enzyme succinate dehydrogenase in active mitochondria and by other cellular dehydrogenases. The assay was used to assess cell viability after treatment of the cells with different flavonoids treatments. The cell lines (25,000 cells/well) were plated in 24-well flat-bottomed plates and cells were exposed to required treatment and cell viability subsequently determined by incubating with 0.5mg/ml MTT at 37 °C for 1h. After that, the medium in each well was carefully aspirated and replaced with 500.0µl of DMSO. The plate was then gently agitated to ensure sufficient dissolution of the water-insoluble purple formazan crystals. After that, 200.0µl of the resultant solution was transferred into a 96-well plate and read at 570nm in CLARIOstar Microplate Reader (BMG-LABTECH),

ii. Live cell imaging:

The effect of kaempferol, morin, and quercetin on the proliferation of HT29 cells was observed using real-time live cell analysis. Cells were seeded in 96-well plates at a seeding density of 10.000 cells per well in completed growth media for24hr. After that, the medium in each well was carefully aspirated and replaced with the growth media with flavonoids (kaempferol, morin, and quercetin) 125µM. Three fields of view were imaged in each well, with a 10× objective every three hours over Three days using the Incucyte S3 Live Cell Analysis System (Essen BioScience) fitted inside an incubator. Confluence percentages were calculated using the integrated Incucyte S3 software.

2.3 Statistical analysis

All graphs and statistics (one-way ANOVA followed by Dunnet's multiple comparison test and two-way ANOVA for group comparison) were performed using GraphPad Prism® software (GraphPad Software, Inc., USA). All sets of data were based on a minimum of three separate experiments and expressed as mean ± standard error of the mean (SEM) and p-values <0.05 were considered statistically significant.

Chapter 3 : Study the effects of flavonoids on the transamidation and deamidation activity of TG2

3.1 Introduction

Flavonoids have been reported in the literature to have a different biological activity such as antioxidant, anti-inflammatory and protective against diseases such as cancers, diabetes, cardiovascular diseases and atherosclerosis (Rzepecka-Stojko et al., 2015; Huang, 2016; Mohana, 2016 and Packzoski et al., 2017). Certain flavonoids have been shown to moderate the esterase activity of trypsin and acetylcholinestears (Xue, G et al., 2017). Screening these natural compounds to moderate TG2 activity is appropriate because TG2 also posses esterase activity (Folket al, 1966). Looking for as potential treatment for coeliac disease (CD) is a sensible option because the only available treatment for CD is a gluten-free diet, which is not a favourable lifestyle because gluten-free options are expensive and are not always easily available (Kelly et al., 2015).

Various therapeutic choices have been considered for CD, such as decreasing intestinal permeability through inhibitors, pro-biotics, genetically modified grains, therapeutic vaccines, acceleration of gastrointestinal degradation of proline-rich gluten by oral enzyme supplementation and TG2 inhibitors (Sollid and Khosla, 2011; Bakshi et al., 2012). Since the activity of TG2 is crucial to the initiation of CD, therefore decreasing the activity of TG2 in CD patients will supply a potential treatment choice. TG2 inhibitors targeted to reduce the TG2 activity have been considered potential therapeutic tools in the treatment of CD (Sollid & Khosla, 2011). However, TG2 is present in every cell and chemical inhibition may stop vital cellular processes such as interference with inflammatory reactions and the ECM (Sollid & Khosla, 2005). Consideration of these factors provides motivation to search for an alternative strategy to treat this condition. Biological active components derived from natural sources are in general considered to be less or non-toxic. Therefore, naturally occurring TG2 inhibitors in food normally ingested may provide an acceptable reagent to be taken orally to moderate the symptoms of CD (General introduction chapter 1.8.7f).

The existence of TG2 inhibitors have been found in several natural products such as garlic (D'Argenio et al., 2010) and milk (De Jong et al., 2003; Aldubyan 2014), but their

structures have not been revealed yet. In this context, another group of naturally occurring compounds the flavonoids which are widely distributed in plants and present in the diet with numerous health-beneficial biological activities (Panche et al., 2016). These natural products (flavonoids) were used in this study to evaluate their effect on TG2 activity as a potential intervention in treating coeliac disease.

3.1.1 History of Flavonoids

Flavonoids have been known as pigments in plants, their chemical structure was not identified until the end of 19th century (Perez-Vizcain et al., 2018). The research was mainly centred on the role of anthocyanin family of flavonoid as pigments. Albert Szent-Györgyi and his workers (1930s) studied the effects of number of flavonoids on human health, they found citrus flavonoids stabilised the biological activity of ascorbic acid. Over the years, research on flavonoids has developed from laboratories to reach clinical studies in humans. The renewed interest in their health properties has been reflected by the number of publications on flavonoids which has increased from 740 per year in 1991 to more than 9000 in 2015, reaching nearly 90,000 in 2018 (Perez-Vizcain et al., 2018).

3.1.2 Chemistry of Flavonoids

The chemical structure of flavonoids is based upon a fifteen-carbon skeleton containing two benzene rings A and B,(Figure 3.1) connected by a heterocyclic pyrone ring (C). The benzene ring is either α-pyrone; (flavonols and flavanones) or its dihydro-derivatives; (flavonols and flavanones) (Kumar and Pandey, 2013). The position of the benzenoid substituent divides the flavonoid class into isoflavonoids (3-position) and flavonoids (2-position). Generally, flavonoids are divided into a different of classes such as flavonols (e.g., quercetin, kaempferol, and myricetin,) flavones (e.g., apigenin, and luteolin), flavanones (e.g., hesperetin, and naringenin), and others. The several classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings (Kumaur, and Pandey, 2013 Popova and Novza, 2016; and Kashlik.2016). Flavonoids exist as aglycones, glycosides, and methylated derivatives. Flavonols differ from flavanones by hydroxyl group at the 3-position and a C2–C3 double bond (Aherne et al., 2002). In glycosides, the glycosidic linkage is normally positioned in positions 3 or 7 and the carbohydrate could be L-rhamnose, D-glucose, glucorhamnose, arabinose or galactose (Xiao et al., 2016).

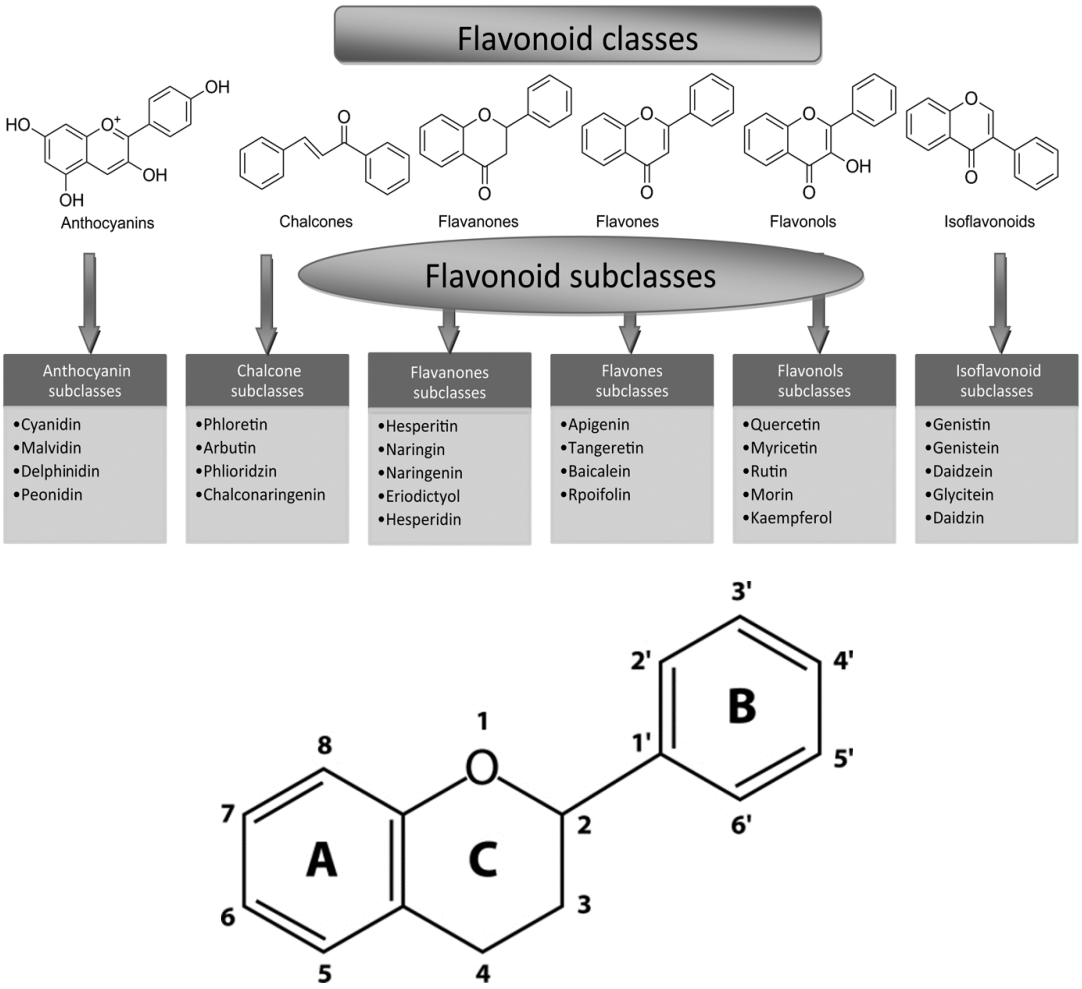


Figure 3-1: Basic flavonoid structure

3.1.3 Classification of flavonoids

Flavonoids are derivatives of 2-phenyl-benzo-γ-pyrone. The carbon atoms in flavonoid molecules are assembled in two benzene rings, commonly known as A and B, that are connected by an oxygen-containing pyrene ring (C). A common part of the chemical structure of all flavonoids is carbon skeleton based on the flavan system (C6-C3-C6) (Brodowska, 2017). Flavonoids in which the B ring is linked in position 4 are called neoflavonoids, whereas when the B ring is linked in position 3 of the C ring they are called isoflavones. When the B ring is linked in position 2 the groups can be further subdivided into many subgroups based upon the structural characteristics of the C ring. These subgroups are flavones, flavonols, flavanones, flavanonols, flavanols or catechins, anthocyanins and chalcones (Panche, 2016) (Figure 3-1).

a) Flavanols

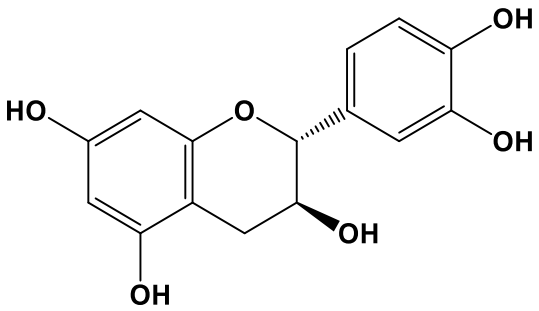


Figure 3-2 Catechin structure (Example for flavanol)

Flavanols are a complex group of polyphenols in the range from the monomeric flavan-3-ols such as catechin (Figure 3-2) epicatechin, and gallocatechin to polymeric procyanidins that known as condensed tannins (Pascual-Teresa et al., 2010). Flavanols mainly occur in fruits, tea, apples, kiwi and cereals (Määttä-Riihinen et al., 2015). It is reported, that they can enhance the levels of nitric oxide in the blood of people who smoke cigarettes and reverse smoking-related disturbances in blood vessels (Brodowska, 2017). The most common representative of the flavanol group is catechines, which are found in black and green tea, chocolate, apricot, apples, and peach (Gramza et al., 2005). Catechin has biological activity such as antioxidation effects (Vinson et al., 2001) anti-atherosclerotic actions (inhibition of the oxidation of low-density lipoprotein) (Rein et al., 2000; Murphy et al., 2003) and anti-carcinogenic activity (Gramza et al., 2005)., green tea catechins have been reported to have antibiotic properties due to their disruptive effect on the bacterial DNA replication process (Brodowska, 2017).-

b) Flavanones

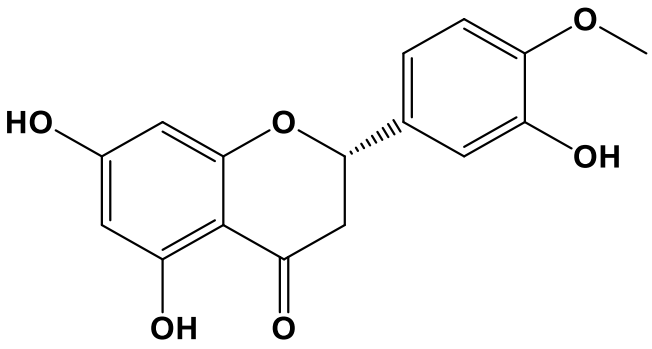


Figure 3-3: Hesperetin structure (Example for Flavanone)

The most important flavanones are naringenin and hesperetin (SeeFigure 3-3) aglycones, which are widespread in foods (Khan et al., 2014). Hesperetin is the distinctive flavanone of orange, lemon, and lime (Goulas et al., 2012). Naringenin can be found in grapefruit

and tomatoes. The main biological activity of flavanones is free radical scavenging, anti-inflammatory, anticancer, cardiovascular, and antiviral effects (Khan et al., 2014).

c) Flavonol

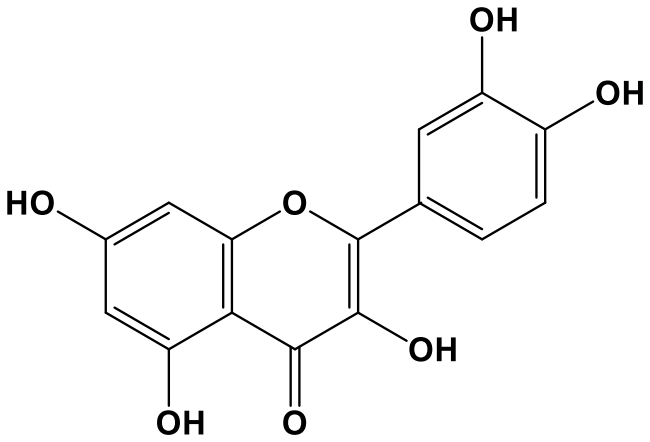


Figure 3-4: Quercetin structure (Example for Flavonol)

Flavonols are an important subgroup of flavonoids and are the most studied subgroup because of their antioxidants activities and other biological effects. Flavonols occur in consumed vegetables and fruits. Major sources of this subgroup are an apple, tomato, onion, grape berries, beverages such as green, black tea and red wine (Makris et al., 2006). The most important members of this subgroup are quercetin (See Figure 3-4), kaempferol, morin, and myricetin (Hollman et al., 1998; Sivaramakrishnan et al., 2008). Quercetin is the most abundant of the flavonol group, which acts as potent antioxidant. It has various biological activity, such as antioxidant, anti-inflammatory activity; it increases blood vessel strength and acts as an antihistamine (Lesjak, et al., 2018). In addition, quercetin can protect against the formation of cataracts, a condition that is also related to the deamidating and cross-linking activities of TG2 (Erlund, 2004). Kaempferol is an antioxidant flavonol occurring in many edible plants (e.g. tea, kale, beans, tomato, strawberries, and grapes) (Calderon-Montano et al., 2011). Several studies have reported the beneficial effects of dietary kaempferol in reducing the risk of stroke, chronic heart disease, and cancer (Brodowska, 2017). Moreover, kaempferol has anti-inflammatory and neuroprotective properties (Hollman et al., 1998). Morin is found in almonds (Wijeratne et al., 2006)], figs and other vegetables (Aggarwal et al., 2006). It has been known to act as a potent antioxidant (Wu et al., 1993; Wu et al., 1994), a protein kinase C inhibitor (Cao, 2005), xanthine oxidase inhibitor (Cao, et al., 2005), cell proliferation inhibitor (Kuo et al., 2007) and an inducer of apoptosis (Manna et al., 2007). Furthermore, morin used in the treatment of oral carcinogenesis had a chemopreventive action (Kawabata et

al., 1999). Myricetin is commonly found in fruits tea, vegetables, red wine, and berries. Myricetin might enhance insulin resistance, in addition to other activities such as anti-hyperlipidemic, anti-inflammation and anti-oxidative stress (Li et al., 2012).

d) Flavones

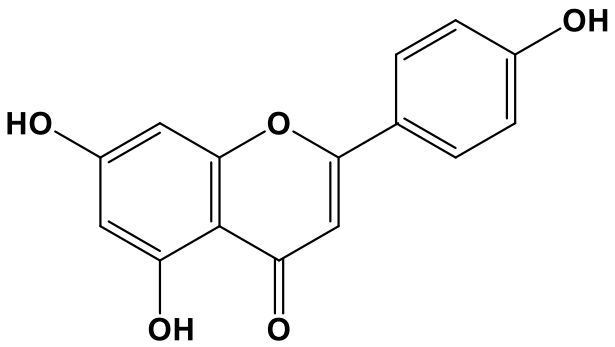


Figure 3-5: Apigenin structure (Example for Flavones)

Flavones are very similar to the structure of flavonol subclass compounds, flavonol possessing an additional hydroxyl substitution at the C 3-position. The most important flavones are luteolin and apigenin (See Figure 3-5). Apigenin is found in onions, parsley, tea and oranges (Patel et al., 2007), while luteolin originates from vegetables and fruits such as broccoli, celery, carrots, parsley, onion leaves and apple skins (Lin et al., 2008). It has been stated that luteolin has several biological properties such as anticancer, anti-allergy, and anti-inflammation (Galati and O'Brien, 2004), whereas apigenin has anti-inflammatory, anti- carcinogenic and antioxidant properties (Nabavi et al., 2018).

e) Isoflavones

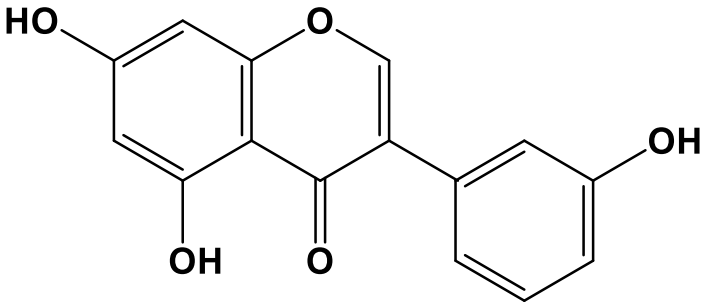


Figure 3-6: Genistein structures (Example for Isoflavones)

Isoflavones are found in soybeans, soy foods, and legumes. They act as phytoestrogens through binding to estrogen receptors in mammals (Barros et al., 2011; Vitale et al., 2013) and they also have anticancer, anti-microbial, antioxidant, and anti-inflammatory activities (Conklin et al., 2007; Chacko et al., 2007) Daidzein and genistein(SeeFigure 3-6) are the most common isoflavones (Yu et al., 2016).

f) Anthocyanidins

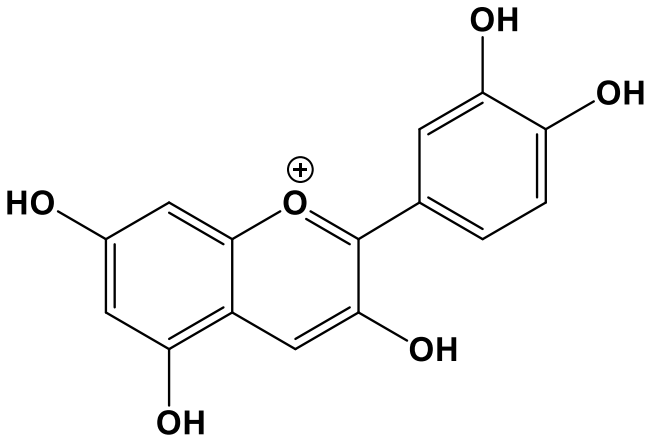


Figure 3-7: Cyanidin structure (Example for Anthocyanidins)

Anthocyanidins are natural pigments in plants and are responsible for the purple, blue, red, and orange colours in many fruits and vegetables. More than 500 diverse anthocyanidins are known and have been defined in the literature (Pascual-Teresa et al., 2010). Anthocyanidins are found in fruits, vegetables, teas, honey nuts, olive oil, black currants, blueberries and strawberries (Roy et al., 2009). The most abundant anthocyanidins occurring in fruits and vegetables are cyanidins (See Figure 3-7), pelargonidin, delphinidin, and peonidin (De Pascual-Teresa et al., 2000). Anthocyanidins have been demonstrated to play an important role in cholesterol decomposition, cardiovascular disease visual acuity, as well as having antioxidant efficacy (Bagchi et al., 2004).

g) Flavonoid analogues

The most important flavonoid analogues are the glycosides, which consist of aglycone part that involves mono- or oligosaccharide moieties bound to the aglycone moiety. The main groups of glycosides are cardiac glycosides, cyanogenic glycosides, anthraquinone, saponins, glucosinolates, and flavonoids glycosides. In addition, flavonoids commonly occur as glycosides. Such as rutinose glycone part with aglycone part either naringin or rutin or hesperidin naringin (Bernhoft et al., 2010; Forbes et al., 2014).

3.1.4 Biological Activities of Flavonoids

Flavonoids are commonly distributed in human's daily diet, such as fruits, vegetables, tea, and others and are considered as bioactive compounds with various biological activities. (Panche et al., 2016). Flavonoids are primarily ingested as a glycone that can be absorbed in the small intestine. However, most of the phenolic compounds in food exist as esters,

glycosides or polymers that can either be absorbed in these forms or hydrolyzed by intestinal enzymes or the colonic microflora; the aglycone or hydrolyzed products can be absorbed.

a) Antioxidant Activity

Flavonoids with antioxidant actions have been proposed to prevent the development of oxidative stress-associated diseases, for example, certain cancers, cardiovascular diseases (CVD) and diabetes (Badmus, 2016 and Martin, 2016). Reactive oxygen species (ROS) have been involved in the etiology of numerous human diseases such as cellular aging, carcinogenesis, mutagenesis, and coronary heart diseases. The ROS are formed during normal metabolism or induced by exogenous factors (Badmus et al., 2016 and Chaudry et al., 2016). The stimulation of antioxidant enzymes scavenging of ROS or metal chelating capability through the flavonoids preventing damage to cellular components such as DNA, proteins, and lipids (Lobo et al., 2010). For example, the oxidation of lipids leads to lipid peroxidation, which is a free-radical mediated propagation of oxidative stress to the polyunsaturated fatty acid component of cell membranes (Heim et al., 2002). The possible health value of flavonoids for the prevention and therapeutic uses has led to the study and identification of a wide range of bioactive principles, to the flavonoids and phenolic compounds in the plants (vegetables, fruits, leaves, seeds, cereal, roots, spices and herbs) (Badmus et al., 2016)

b) Anti-Inflammatory Activity.

In tissue injury, microbial pathogen infection, and chemical irritation, there is a biological process known to occur which is inflammation. Movement of immune cells from blood vessels and the release of mediators at the site of damage initiate inflammation. This process followed by a release of ROS (reactive oxygen species), RNS (reactive nitrogen species) and proinflammatory cytokines helps to eliminate foreign pathogens and repair the injured tissue (Kumar et al., 2013). Various flavonoids have a significant effect on the function of the immune system and inflammatory cells. Flavonoids such as hesperidin, apigenin, luteolin, and quercetin have been reported to have anti-inflammatory and analgesic effects. Flavonoids may affect the function of enzyme systems that involved in the generation of inflammatory processes, especially tyrosine and serine-threonine protein kinases (Kumar et al, 2013; Vezza, et al., 2016 and Zhang, 2016,).

c) Anticancer Activity

Dietary flavonoids have been reported as cancer chemo preventive agents. Consumption of onions and/or apples, two major sources of the flavonol quercetin, is inversely associated with the incidence of cancer of the prostate, lung, stomach, and breast (Kumar et al., 2013 and Hua et al., 2016). Numerous mechanisms have been suggested for the effect of flavonoids on the initiation stages of cancer relating to impacts on development and hormonal activities (Duthie et al., 2000). Quercetin has been shown to arrest the cell cycle in proliferating lymphoid cells. In addition, quercetin exerted growth-inhibitory effects on several malignant tumour cell lines *in vitro* including; P-388 leukemia, colon cancer, gastric cancer, human squamous, human breast cancer, and ovarian cancer cells (Kumar et al., 2013 and Parvaresh et al., 2016).

d) Biochemical effects of Flavonoids on enzymes:

Flavonoids are known to inhibit a number of enzymes such as xanthine oxidase (implicated in oxidative injury to tissue by ischemia-reperfusion), phosphodiesterase (degradation of cyclic nucleotides into inactive metabolites), aldose reductase (responsible for the first steps in the polyol cycle), Ca^{2+} ATPase (involved in excitation/contraction of cardiac muscle), and lipo-oxygenase (catalysing the oxygenation) (Agrawal et al., 2011). Flavonols (e.g. quercetin and kaempferol) inhibit adenosine deaminase of endothelial cells, whereas, flavones are inactive against this enzyme (Hayashi et al., 1993). In addition, a study by Kyo et al (1998) who reported that the baicalin and baicalein, flavonoids isolated from a medicinal plant (roots of *Scutellaria baicalensis Georgi* (Lamiaceae)) inhibit intracellular Ca^{2+} levels rising by reducing phospholipase C activity and ability of these flavonoids to inhibit several cellular enzymes linked to cell signalling e.g. protein kinase C, phospholipase A_2 and protein tyrosine kinase (Musci and Pragathi, 1985).

e) Flavonoids effects on transglutaminase 2 activity:

It can be seen from the literature that, there are few studies about the effects of flavonoids on transglutaminase 2 activity. Some studies have shown that flavonoids could have an effect on the role of TG2 in some diseases, such as, the TG2 cross-linking activity has enhancing role in the formation of proteins complex that helps in stabilising the ECM. Malla et al., (2016) discussed the effect of flavonoids extracts on the role of TG2 in the wound healing process. Beazley and Nurminskaya (2015) showed in their study that,

dietary quercetin could be the reason for the reduction of reproductive capacity in female mice, and suggest that TG2 may regulate ovarian aging (Beazley and Nurminskaya, 2015). Studies show that some flavonoids could influence the role of TG2 in certain diseases. For example, as mentioned previously, by enhancing the formation of stable protein complexes, which helps in stabilizing the ECM (Malla, 2016). Additionally, Al-basher and Al-otibi, 2018 reported in their study, that the olive extract containing flavonoids accelerates wound healing process, through encouraging antioxidant capacity and expression of TG2, which is required for collagen deposition and re-epithelialization process. From the literature, there are no studies describing the effects of flavonoids on TG2 activity using transamidating or deamidating assay. Therefore, one of the aims of this research was to study the effect of dietary flavonoids on the peptide/protein cross-linking, amine incorporating, and the deamidating activity of TG2 as a potential method to moderate the initiation of coeliac disease (CD) and to fully evaluate any influence the dietary flavonoids can have on the enzyme.

3.2 Aims:

As discussed previously, Screening the natural compounds to moderate TG2 activity as potential intervention in treatment of coeliac disease (CD) is a functional option because the only available treatment for CD is a gluten free diet, which is not favourable for number of suffrers because gluten free options are expensive and are not always easily available. These natural products (flavonoids) were used in this study to evaluate their effect on TG2 activity as a potential intervention in treating coeliac disease.

This chapter had the following experimental aims:

- Study the effect of flavonoids on the amine incorporation activity of TG2 using the biotin cadaverine assay (Slaughter et al., 1992).
- Study the effect of flavonoids on the TG2 peptide cross-linking assay (Trigwell et al. 2004).
- Investigate the effect of flavonoids on TG2 deamidation activity using an optimised OPA assay (See Chapter 6).

- **Methods and Materials:**

See Methods and materials chapter:

- Transglutaminase transamidation activity assays (2.2.6c).
- Transglutaminase deamidation activity assay (2.2.6b).

3.3 Results

3.3.1 Effect of flavonoids on the amine incorporation activity of TG2

a) Flavonoids in DMSO

To observe the effect of the flavonoids on the amine incorporation activity of TG2, the flavonoids standards were dissolved in DMSO, the reason for using DMSO is that it was also used to study *in situ* TG2 activity in cell lines (See cell culture chapter 5). Thirteen flavonoids at different concentrations (1.25, 12.5 and125µM) were subjected to an initial screen using the biotin cadaverine assay (Slaughter et al, 1992) using hrTG2 as it is relevant to CD. In addition, gpl TG2 which is routinely used in TG2 assays in the literature because it is seen as being equivalent to human TG2 and is used to moderate the assay costs (Figure 3-8 and Figure3-9).

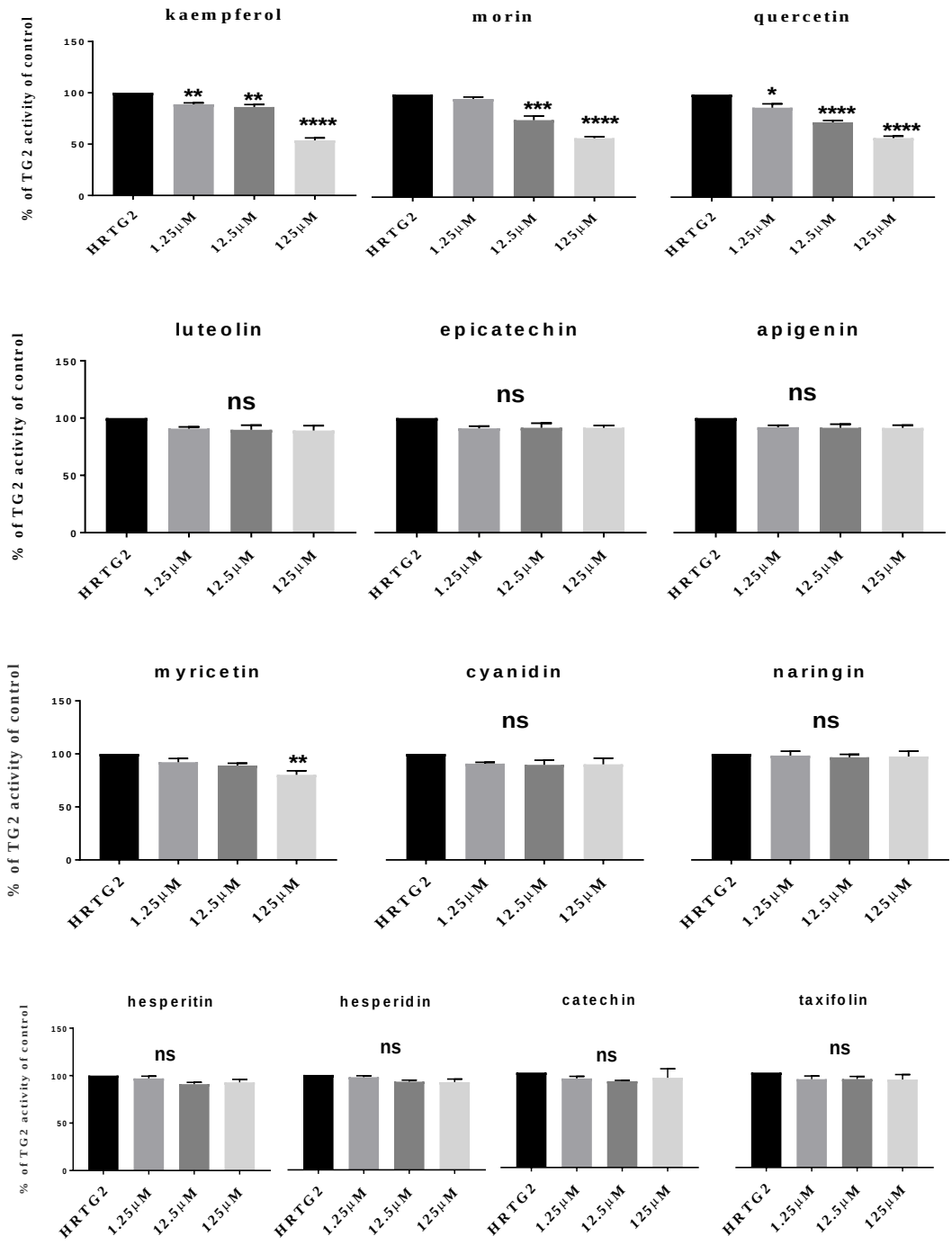


Figure 3-8: The effect of flavonoids /DMSO on the amine incorporation activity of hrTG2:

Flavonoids standards (1.25, 12.5 and 125 µM) dissolved in DMSO then incubated with 5µgml^{-1} hrTG2 after which the TG2 activity was measured according to the method of Slaughter et al (1992)(see the Materials and method chapters, section 2.2.6c). The data points represent the mean± S.E.M from three independent experiments (n=3, P-value * < 0.05, **< 0.01, *** < 0.001, **** < 0.0001). All the values are as compared to the control(HRTG2 column).

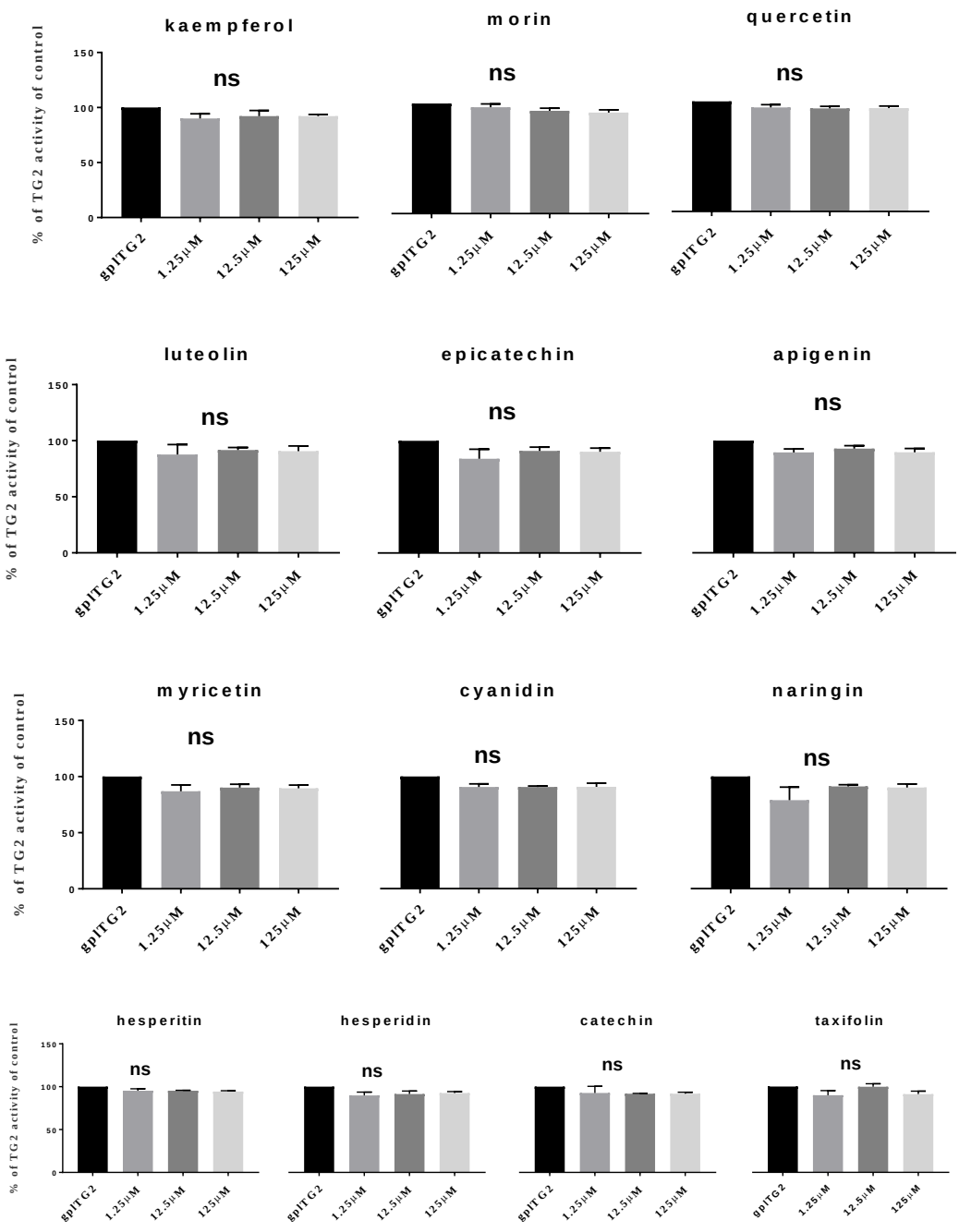


Figure 3-9: The effect of flavonoids /DMSO on the amine incorporation activity of gplTG2:

Flavonoids standards (1.25, 12.5 and 125 µM) dissolved in DMSO then incubated 10µgml^{-1} gpl TG2 after which the TG2 activity was measured according to the method of Slaughter et al (1992).)(see the Materials and method chapters, section 2.2.6c).The data points represent the mean± S.E.M from three independent experiments (n=3, P-value * < 0.05, **< 0.01, *** < 0.001, **** < 0.0001). All the values are as compared to the control (gplTG2 column).

The three flavonoids (kaempferol, morin, and quercetin), which have a significant inhibitory effect on hrTG2, were selected for further investigation (Figure 3-8). These three flavonoids were used to study the effect of mixing flavonoids on the TG2 activity and for kinetic analysis of the inhibition of TG2.

i. Effect of mixing flavonoids/DMSO on amine incorporation activity of TG2:

Flavonoids are present in foods not as a single component but as a mixture of many flavonoids at different concentrations. Therefore, it was crucial to study the effect of combining flavonoids to look for synergism or antagonism in their effects on gplTG2 and hrTG2. The combination effect between flavonoids on their inhibitory action was done by mixing kaempferol, morin, and quercetin in two different concentration 12.5 and 125 µM in DMSO. Then, subject to the TG2 amine incorporation assay (Figure 3-10).

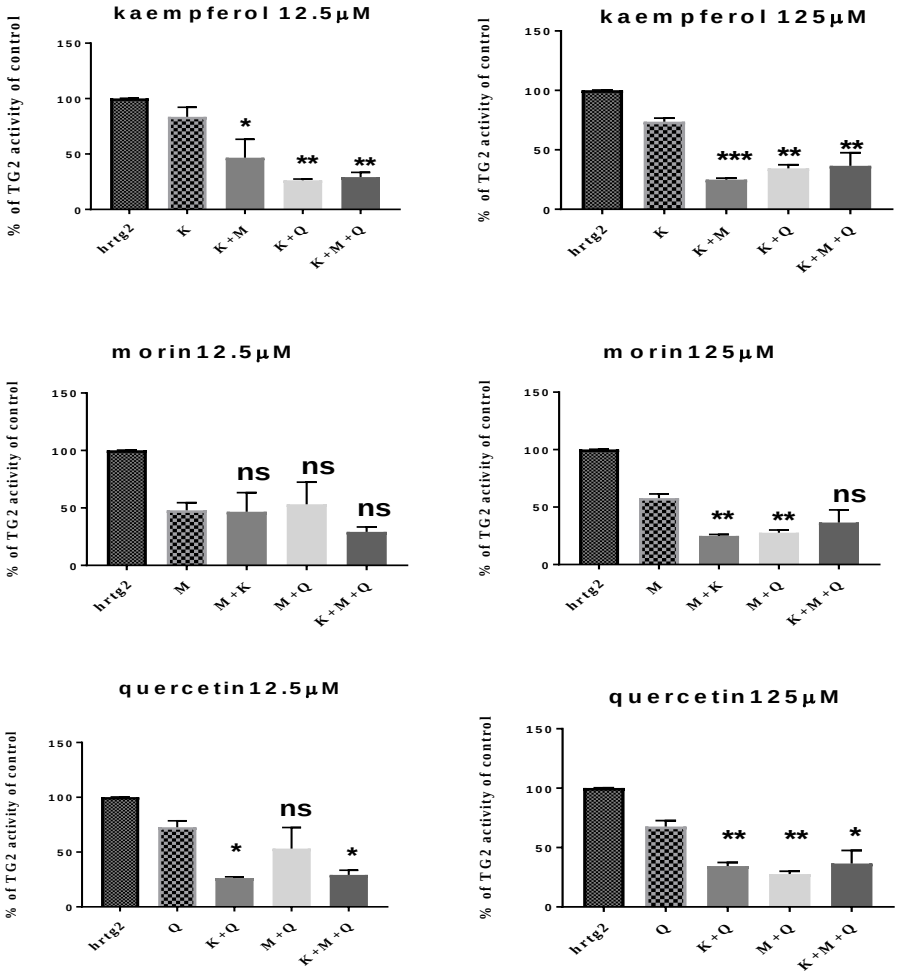


Figure 3-10: Effect of mixing of flavonoids/DMSO on hrTG2 amine incorporation activity

Flavonoids are mixed in different mixtures (12.5 and 125 µM) dissolved in DMSO then incubated with 5µgml^{-1} hrTG2. After which the TG2 activity of each mixture was measured according to the method of Slaughter et al (1992). (see the Materials and method chapters, section 2.2.6c). The data points represent the mean (± S.E.M) from three independent experiments. (n=3, P-value * < 0.05, **< 0.01, *** < 0.001, **** < 0.0001). All the values of the mixtures were compared to the flavonoids as an individual.

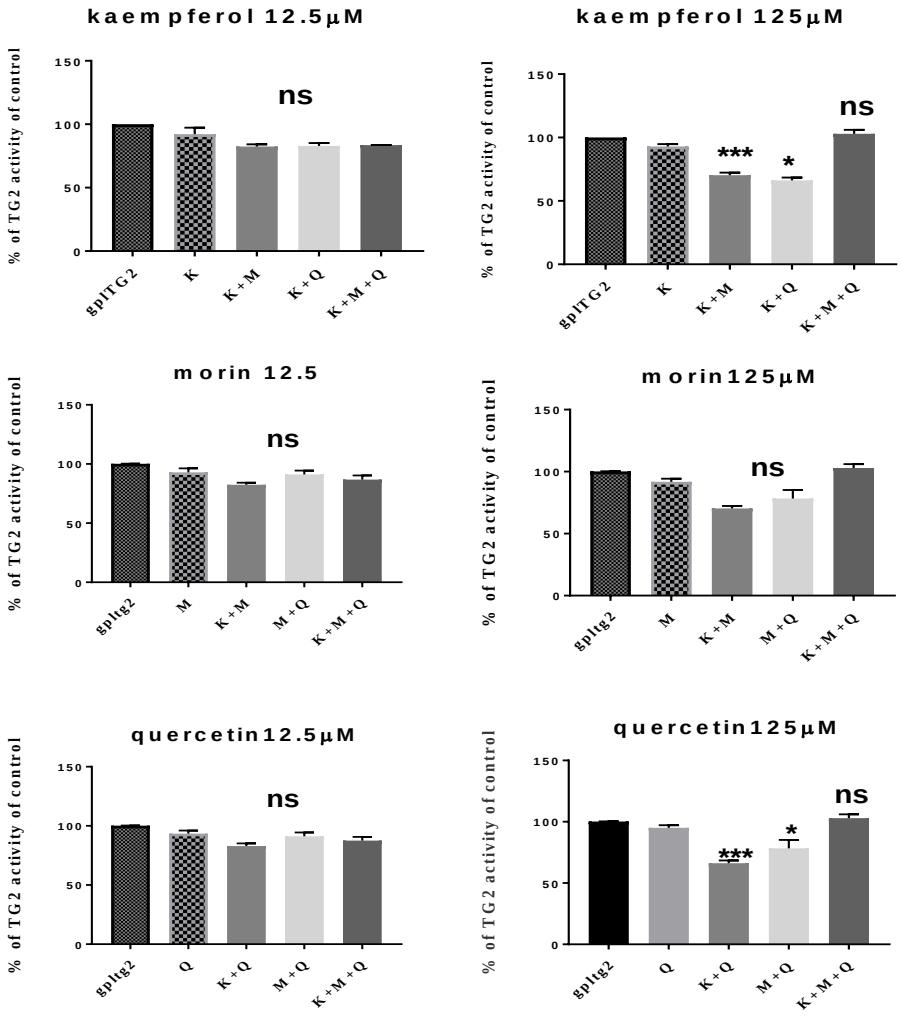


Figure 3-11: Effect of mixing of flavonoids/DMSO on gpl TG2 amine incorporation activity

Flavonoids are mixed in different mixtures (12.5 and 125 µM) dissolved in DMSO then incubated with 10µgml^{-1} gplTG2, after which the TG2 activity of each mixture was measured according to the method of Slaughter et al (1992). (see the Materials and method chapters, section 2.2.6c).The data points represent the mean (± S.E.M) from three independent experiments. (n=3, P-value * < 0.05, **< 0.01, *** < 0.001, **** < 0.0001). All the values of the mixtures were compared to the flavonoids as individual.

The combination of flavonoids exert inhibition behavior against hrTG2 activity higher than individually, the exception was the effect of morin on gplTG2, which the data suggest that statistically no significant effect between their individual and combination effect. There is a necessity to examine this inhibition mechanism, so the kinetics of the inhibition of TG2 by flavonoids were studied (See next section).

ii. Kinetics study on the significant flavonoids:

In order, to understand the mechanism of the inhibition of TG2 by the three flavonoids dissolved in DMSO, the Michaelis-Menten constant (Km) was derived using Graph pad prism 7.

Table 3-1: The kinetics study of flavonoids in DMSO on amine the amine incorporation activity of hr TG2

	Km (mM)	Vmax Rate of reaction (Ab min^{-1})
hrTG2	0.050 (±0.004)	0.030
kaempferol	0.250 (±0.001	0.013
Morin	0.001 (±0.0002)	0.020
Quercetin	0.120 (±0.002)	0.020

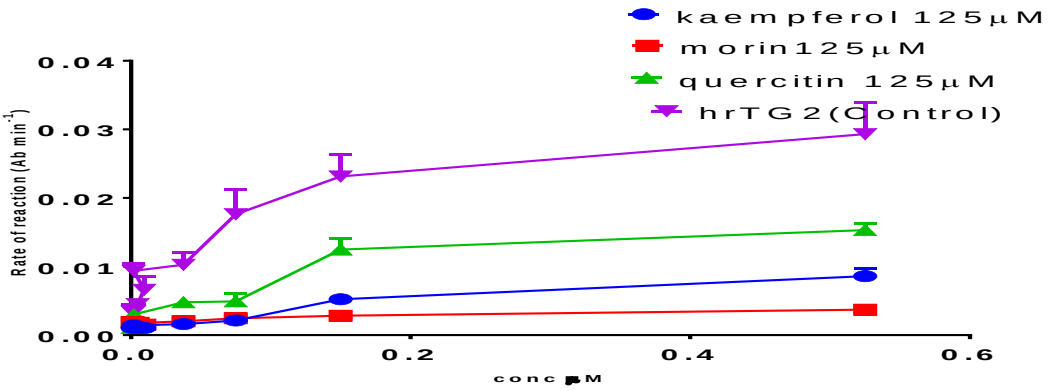


Figure 3-12: The study of kinetics for significant flavonoids /DMSO on amine incorporation activity of hrTG2

Kaempferol, morin, and quercetin (125 µM) dissolved in DMSO then incubated with 5µgml^{-1}hrTG2, in different concentrations of substrate (biotin cadaverine), after which the TG2 activity was measured according to the method of Slaughter et al (1992) (see the Materials and method chapters, section 2.2.6c). Then the rate of reaction was calculated. The data points represent the mean± S.E.M from three independent experiments.

In order, to understand the mechanism of the inhibition of TG2 by the three flavonoids dissolved in DMSO, the Michaelis-Menten constant (Km) was derived using Graph pad prism 7.

Table 3-1Table3-1 and Figure3-12 show that the Km value of the substrate with hrTG2 in presence of morin decreased. The other flavonoids (kaempferol and quercetin) increased the Km value with the substrate and hrTG2.

b) Flavonoids in Sodium deoxycholate

As the aim of this part of the research was to study the effect of flavonoids on the activity of TG2 as a potential treatment for coeliac disease (CD). Sodium deoxycholate

0.5 %(w/v) was used as a means to disperse thirteen flavonoids, and they were subjected to an initial screen using the biotin cadaverine assay to measure the transamidating activity of TG2 (Slaughter et al, 1992). Previous work at NTU has shown that TG2 is unaffected by sodium deoxycholate and this detergent is similar in structure to the detergents released by the bile duct in the digestion of food (Negretti et al., 2017), which makes this more relevant to CD.

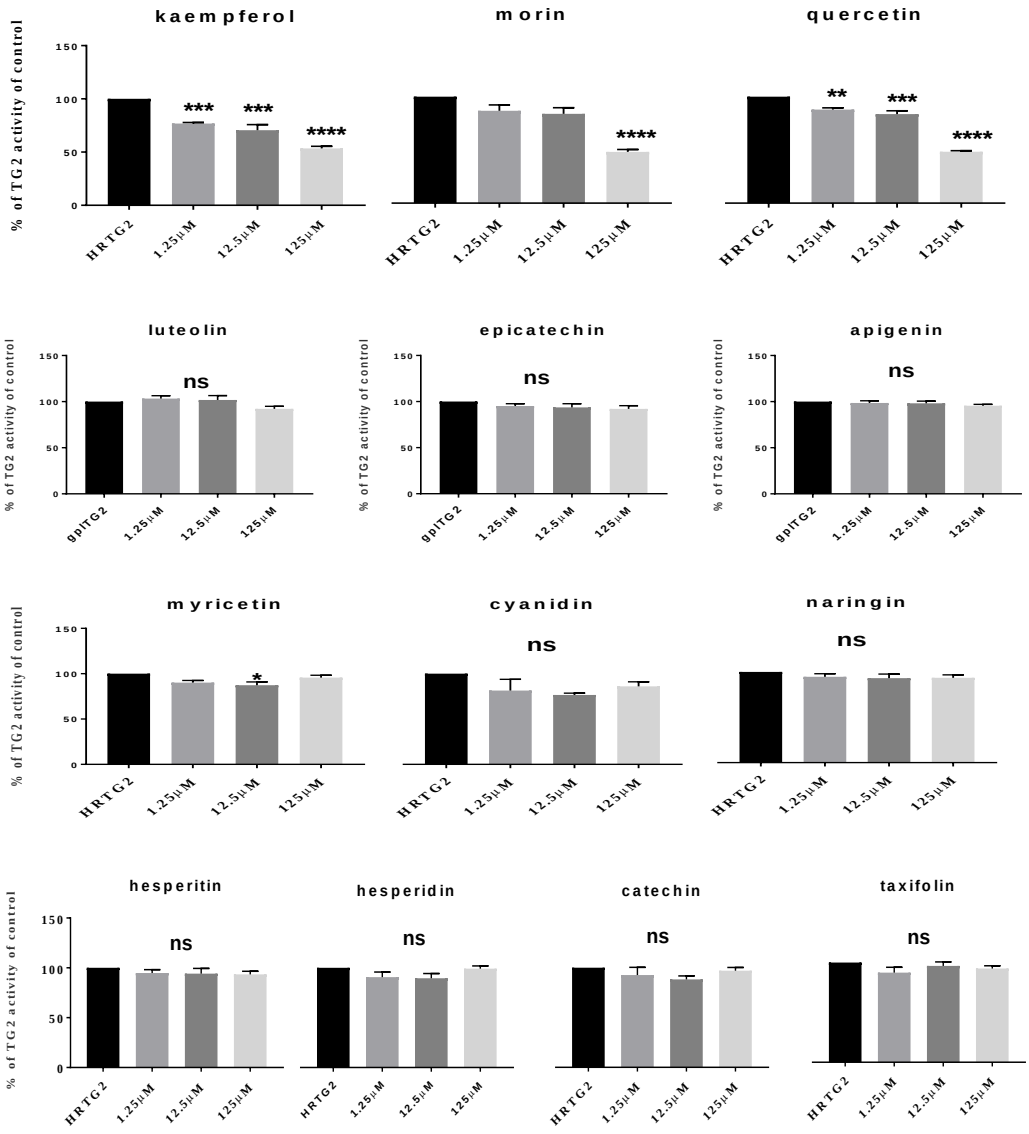


Figure3-13: The effect of flavonoids dissolved in sodium deoxycholate on the amine incorporation activity of hrTG2.

Pure flavonoids (1.25, 12.5 and 125 µM) dissolved in sodium deoxycholate 0.5 %(w/v) then incubated with 5µg ml^{1}hrTG2, The TG2 activity was measured according to the method of Slaughter et al (1992) described in methods and materials chapter. (see the Materials and method chapters, section 2.2.6c)The data points represent the mean± S.E.M from three independent experiments (n=3, P-value * < 0.05, **< 0.01, *** < 0.001, **** < 0.0001). All the values are as compared to the control untreated TG2.

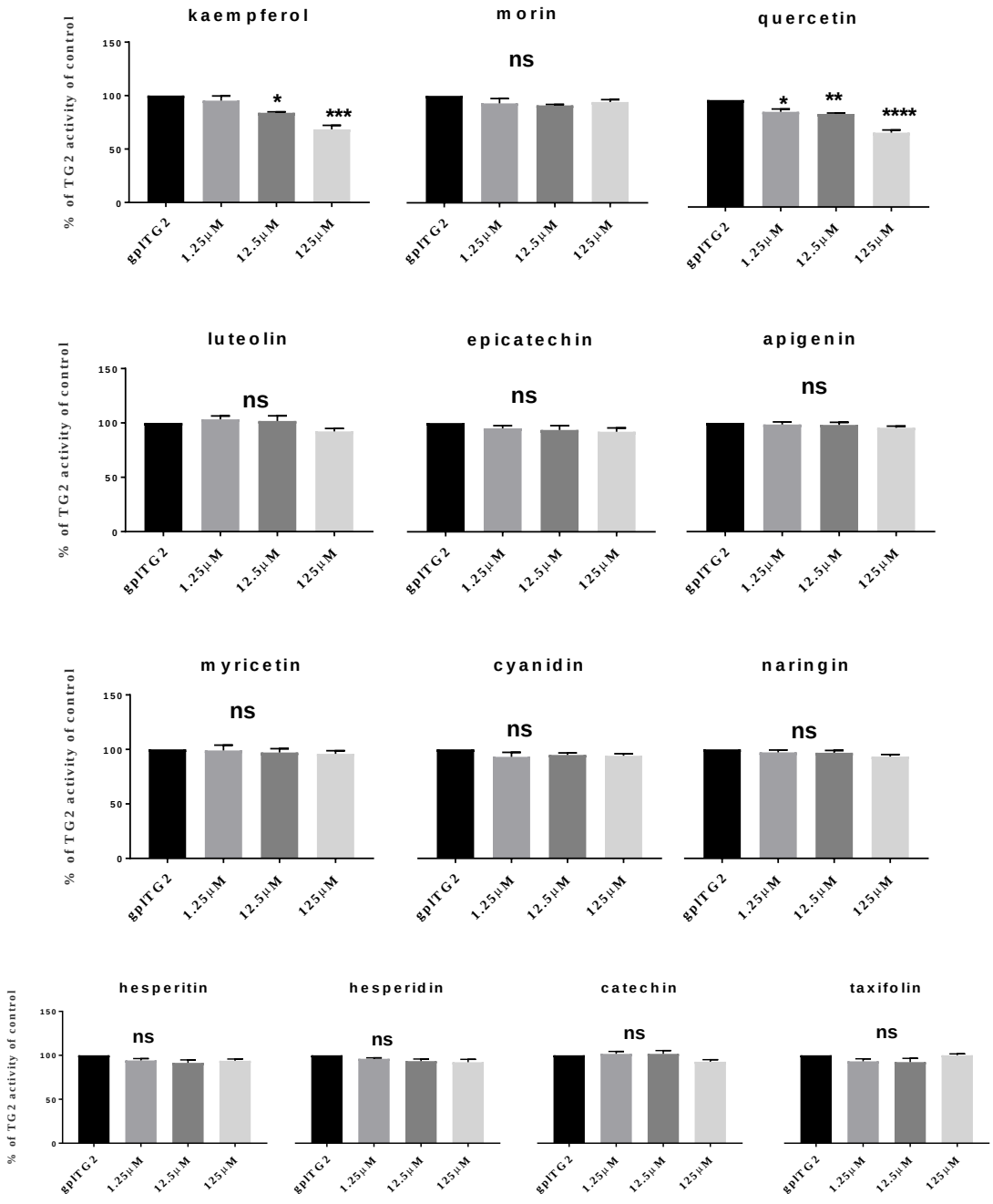


Figure 3-14: The effect of flavonoids /sodium deoxycholate on gpl TG2 amine incorporation activity.

Pure flavonoids (1.25, 12.5 and 125 µM) dissolved in sodium deoxycholate 0.5 %(w/v) then incubated with 10µg ml^{-1} gpl TG2. The TG2 activity was measured according to the method of Slaughter et al (1992) described in methods and materials chapter. (see the Materials and method chapters, section 2.2.6c). The data points represent the mean± S.E.M from three independent experiments (n=3, P-value * < 0.05, **< 0.01, *** < 0.001, **** < 0.0001). All the values are as compared to the control untreated TG2.

Three flavonoids (kaempferol, morin, and quercetin) at 125 µM showed significant inhibition for the hr TG2 amine incorporation activity (P>0.0001), while, only quercetin

and kaempferol show significant inhibitory (kaempferol p>0.001; quercetin >0.0001) effect against gpl TG2.

i. Effect of mixing flavonoids/deoxycholate on the amine incorporation activity of TG2:

In order to study the combination effects, kaempferol, morin, and quercetin were mixed at two different concentration 12.5 and 125 µM in 0.5 %(w/v) sodium deoxycholate then subjected to the biotin amine incorporation TG2 assay (See Figure 3-15 and 3-16).

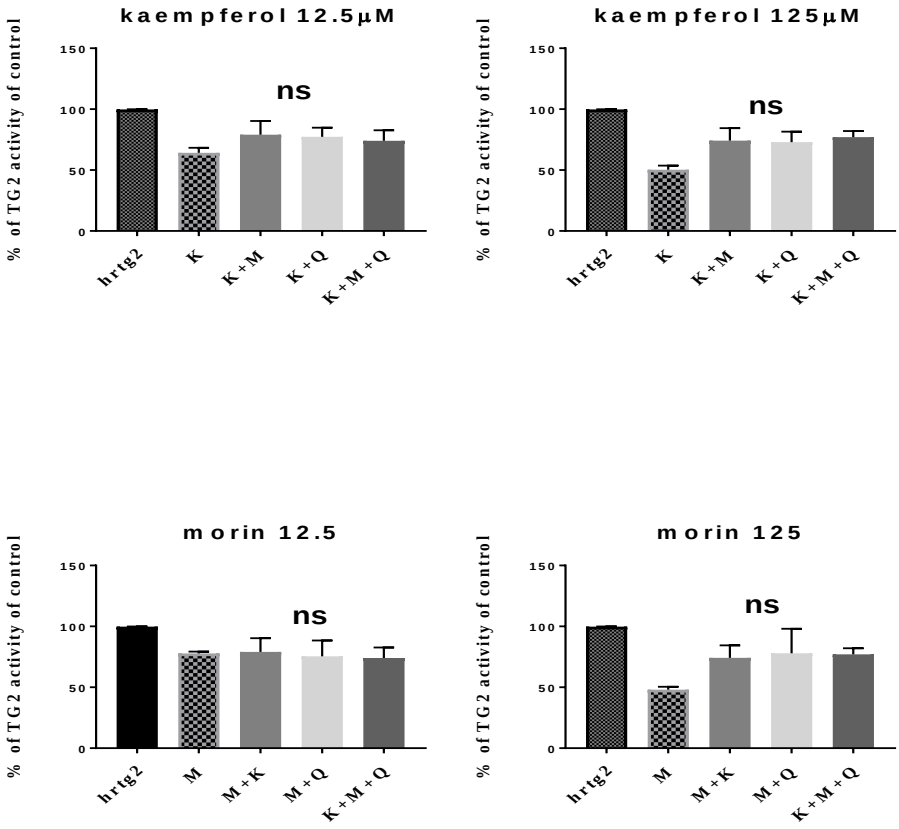


Figure 3-15: The effect of flavonoids mixture/deoxycholate sodium on amine incorporation activity of hrTG2.

Flavonoids mixtures (12.5 and 125 µM) dissolved in 0.5% (w/v) sodium deoxycholate then incubated with 5µgml^{-1} hrTG2 10µgml^{-1}, after which the TG2 activity measured according to the method of Slaughter et al (1992). (see the Materials and method chapters, section 2.2.6c)The data points represent the mean± S.E.M from three independent experiments (n=3, P-value * < 0.05, **< 0.01, *** < 0.001, **** < 0.0001). All the values of the mixtures were compared to the flavonoids as an individual.

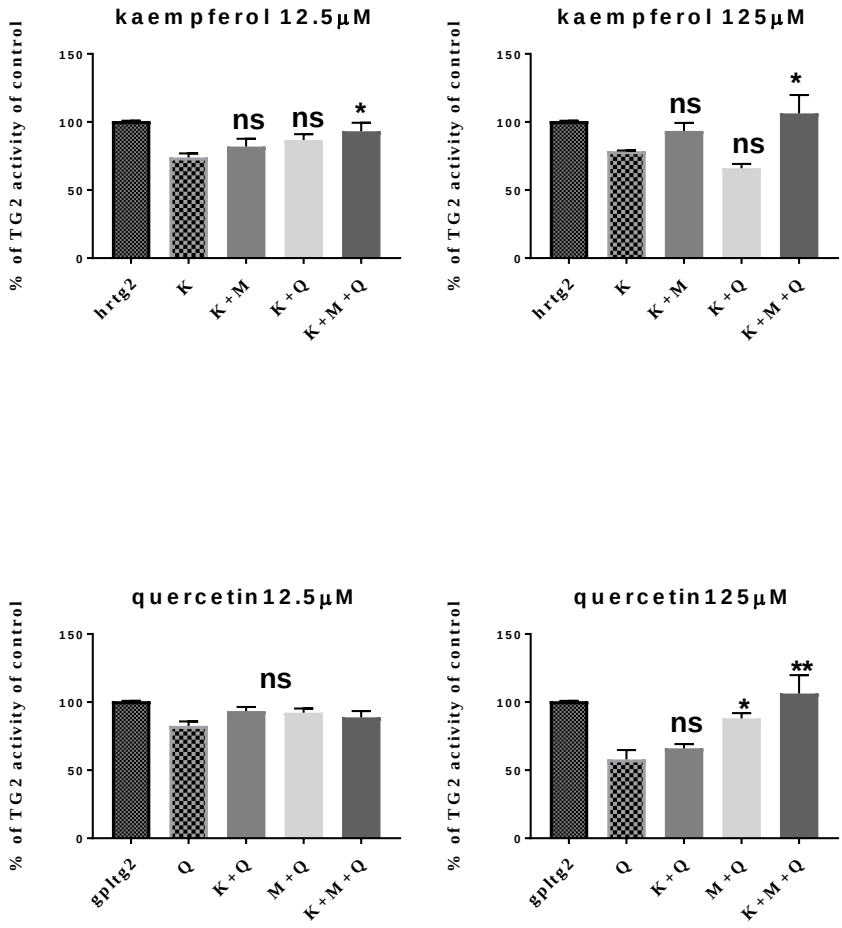


Figure 3-16: The effect of flavonoids mixture/deoxycholate sodium on amine incorporation activity of gplTG2.

Flavonoids mixtures (12.5 and 125 µM) dissolved in 0.5% (w/v) sodium deoxycholate then incubated with 10µgml^{-1} gplTG2, after which the TG2 activity measured according to the method of Slaughter et al (1992). (see the Materials and method chapters, section 2.2.6c). The data points represent the mean± S.E.M from three independent experiments (n=3, P-value * < 0.05, **< 0.01, *** < 0.001, **** < 0.0001). All the values of the mixtures were compared to the flavonoids as an individual.

The data in Figure 3-15 and 3-16 display the effect of mixing morin; quercetin and kaempferol on the inhibition of TG2.The finding suggests that the individual inhibitory effect of the flavonoids was overcome after mixing.

ii. Kinetic analysis for the three significant flavonoids:

The kaempferol, morin, and quercetin, which have a significant inhibitory effect on the amine incorporation activity of TG2 (See Figure 3-14), were used to study the mechanism of their inhibitory action. Kinetic plots for the three flavonoids were determined using the biotin amine incorporation assay with analysis by Graphpad Prism 7 software (See Table 3-2 and Figure 3-17).

Table 3-2: The study of kinetics for significant flavonoids /Deoxycholate sodium on amine incorporation activity of TG2.

	Km (mM)	Vmax rate of interaction (Ab min^{-1})		Km (mM)	Vmax rate of interaction(Ab min^{-1})
hrTG2	0.03 (±0.003)	0.041	gplTG2	0.19 (±0.001)	0.027
Kaempferol	0.11 (±0.003)	0.040	Kaempferol	0.008 (±0.003)	0.006
Morin	0.01 (±0.002)	0.021	Quercetin	0.002 (±0.002)	0.005
Quercetin	0.13 (±0.001)	0.020			

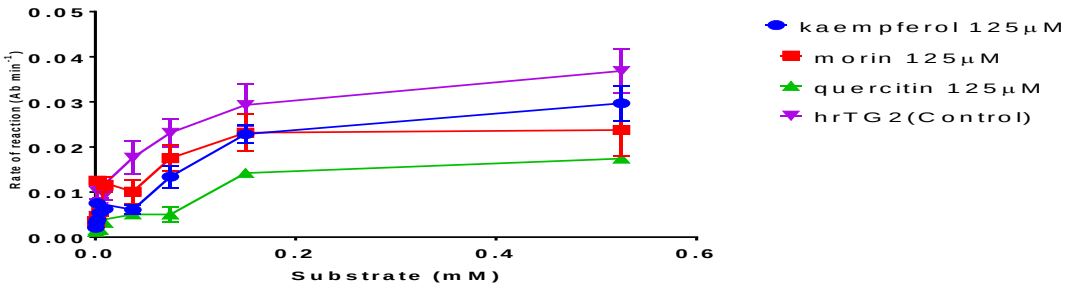


Figure 3-17: The study of kinetics for significant flavonoids /Deoxycholate sodium on amine incorporation activity of hrTG2.

kaempferol, morin, and quercetin (125 µM) dissolved in sodium deoxycholate 0.5%(w/v) then incubated with 5µgml^{-1} hrTG2, in different concentrations of substrate (biotin cadaverine), after which the TG2 activity was measured according to the method of Slaughter et al (1992). (see the Materials and method chapters, section 2.2.6c). Then the rate of reaction was calculated using graph pad prism 7. The data points represent the mean± S.E.M from three independent experiment

The findings display that the action of kaempferol and quercetin against hrTG2 increased the Km of substrate with hrTG2. Morin displayed a reduction in the Km of the substrate with the hrTG2. Whereas, kaempferol and quercetin reduced the Km value of the substrate with gplTG2 enzyme.

3.3.2 Effect of flavonoids to the TG2 peptide cross-linking assay

In order to observe the effect of the flavonoids on the protein/peptide cross-linking the activity of TG2, the flavonoids standards were dissolved in sodium deoxycholate 0.5% (w/v) and DMSO then applied to the TG2 activity assay using gpl and hrTG2.

a) Flavonoids in DMSO

Thirteen flavonoids were subjected to an initial screen using the cross –linking activity assay (Trigwell et al., 2004) (Figure 3-18and Figure 3-18). All flavonoids show potent inhibition action against the cross-linking activity of hrTG2 and gplTG2.

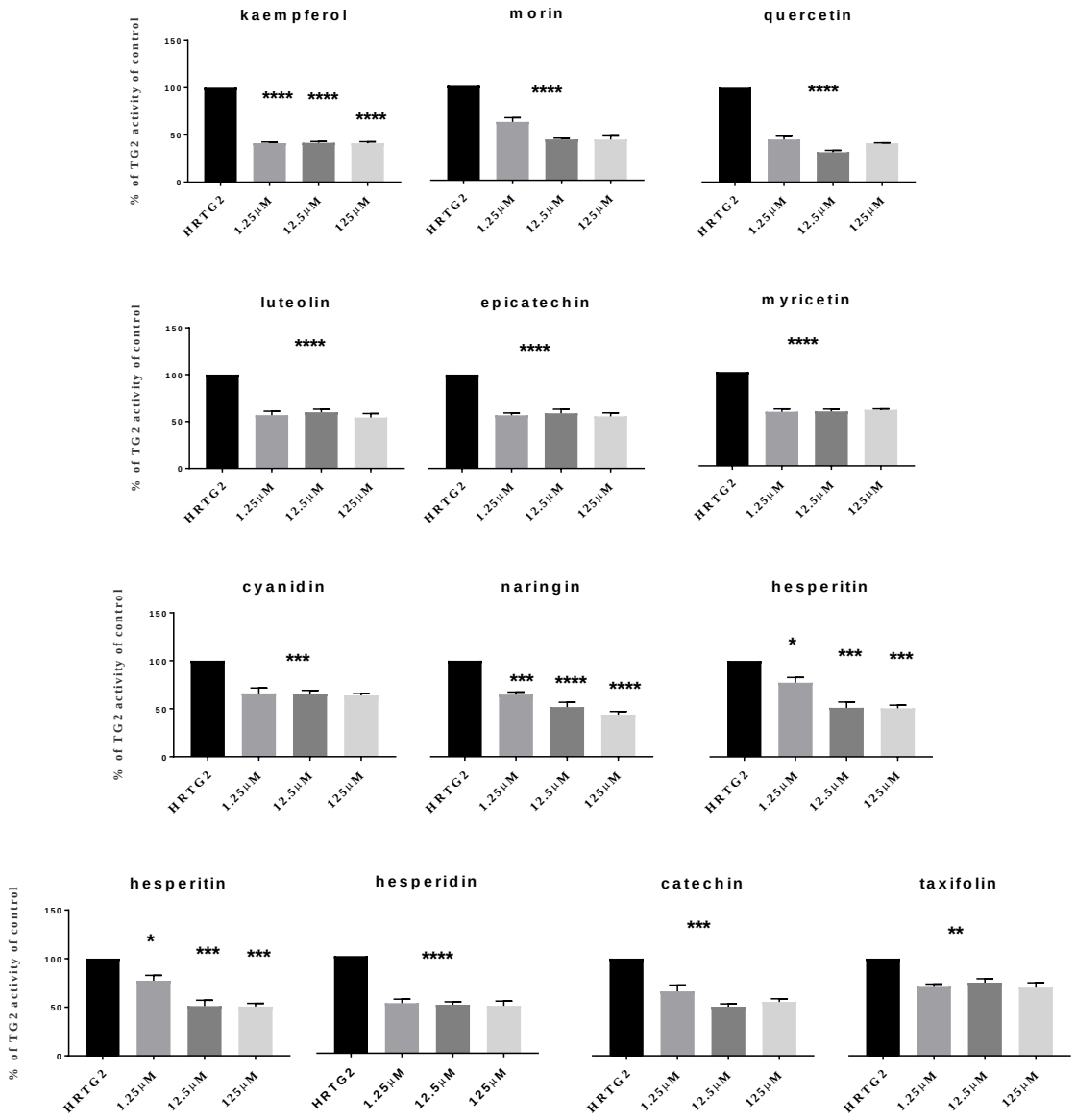


Figure 3-18: The effect of flavonoids /DMSO on the cross-linking activity of hrTG2.

Flavonoids standards (1.25, 12.5 and 125 µM) dissolved in DMSO then incubated with 5µgml^{-1} hrTG2. and subject to the screen their effect on cross-linking activity according to the method of Trigwell et al (2004). (see the Materials and method chapters, section 2.2.6c)The data points represent the mean± S.E.M from three independent experiments (n=3, P-value * < 0.05, **< 0.01, *** < 0.001, **** < 0.0001). All the values are as compared to the control.

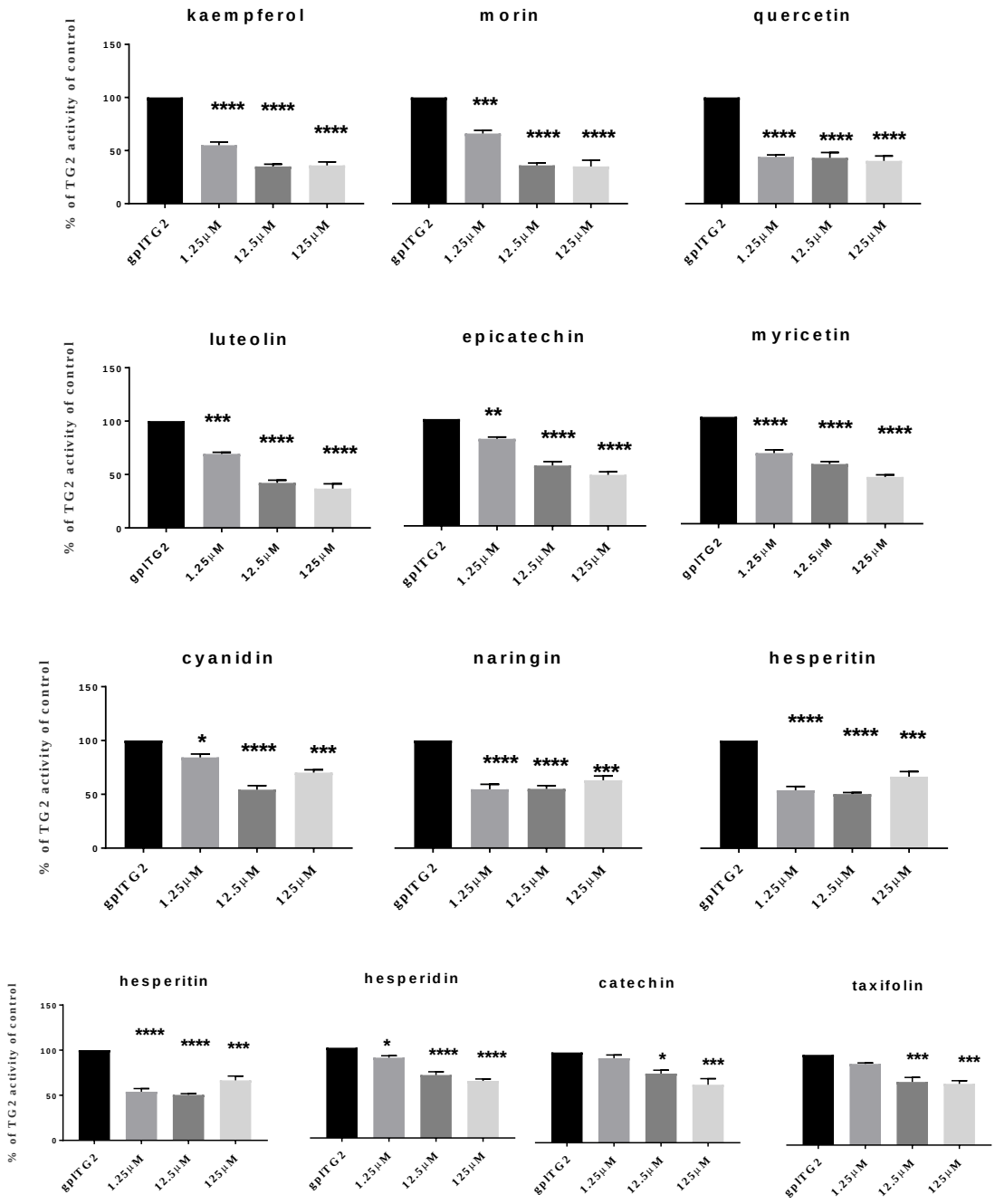


Figure 3-19: The effect of flavonoids /DMSO on the cross-linking activity of gplTG2.

Flavonoids standards (1.25, 12.5 and 125 µM) dissolved in DMSO then incubated with 10µgml^{-1} gpl TG2 and subjected to the screen their effect on cross-linking activity according to the method of Trigwell et al (2004). (see the Materials and method chapters, section 2.2.6c)The data points represent the mean± S.E.M from three independent experiments (n=3, P-value * < 0.05, **< 0.01, *** < 0.001, **** < 0.0001). All the values are as compared to the control.

The data in Figures 3.18 and 3.19 display the significant inhibition from most flavonoids used, this effect was more potent when using hrTG2 than gplTG2, and related to the concentration used.

i. Effect of mixing flavonoids on the cross-linking activity of TG2:

To be in line with the amine incorporation study, the combination effect between flavonoids on their inhibitory action was done by mixing kaempferol, morin, and quercetin in two different concentration 12.5 and 125 µM in DMSO. Then, subject to the TG2 cross-linking assay.

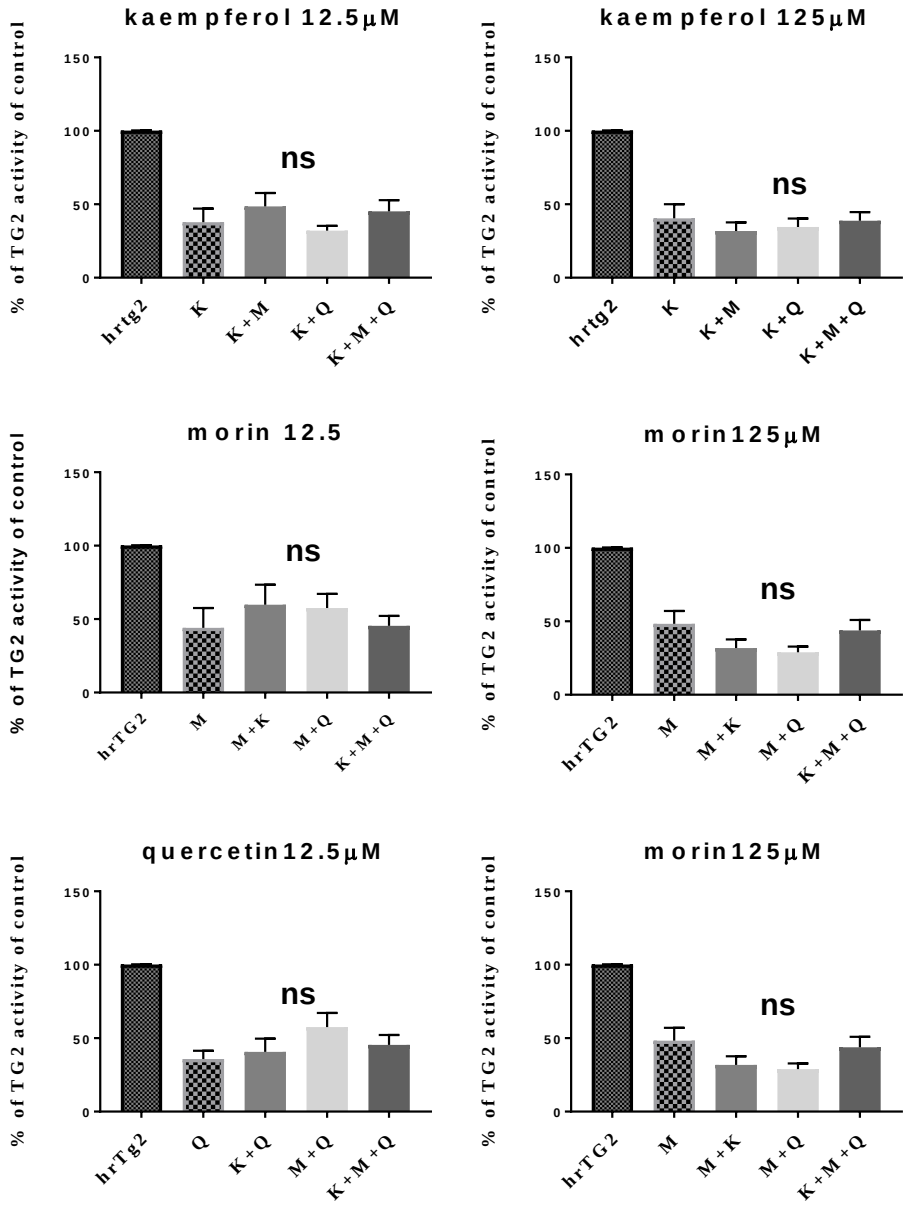


Figure 3-20: Effect of mixing of flavonoids/DMSO on hrTG2 cross-linking activity.

Flavonoids mixtures (12.5 and 125 µM) dissolved in DMSO then incubated with 5µgml^{-1}hrTG2, after which the TG2 activity was measured according to the method of Trigwell et al (2004). (see the Materials and method chapters, section 2.2.6c)The data points represent the mean± S.E.M from three independent experiments (n=3, P-value**** < 0.0001, ***< 0.001, **< 0.01, *< 0.05). All the values of the mixtures were compared to the flavonoids as an individual.

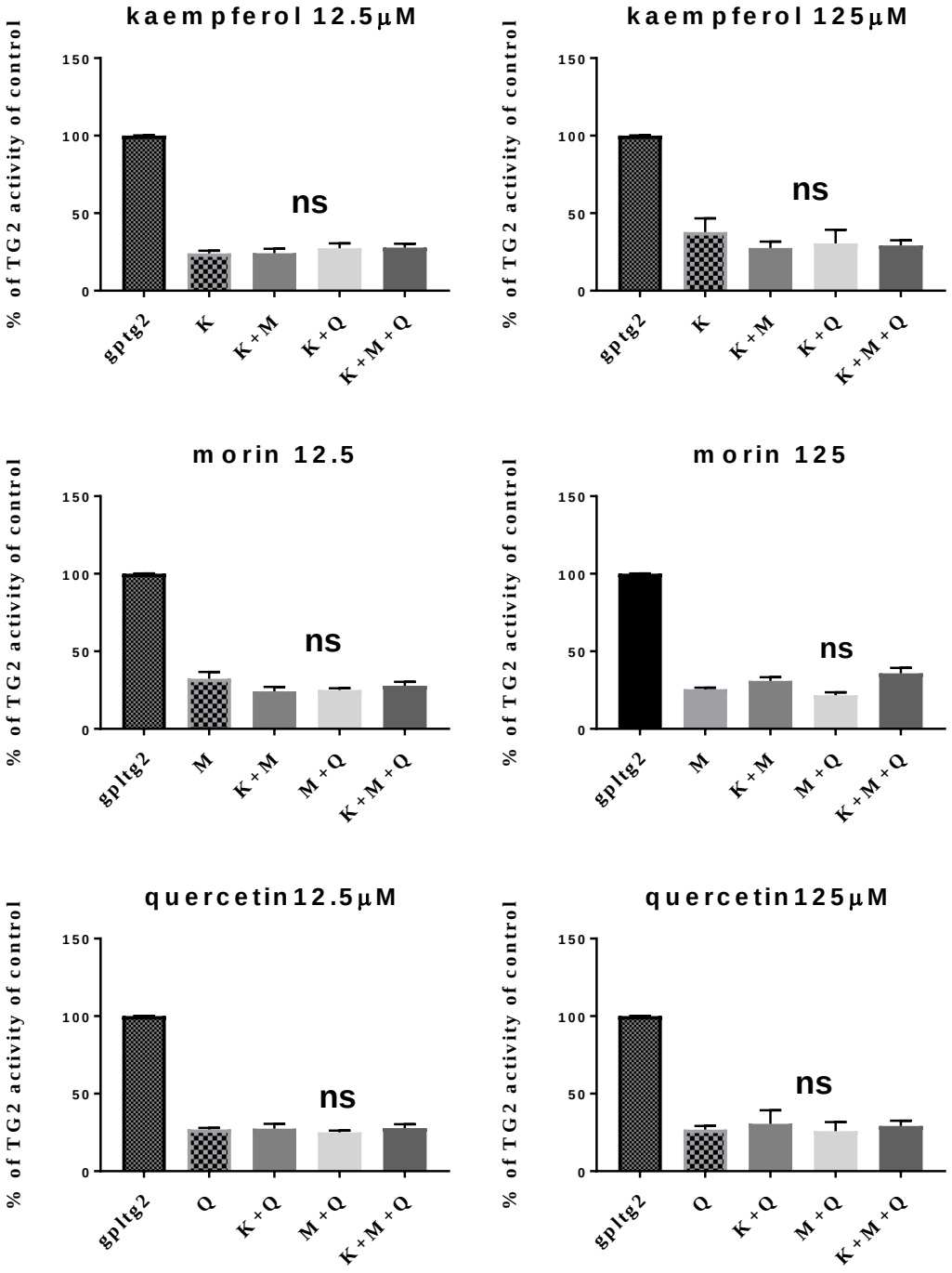


Figure 3-21: Effect of mixing of flavonoids/DMSO on gpTG2 cross-linking activity.

Flavonoids mixtures (12.5 and 125 µM) dissolved in DMSO then incubated with 10µgml^{-1}gplTG2 after which the TG2 activity was measured according to the method of Trigwell et al (2004). The data suggest that there is no effect of combination the flavonoids on their individual effect. The data points represent the mean± S.E.M from three independent experiments (n=3, P-value**** < 0.0001, ***< 0.001, **< 0.01, *< 0.05). All the values of the mixtures were compared to the flavonoids as an individual

The finding shows no change in their cross-linking inhibition action when the flavonoids are mixed and compared to the flavonoids as an individual (Figure 3-20 and 3-21).

ii. Kinetics study for the selected flavonoids in DMSO:

In order to study the mechanism of action of significant flavonoids in DMSO the Michaelis-Menten constant (Km) was analysed using graph pad prism 7.

Table 3-3: The study of kinetics for selected flavonoids in DMSO on the cross-linking activity of TG2.

	Km (mM)	Vmax rate of interaction (Ab min-1)		Km(mM)	Vmax rate of interaction (Ab min-1)
hrTG2	2.41 (±0.003)	0.11	gplTG2	0.244 (±0.001)	0.013
Kaempferol	1.41 (±0.001)	0.02	Kaempferol	0.051(±0.002)	0.006
Morin	1.14 (0.001)	0.02	Morin	0.045 (±0.001)	0.005
Quercetin	1.818 (±0.002)	0.05	Quercetin	0.454 (±0.003)	0.007

Table 3.3: Kaempferol, morin, and quercetin (125 µM) dissolved in DMSO, then incubated 5µgml^{-1} hrTG2 and 10µgml^{-1} gpl TG2, in different concentrations of substrate (TVQQEL) after which the TG2 activity was measured according to the method of Trigwell et al (2004).Then the rate of reaction was calculated using graph pad prism 7. All the values are as compared to the control.

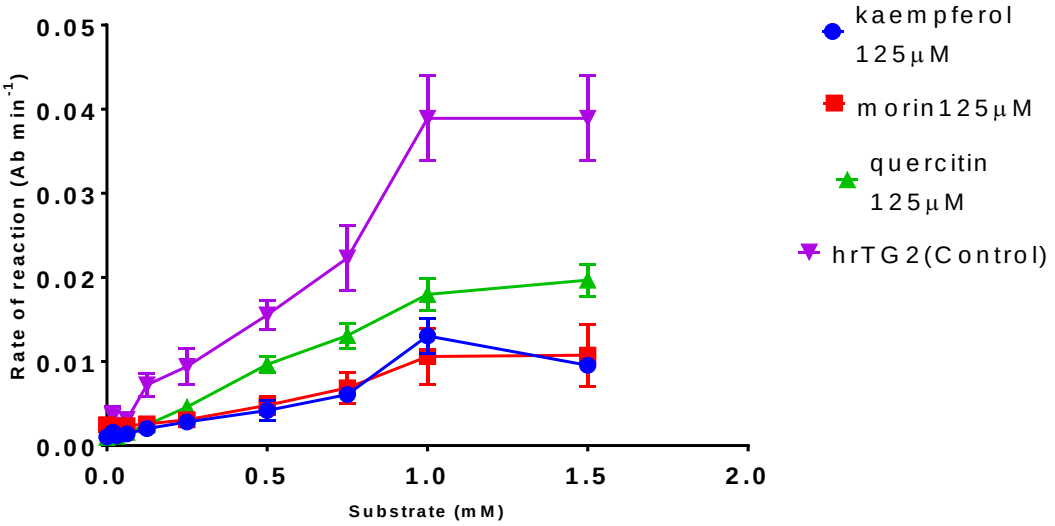


Figure 3-22: Kinetics study for the selected flavonoids in DMSO

Kaempferol, morin, and quercetin (125 µM) dissolved in DMSO then incubated with 5µgml^{-1}hrTG2, in different concentrations of substrate (TVQQEL), after which the TG2 activity measured according to the method of Trigwell et al (2004). (see the Materials and method chapters, section 2.2.6c). Then the rate of reaction was calculated using graph pad prism 7.

The findings display that the inhibition effect of all flavonoid reduces the Km value of the substrate with the gplTG2 enzyme, except quercetin, which increased the Km value of substrate with the gplTG2 (Table 3 3 and Figure 3-22).

b) Flavonoids in sodium deoxycholate

Thirteen flavonoids were subjected to an initial screen using the TG2 peptide/protein cross-linking assay (Trigwell et al., 2004).

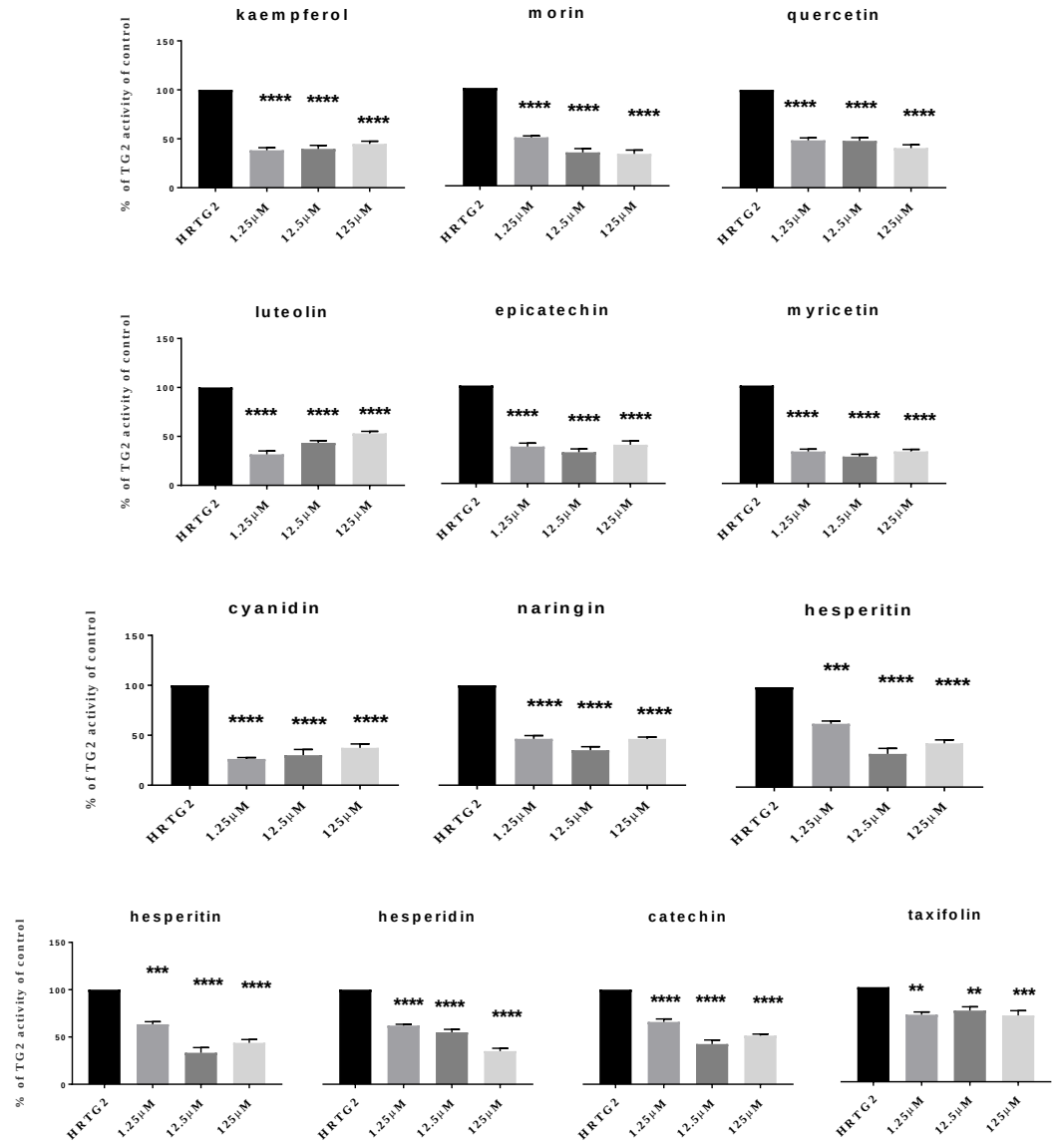


Figure 3-23: The effect of flavonoids/ sodium deoxycholate on the cross -linking activity of hrTG2.

Flavonoids standards (1.25, 12.5 and 125 µM) dissolved in sodium deoxycholate then incubated with 5µgml^{-1} hrTG2 and subjected to the screen their effect on cross-linking activity according to the method of Trigwell et al (2004). (see the Materials and method chapters, section 2.2.6c). The data points represent the mean± S.E.M from three independent experiments (n=3, P-value * < 0.05, **< 0.01, *** < 0.001, **** < 0.0001).

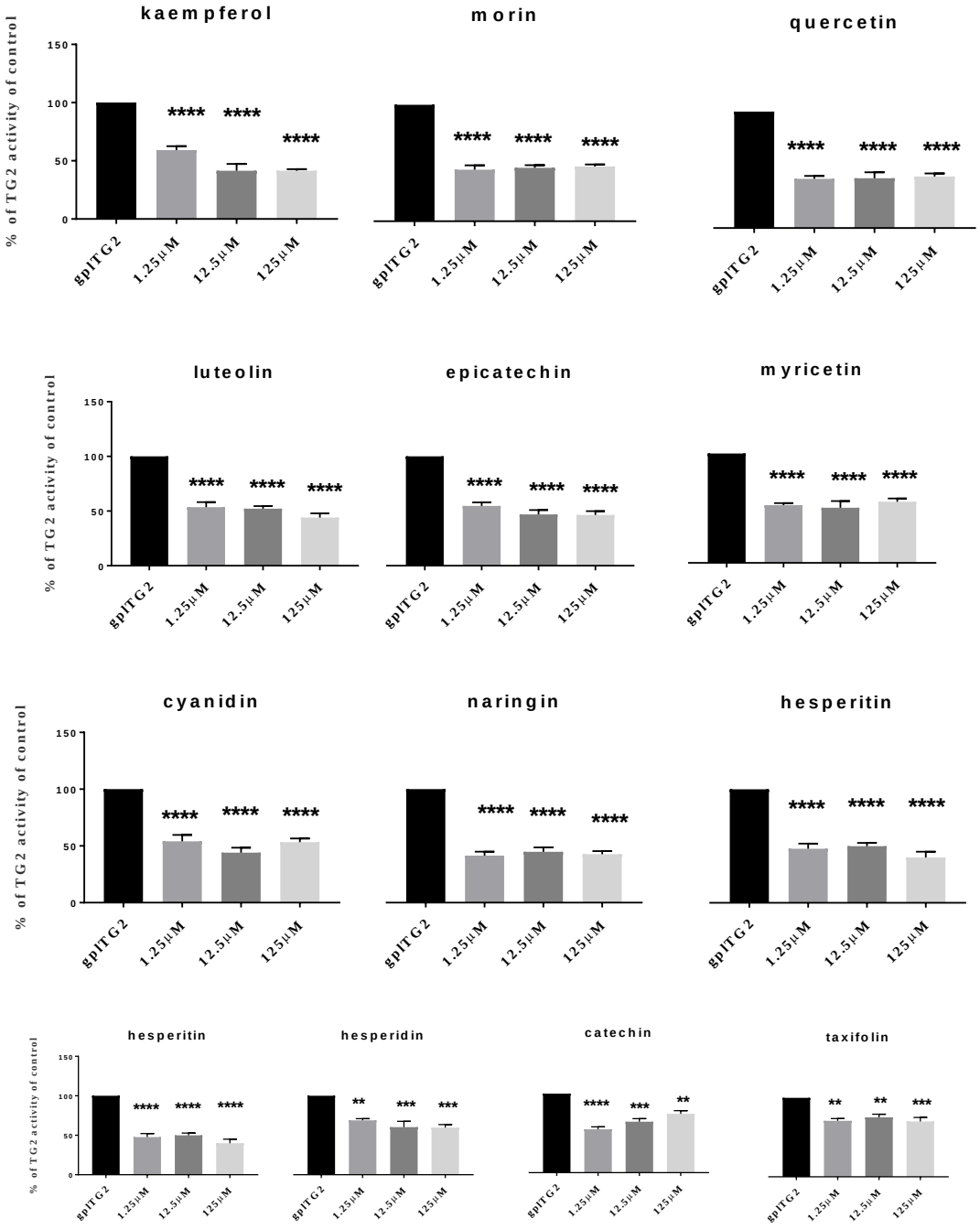


Figure 3-24: The effect of flavonoids/ sodium deoxycholate on the cross -linking activity of gpl TG2.

Flavonoids standards (1.25, 12.5 and 125 µM) dissolved in sodium deoxycholate then incubated with 10µgml^{-1} gpl TG2 and subjected to the screen their effect on cross-linking activity according to the method of Trigwell et al (2004) (see the Materials and method chapters, section 2.2.6c). The data points represent the mean± S.E.M from three independent experiments (n=3, P-value * < 0.05, **< 0.01, *** < 0.001, **** < 0.0001).

Most of the flavonoids have an inhibitory action on both of the forms of TG2 and their activities were more potent on hrTG2 than gplTG2 (Figure3-23 and Figure 3-24).

i. Effect of mixing flavonoids on the cross-linking activity of TG2:

In order to study the effect of mixing flavonoids on the protein/peptide cross-linking TG2 activity, three flavonoids (kaempferol, morin, and quercetin) were mixed in two different concentration 12.5 and 125 µM in sodium deoxycholate 0.5%(w/v), then subjected to cross-linking assay.

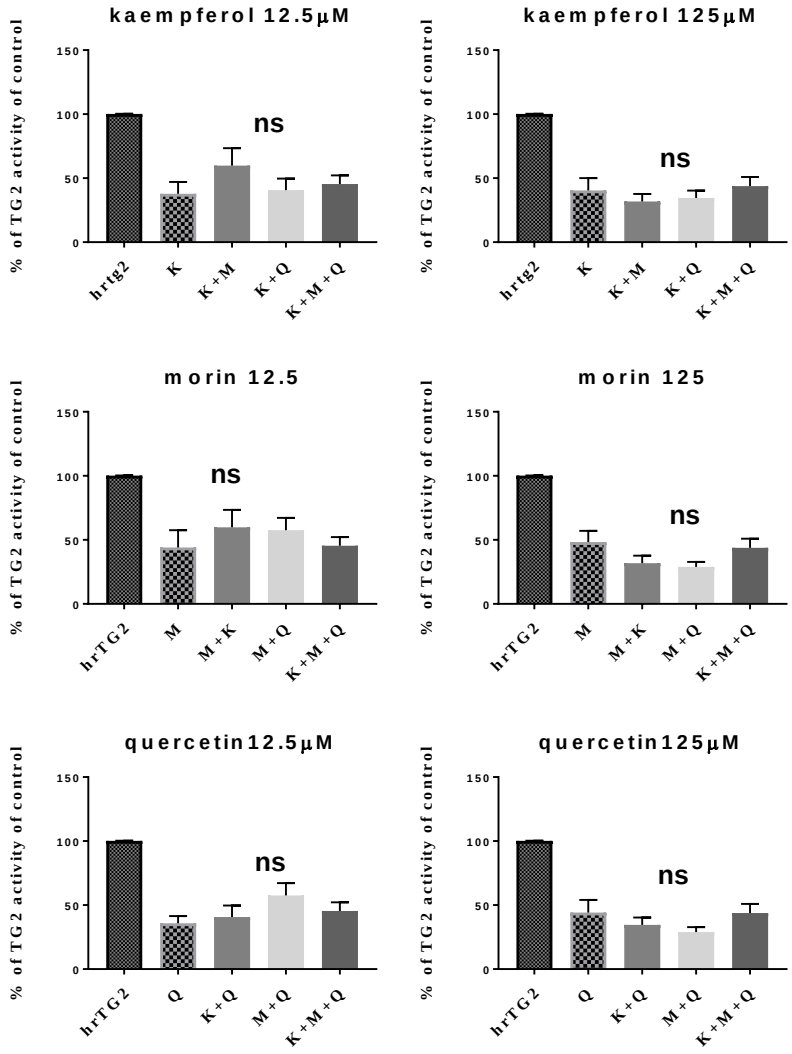


Figure 3-25: Effect of mixing of flavonoids/Deoxycholate on hrTG2 cross-linking activity.

Flavonoids mixtures (12.5 and 125 µM) dissolved in Sodium deoxycholate 0.5 %(w/v) then incubated with 5µgml^{-1}hrTG2, after which the TG2 activity was measured according to the method of Trigwell et al (2004) (see the Materials and method chapters, section 2.2.6c). The data points represent the mean± S.E.M from three independent experiments (n=3, P-value**** < 0.0001, ***< 0.001, **< 0.01, *< 0.05). All the values of the mixtures were compared to the flavonoids as an individual.

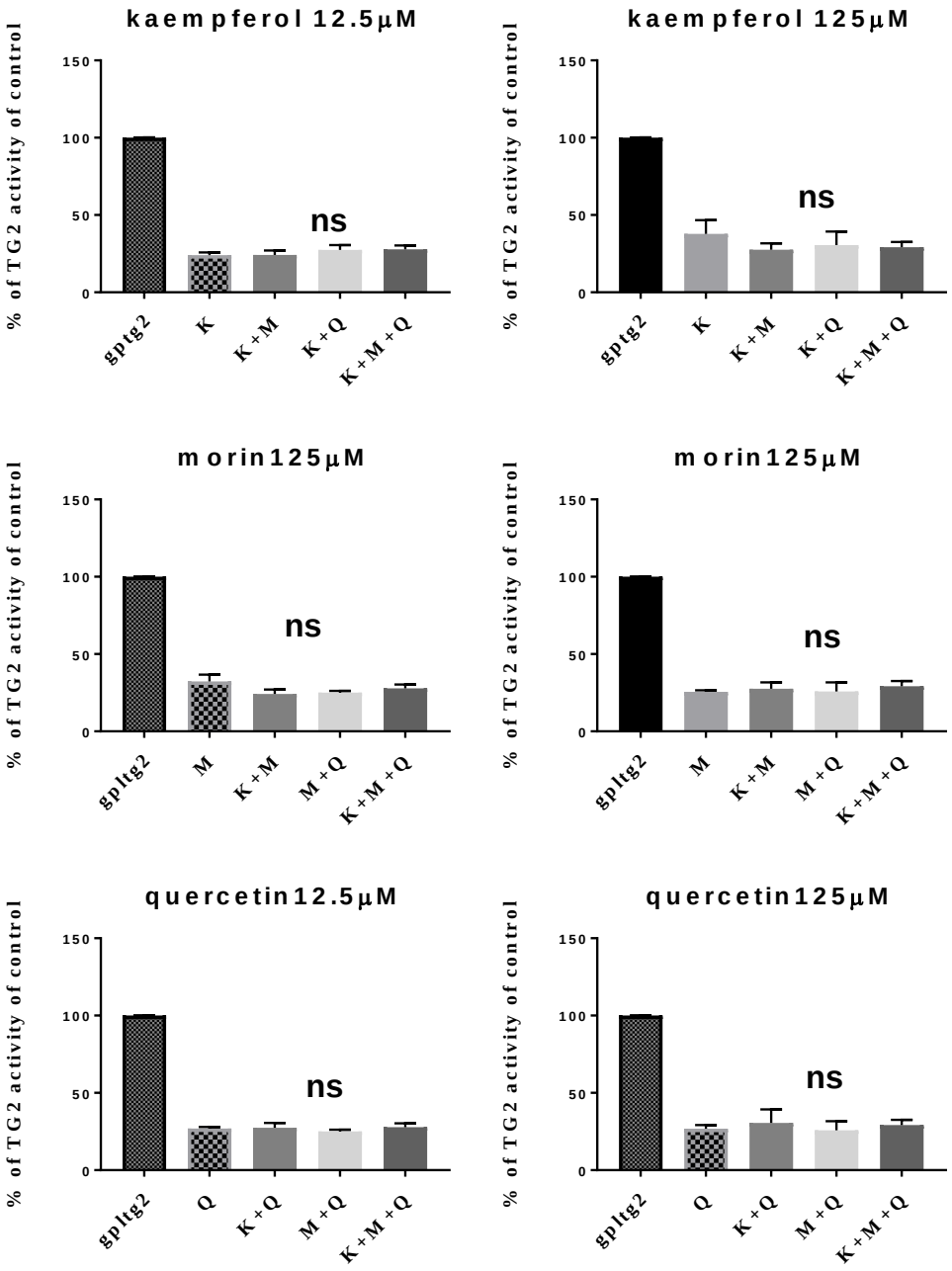


Figure 3-26: Effect of mixing of flavonoids/Deoxycholate on gpl TG2 cross-linking activity.

Flavonoids mixtures (12.5 and 125 µM) dissolved in Sodium deoxycholate 0.5 %(w/v) then incubated with 10µgml-1gplTG2 after which the TG2 activity was measured according to the method of Trigwell et al (2004). (see the Materials and method chapters, section 2.2.6c).The data points represent the mean± S.E.M from three independent experiments (n=3, P-value**** < 0.0001, ***< 0.001, **< 0.01, *< 0.05). All the values of the mixtures were compared to the flavonoids as an individual.

The finding shows no synergistic inhibition effects between three flavonoids on TG2 protein/peptide cross-linking activity (Figure 3-25 and 3-26). Worth mention that most of the combinations are significant compared to the control (hr TG2 or gplTG2) but they may not be significant compared to the single flavonoid.

ii. Kinetics study for the selected flavonoids:

To study the mechanism of action of significant flavonoids in deoxycholate, the Michaelis-Menten constant (Km) measured by using graph pad prism 7. The finding display that all the flavonoids reduce the Km of the substrate with the TG2 enzyme (Table 3-4 and Figure 3-27).

Table 3-4: The study of kinetics for selected flavonoids /deoxycholate on the cross-linking activity of TG2.

	Km (mM)	Vmax Rate of reaction (Ab min-1)
hrTG2	2.41 (±0.002)	0.11
Kaempferol	0.12 (±0.003)	0.04
Morin	0.59 (±0.003)	0.02
Quercetin	0.13 (±0.004)	0.02

Table 3.4: Kaempferol, morin, and quercetin (125 µM) dissolved in Sodium deoxycholate 0.5%(w/v) then incubated with 5µgml^{-1}hrTG2, in different concentrations of substrate (TVQQEL), after which the TG2 activity measured according to the method of Trigwell et al (2004) (see the Materials and method chapters, section 2.2.6c).Then the rate of reaction was calculated using graph pad prism 7.

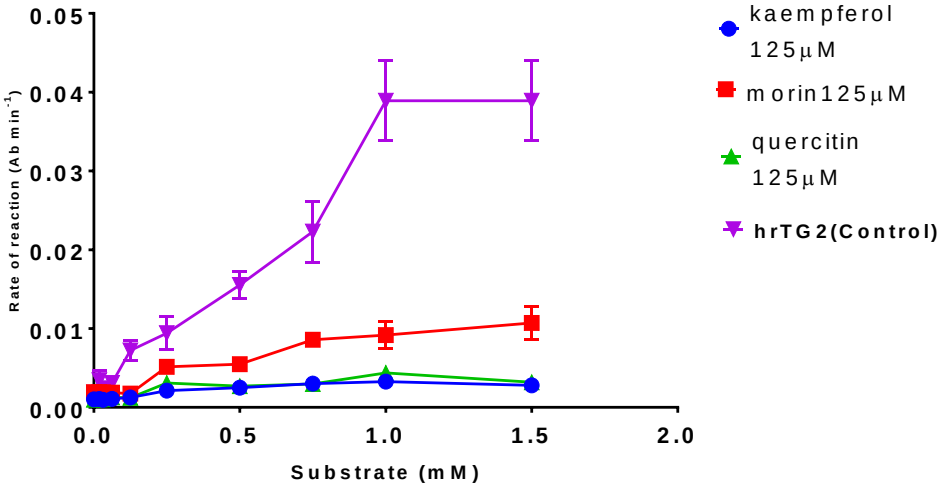


Figure 3-27: Kinetics study for the selected flavonoids in sodium deoxycholate

Kaempferol, morin, and quercetin (125 µM) dissolved in Sodium deoxycholate then incubated with 5µgml-[1]hrTG2, in different concentrations of substrate (TVQQEL), after which the TG2 activity measured according to the method of Trigwell et al (2004) (see the Materials and method chapters, section 2.2.6c). Then the rate of reaction was calculated using graph pad prism 7.

3.3.3 Application of flavonoids to deamidation assay:

As the main aim of this project was related to the identification of natural products which can act as an inhibitor for TG2 in coeliac disease. Pure flavonoids were applied to the optimised deamidation assay (See chapter 6), using a gliadin protein as a substrate for hrTG2.

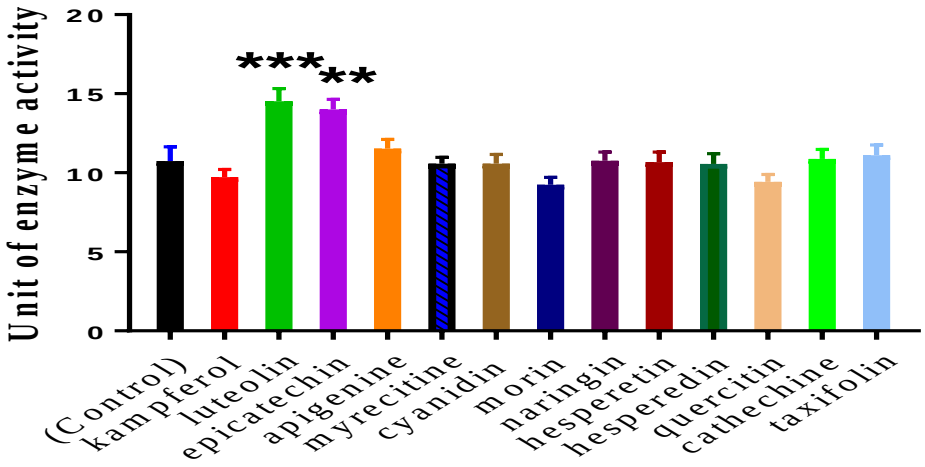


Figure 3-28: Effect of flavonoids on deamidation activity of TG2.

Pure Flavonoids, **at 125µM** dissolved in Sodium deoxycholate 0.5 %(w/v) then incubated with 5µgml^{-1}hrTG2, then applied to OPA assay for measurement ammonia released as described in materials and methods chapter, section 2.2.6b)ii. The data points represent the mean± S.E.M from three independent experiments (n=3, P value * < 0.05, **< 0.01, *** < 0.001, **** < 0.0001). All the values are as compared to the control (hrTG2+ gliadin+Ca^{+2}).

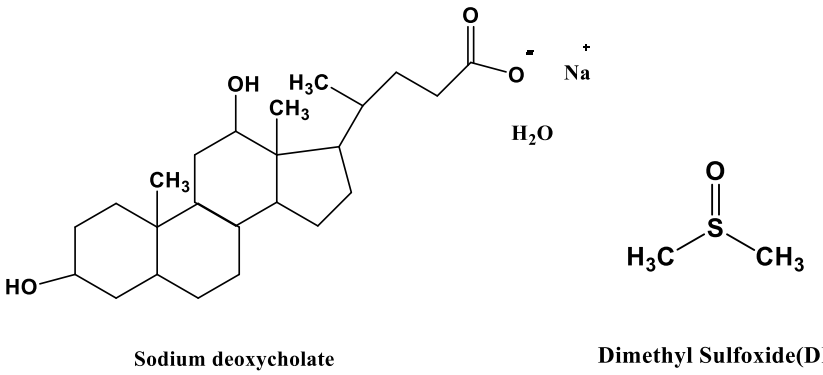
The data display that at 125µM no flavonoids show a significant reduction in TG2 deamidation when comparing to the control (Figure 3-28).

3.4 Discussion:

The assessment of potential therapeutics to treat the coeliac disease are typically achieved on simplified biological systems e.g. enzyme assays. Although, Stuven et al (2013) have reported that some *in vitro* and *in vivo* models such as intestinal cell lines and human mucosal biopsy cultured have helped in understanding of the pathophysiology of the CD, but not all that models have been applied in testing new therapeutics for the disease. Currently, adherence to a gluten-free diet represents the only therapy for coeliac disease that has been confirmed to alleviate the symptoms and prevent potential complications (Peräaho, M et al., 2003and Bascunan et al., 2017). However, the gluten-free diet, despite being safe and most effective, is not always ideal due to the cost implications, which has an impact on the quality of life of the sufferer. A study from England documented

dissatisfaction with a gluten-free diet by the majority of patients with coeliac disease (Rashtak et al., 2012). Therefore, research for developing novel therapeutic strategies appears to be a reasonable alternative treatment option. The crucial role of the activity of TG2 in initiation of CD has introduced a new approach in treatment of this disease by exploring substances have an inhibition effect on the activity of TG2. The side effects of using chemical TG2 inhibitors as mentioned previously, (See introduction chapter, section 3-1) has directed this research towards the screening of TG2 inhibitors from natural sources. The natural products chosen for this study are dietary flavonoids due to their potent biological activity. Therefore, the aim of this study was to test the effect of flavonoids on the activity of gplTG2 and hrTG2.

Thirteen pure flavonoids were selected to cover most of flavonoids subclasses, they divided into a different of classes such as flavonols (e.g., quercetin, kaempferol, morin, epicatechin, and myricetin,) flavones (e.g., apigenin, and luteolin), flavanones (e.g., hesperetin, and naringin), anthocyanin (cyanidin chloride), catechins (catechin and epicatechin), flavonolignans (taxifolin). All 13 flavonoids were dispersed either in sodium deoxycholate 0.5 %(w/v) or in DMSO and applied to the TG2 assays in the final concentrations of 1.25, 12.5 and 125µM. The reason for using sodium deoxycholate bile detergent was to try and mirror the environment of the human lower intestine where TG2 is located and initiates CD. As the bile salts occur in the duodenum of the small intestine which is the main part of intestine where lipid digestion happens, bile salts help in emulsifying the fatty foods adsorbing on oil-water interfaces (Malik, 2016).This would also help to solubilise the flavonoids in food released by maceration in the mouth and digestion in the stomach.



Sodium deoxycholate **Dimethyl Sulfoxide(DMSO)**

Figure 3-29: Sodium deoxycholate and DMSO chemical structures.

The screening of the TG2 modulatory effects was done using biotin cadaverine incorporation assay (Slaughter et al. 1992) and protein/peptide cross-linking assay

(Trigwell et al. 2004). These assays are the most commonly used assays to study the transamidation activity of transglutaminases. These assays use the incorporation of a biotinylated amine or peptide into such protein substrates as N′, N dimethyl casein or casein (Slaughter et al., 1992 and Trigwell et al., 1990), (For a more detailed explanation see materials and methods chapter 22.2.6c)i).

Three flavonoids (kaempferol, morin, and quercetin) in both solvents sodium deoxycholate and DMSO, demonstrated inhibitory behavior against hrTG2 and to a lesser extent on gplTG2 amine incorporation activity. At 125µM, the reduction in activity was around 50% for the 3 flavonoids against hrTG2 activity, whereas, kaempferol and quercetin in sodium deoxycholate displayed an inhibition effect for gplTG2 activity.

Those three flavonoids have a similar chemical structure and they are in the same subclass which is the flavonol(Figure 2-3). The detailed structures of the flavonoids differ structurally in the position of their hydroxyl group. The unexpected finding is that myricetin which is flavonol has moderate inhibitory effect which is statistically insignificant compared to other flavonols as the rings A and C are same for the 4 flavonols used, but the main difference in myricetin structure is that it contains an OH group at position 5- (Figure 2-3). As mentioned in the literature, the B-ring hydroxyl configuration is the most significant determinant of the biological effects of the flavonoids in general (Pannala et al., 2001and Burda and Oleszek, 2001). Therefore, it was hypothesized that the affinity binding between flavonoids and transglutaminase was related to the position of the hydroxyl group within the flavonoids. In the presence of calcium ions, TG2 exposes hydrophobic areas within its structure (Pinkas, 2007). This suggests that the interaction between the flavonol may rely on the presence of calcium ions. If the flavonols disturb the binding of calcium to TG2, preventing the enzyme from completely opening up this, in turn, could alter the substrate from binding to the active site cys377.

These findings show that similar results were obtained from flavonoids with using two solvents (DMSO and sodium deoxycholate), this suggests that only kaempferol, morin, and quercetin which belongs to flavonol subclass of flavonoids at this concentration using the amine incorporation assay have inhibition effect on the amine incorporation activity of hrTG2. The major exception was there was a less inhibitory effect on gplTG2 suggesting that although the two enzymes have similar properties and homology that there

are subtle differences between the two enzymes in terms of their interaction with these natural products.

In an attempt to understand the mechanism of the inhibition that the flavonoid performed. The Michaelis-Menten kinetic constant (Km) and Vmax were graphically determined to measure the kinetic properties of the flavonols on TG2 (Alexander and Griffiths, 1993 and Chambers and Rickwood, 1993). The Km refers to the concentration of the substrate when the reaction velocity is equal to one half of the maximal velocity for the (50% Vmax) (Palmer and Bonner 2007 and Roskoski, 2015). However, enzyme inhibition refers to the interference of the inhibitors with the enzyme, which usually provides information about the mechanism of enzyme action. Inhibition often classified as competitive, non-competitive, mixed and uncompetitive depending on the way the inhibitor interferes with the enzyme activity and alters the kinetic parameters (Boyer, 2011).

Competitive inhibitors are substances that be similar to the substrates and they bind to the active site causing lateness to the reactions. Consequently, competitive inhibitors increase Km value (decrease the affinity, less chance the substrates can go to active site), and Vmax stays the same. Where the uncompetitive inhibitors can bind near to the active site but not occupy the active site. As a result, uncompetitive inhibitors minimise the Km value (increase affinity) and lower Vmax.

Data in Table 3 1, Table 3 2, , Figure 3 12 and Figure 3 17 demonstrate that the Km value of the substrate with hrTG2 was increased in presence of kaempferol and quercetin flavonoids. While the same flavonoids (kaempferol and quercetin) in sodium deoxycholate exerted a different mechanism, in presence of gplTG2, as they reduce the Km value of substrate with gplTG2. For more explanations, in the absence of flavonoids the mean Km values for the biotin cadaverine as a substrate to hrTG2 was 0.05 mM (±0.004) and for gpl TG2 0.19 mM (± 0.003), the flavonoids causing a raise in Km value in presence of the enzyme affected. The exception was morin; in presence of hrTG2 has a low Km value for substrate 0.01 mM (±0.003), in sodium deoxycholate; 0.001 (±0.001) in DMSO. The results show that the flavonoids (kaempferol and quercetin) inhibit hrTG2 via competitive inhibition because the Km increases in competitive inhibition (Boyer, 2011). The competitive mechanism of inhibition refers to binding of inhibitor at the active site on the enzyme.

These findings assume that this, at least in part, describes the situation of the inhibition of the TG2 enzyme by flavonoids using this substrate. Whereas, morin exhibits uncompetitive inhibition of hrTG2 with the biotin cadaverine substrate. Uncompetitive inhibitors differ from competitive inhibitors in that they have a separate binding site on the enzyme and they only bind to the enzyme when substrate is bound to the enzyme. The kaempferol finding agreed with study done by Isao and Ikuyo 1999, they reported that kaempferol act as competitive inhibitor for the oxidation of L-DOPA catalyzed by mushroom tyrosinase. A study by Chun et al (2002) is another study which agreed with these finding, it was reported that the flavonoid act as competitive inhibitors to the activity of xanthine oxidase enzyme. As mentioned previously, there are no published reports about the effect of flavonoids on activity of TG2 or their mechanism. Whereas, this finding is contradictory to previous report by Martinez-Gonzalez and coworkers (2017), which mentioned that the main inhibition mechanism determined for polyphenolic compounds against digestive enzymes is non-competitive.

In the literature, it has been suggested that the inhibition of digestive enzymes (e.g trypsin) by flavonoids could be dependent on the location of their OH-groups (Gonzaliz et al., 2017). Moreover, a study by Xie et al (2015) has concluded that the activity of flavonoids as antibacterial is depending on the sites of hydroxyl groups on the aromatic rings of flavonoids. However, the methylation of the active hydroxyl groups tended to decreases the antibacterial activity of flavonoids (Xie et al., 2015). This could suggest that these flavonoids exert their inhibitory effect on TG2 by a similar mechanism. The structural variations between flavonoids generally are in the number and the location of OH group in B and C rings (Figure2-3), which might affect their hydrophilicity and their interaction with transglutaminase. In flavonol subclasses, the number of OH group in ring B and C varied from two in kaempferol to three in morin and quercetin, and four in myricetin (Figure 2-3). In addition, the study by Fan, J et al (2013) has reported that the flavonoids (flavonol) act as naturally occurring inhibitors for dipeptidyl peptidase IV (DPP-IV), which is a serine aminopeptidase that is a target for type 2 diabetes therapy. The comparison between these effects of flavonoids on the peptidase with the finding of this study could speculate the importance of flavonoids in treatment of CD as by inhibiting of peptidase will lead to generating of larger peptides which will be too big to go through the occludin gaps between the epithelial cells. The transglutaminases are believed to have been derived from a common ancestral gene for a cysteine peptidase, as

the catalytic core of transglutaminases appears to derive from the minimal ancestral structural unit of the thiol-protease fold (Fernandes et al., 2015). Additionally, many studies have reported the inhibition effect of flavonoids on the activity of esterase enzymes such as acetylcholinesterase (Dzoyem et al., 2017). For example, a series of flavonoid were used as potential multifunctional ligands against Alzheimer disease with inhibitory influence against cholinesterases (Faraji et al., 2019). These effects of the flavonoids on esterase enzymes might be helpful in the speculation of the importance of these study findings, additional experiments needed in future to explain the mechanism of the inhibition of TG2 activity by flavonoids.

The question here is whether purified flavonoids have the same health benefit as the flavonoids present in whole food. It was decided to study the effect of the combinations of flavonoids on hrTG2 and gplTG2 transamidation activity to mimic the mixtures that could be present in food. The three flavonoids (kaempferol, morin, and quercetin) were dispersed in sodium deoxycholate or in DMSO in different mixtures and then applied to biotin cadaverine incorporation assay (Slaughter et al., 1992). The Figure 3-10 for the flavonoids in DMSO show the improvement in the effect of flavonoids when mixed, for example, the inhibition effect of kaempferol on the hrTG2 activity was improved by the addition of quercetin. As kaempferol alone at 125µM reduce 23.03% of the hrTG2 activity, whereas, when mixed with morin the reduction in the activity of the hrTG2 was 75.06% and 65.51% with quercetin. The combination of three flavonoids has higher inhibition action than individually, 63.46 %. Another curious finding is that the combination of flavonoids has significant inhibitory action at a lower concentration (12.5µM). For gpl TG2 activity even mixtures of the three flavonoids have not improved the action of individual flavonoids effect on amine incorporation activity of gplTG2 (Figure 3-11). The finding in Figure 3-15 and Figure 3-16 display that the flavonoids dispersed in sodium deoxycholate have no synergistic effect among all mixtures used. Although, this finding agrees with the literature, which reports that the individual antioxidants do not appear to have consistent preventive effects with the mixture of food contain antioxidant (Liu, 2003). For example, several studies have shown that the consumption of yellow and green fruits and vegetables inversely related to the risk of cancer, due to the presence of β-carotene in plenty in these fruits and vegetables. However, the incidence of non-melanoma skin cancer was unaltered in patients receiving a β-carotene supplement (Hennekens et al., 1996).

To measure the effect of flavonoids on the TG2 protein cross-linking activity *in vitro* according to the method of Trigwell et al (2004), the 13 flavonoids in DMSO and sodium deoxycholate were applied to the assay using gpl and hrTG2. Most of flavonoids display –(particularly that dissolved in DMSO)- a significant inhibition effect for the TG2 protein cross-linking activity at concentration 1.25, 12.5 125µM. The hrTG2 was more sensitive to the effect of flavonoids than gpl TG2 (Figure 3-18, Figure 3-19, Figure 3-23 and Figure 3-24). The reduction of TG2 activity ranged from50 to 70% for most of flavonoids. This finding is inconsistent with the effect of the flavonoids on amine incorporation assay. However, in agreement with a study by Wu, C., and Yen, G., (2005) which demonstrated the protection of flavonoids against advanced glycation end products formation that is involved in hyperglycemia-mediated oxidative stress through extensive anti-cross-linking activities. Moreover, another study by Urios, P et al., (2007) has reported the very potent inhibition effect of flavonoids at micromolar concentrations on the formation of the cross-linking AGE (pentosidine) in collagen, which is involved development of vascular complications in diabetes. This effect of flavonoids as mentioned by Urios, P et al., (2007) was due to the ability of the flavonoid to binding the transition metal ions that play a role in glycoxidation. Further experiments are warranted to explain the mechanism of that flavonoids inhibition for the cross-linking TG2 activity. In addition, to the role of flavonoids in binding of the transition of metal ions (Ca^{2+}), which play a role in the activity of TG2 (Urios et al., 2007). To consistent with the amine incorporation study, the same three flavonoids (kaempferol, morin, and quercetin) were selected to study the effect of the combination of flavonoids on their inhibition of TG2 cross-linking activity.

Data in Figure 3-20,Figure 3-21,Figure 3-25 and Figure 3-26 did not show synergism effects between three flavonoids,compared to the inhibition action of the flavonoid individually. The inhibitory effect of the flavonoid individually was more pronounced than in a mixture. Based on these data, the interactions of flavonoids present in a mixture can affect the total cross-linking inhibitory action. These findings are consistent with the study of Maria et al (2009), where they demonstrated that the antioxidant action of individual flavonoid was changed when compared with mixtures of flavonoids. While that flavonoid –flavonoid interactions have effects in the inhibition behavior of TG2 activity of flavonoids mixtures, there are requirements for more detailed study for flavonoid combinations to better understand the mechanisms involved in these interactions.

The data in Table 3-3, Table 3-4, Figure 3-22 and Figure 3-27, indicate that kaempferol, morin, and quercetin in both solvents reduced the Km value of the substrate with hrTG2. Therefore, the Km values suggest that the kaempferol, morin and quercetin flavonoids exert their inhibition action by un-competitive mechanisms, which disagrees with their effect against the amine incorporation activity of TG2. (Figure3-12 and Figure 3-17). On the other hand, with the gplTG2,the quercetin works as competitive inhibitor for cross linking activity of gplTG2, because it increases the Km value of substrate with the enzyme. A study by Ribeiro and coworkers (2014) has mentioned that the quercetin flavonoids have uncompetitive inhibitory effect on the activity of the lipoxygenase enzymes (inflammatory diseases mediators). As aforementioned, the human recombinant TG2 was used to mimic the situation of CD, however, guinea pig TG2 was used because is routinely used in TG2 assay in the literature to help reduce assay costs. The comparison in these study between two forms of TG2 aimed to investigate if there difference in response between two forms toward the biomolecules in the assays. The finding suggests that there is variation in the response between hrTG2 and gplTG2, which suggests that researchers should use hrTG2 in a study to look at proposed modulators of TG2 activity in relation to CD. This finding, show the hrTG2 was more sensitive to flavonoids than gplTG2. The subtle difference in homology between gpl and hr TG2 could be the reason for these differences, as between human and guinea pig TG2 there is an 80% homology (Aeschlimann et al., 1994). Generally, the flavonoid inhibition effect is related to their structures, the nature of the enzyme and substrates. These factors could help in explaining the difference in the mechanism of inhibition between hrTG2 and gplTG2, many of studies have confirmed the structural activity inhibitory relationship of flavonoids on different enzymes (Xie et al., 2003; Phan et al., 2013 and Daniela et al., 2014).

As the deamidating activity of TG2 is crucial in initiation of CD and because one of the main aims of this research optimised a simple assay for the measurement of the deamidating activity of TG2 (See Chapter 6). The pure flavonoids were applied to the novel OPA assay to measure their effect on the ammonia released after incubation of hrTG2 using gliadin mixture peptides as the substrate (Deamidating activity of hrTG2). The concentration of flavonoids used in the assay was 125µM in sodium deoxycholate. At this concentration, the flavonoids, exert inhibition for transamidation activity of TG2. The data in Figure 3-28 show that not all of the tested flavonoid showed significant inhibition effect. The luteolin, epicathecine and hesperidine flavonoids show activation

effect on the activity of hrTG2, as the release of ammonia were high. Further experiments warranted to study the mechanism of those flavonoids on the activity of TG2 to in relation to coeliac disease. The curious finding that the lowest ammonia amount was found in the samples with same flavonoids that have inhibitory action on the transamidation activity of hr TG2 (kaempferol, morin, and quercetin, See Figure 3-28). Further experiments needed to study the link between the effect of the flavonoids on the transamidation and deamidation activity of TG2.

3.5 Summary of key findings

- From 13 pure flavonoids used in the study, only kaempferol, morin and quercetin at concentration 125µM in Sodium deoxycholate or DMSO have a significant inhibition effect on the amine incorporation activity of TG2.
- The Kinetics analysis data suggested that flavonoids (quercetin and kaempferol) inhibit hrTG2 via competitive inhibition, whereas, morin exhibits uncompetitive inhibition of hrTG2 and gplTG2 with the biotin cadaverine substrate.
- Combination of flavonoids improves the hrTG2 amine incorporation inhibitory impact for the flavonoids dissolved in DMSO, however, no effect from the combination, which are dispersed in sodium deoxycholate.
- Most of the flavonoids are displayed inhibition effect on the cross-linking activity of hrTG2 and in low extent gplTG2 and in DMSO more than in Sodium deoxycholate.
- Findings of a combination of flavonoids on the cross-linking activity of TG2, show no synergism effects between three flavonoids were their action individually effect is more than in mixture, the exception was in a mixture of kaempferol and quercetin, which show improvement in the inhibitory action of kaempferol in deoxycholate against gplTG2.
- Km values suggest that the kaempferol, quercetin, and morin exert their inhibition action on the cross-linking activity of hrTG2 by un-competitive mechanisms. Whereas, quercetin act by competitive inhibition for gpl TG2 activity.
- Flavonoid at 125µM concentration did not show significant reduction effect on the amount of ammonia released (the deamidating activity of hrTG2) comparing to the control.

Chapter 4 : Extraction and purification of flavonoids from food and to study the effects of the extracted flavonoids on the TG2 activity

4.1 Introduction:

This study, as aforementioned(See Chapter3 introduction section), focused on flavonoids as a natural source for the potential TG2 inhibitors in relation to coeliac disease. As the selection of the appropriate extraction method is crucial to produce a safe and pure biological component. Therefore, this chapter displays attempts to select the optimum extraction and purification method for the flavonoids from a food source.

4.1.1 Flavonoid extraction;

The extraction of bioactive compounds from natural sources is a very important step. This step must meet requirements such as ease of use, efficiency, flexibility, and cost-effectiveness. In addition, it should extract and preserve the major fraction of the natural bioactive substances in the natural sources. Recently, numerous research has been focused on the extraction of valued compounds using both classical and innovative technologies, including the use of green solvents (Chua 2013). The selection standard of the best extraction method depends on the highest recovery, to minimise the degradation of the compounds that occur as a result of different process steps (Routray and Orsat 2012). In the literature, there have been different extraction techniques used for bioactive molecules from natural sources, such as;

- Maceration, infusion, percolation, and decoction: These techniques are the easiest methods to extract flavonoids. The alteration in temperature and choice of solvents improve the extraction process .
- Soxhlet extraction, which has been widely used for the extraction of bioactive compounds, this method has some disadvantages due to the use of high temperature, (Uppugundla et al., 2009; Chua, 2013).
- Microwave-assisted extraction: depends upon the use of microwave energy to facilitate the partition of analytes into the solvent (Trusheva et al., 2007).

- Ultrasound-assisted extraction or sonication extraction involves using the mechanic influence of the ultrasound to increase the surface contact between solvents and samples (Handa, et al., 2008).
- Other methods such as accelerated solvent extraction and supercritical fluid extraction have also being used in the extraction of flavonoids from plant and food materials (Azwanida, 2015).

4.1.2 Flavonoid purification

Flavonoid purification procedures are generally used to clean-up extracts prior to any further analysis by analytical methods such as HPLC and TLC. The purification step aims to remove potential interfering components (e.g. macromolecules) that may interfere with the analysis. These purification methods include liquid-liquid partitioning with non-miscible solvents and column chromatography on Sephadex LH-20, preparative HPLC, and solid-phase extraction (SPE) using commercially available disposable cartridges (Markham et al., 1998and Grayer, 2012). Based on the affinity between flavonoids and absorbents, a macro-porous adsorbent has been used in the purification of flavonoids. Purification by solid-phase extraction (SPE) is a relatively simple method used for elimination of polar, non-phenolic impurities in one-step. However, components of products or impurities are irreversibly bonded to the chromatographic surfaces, which may affect the selectivity of this method. Which also causes a reduction in the binding capacity of the stationary phase (Kraemer-Schafhalter, et al., 1998).

In this study, the IMAC (Immobilized metal ion affinity chromatography) technique was suggested as purification method for flavonoids from their natural sources. The IMAC has been used for purification of proteins. The concept of IMAC has first been produced by Porathet al (1975). It was based on the known affinity of transition metal ions such as Cu^{2+}, $Zn2^{+}$, $Ni2^{+}$, and Co^{2+} to form co-ordinate covalent complexes with histidines and cysteines in aqueous solutions (Hearon 1948) and extended to the idea to use metal ions to support the fractionation of proteins in solutions. Initially IMAC developed for purification of native proteins with an intrinsic affinity to metal ions (Porath, et al. 1975), and then the method has been expanded with a broad portfolio of applications including; antibodies, phosphorylated proteins, and recombinant His-tagged proteins (Block, et al., 2009). There are numerous advantages of IMAC for purification of His-tagged proteins

compared to other affinity chromatography methods such as the low costs and the simplicity of use.(Derewenda, 2004).

It is known that flavonoids form coordinate covalent bonds with the copper ions held on the IMAC resin due to the presence of hydroxyl groups with oxygen atoms rich with electrons, the IMAC method was suggested as purification method for the flavonoids from different food extracts. The reason for using this technique is attempt to obtaining more purified components from the food extract that the utilising of copper chelating resins to capture flavonoids prior to analysis allows the removal contaminants including TBHQ and sugars which may subsequently interfere with colorimetric assays (see discussion section and Materials and method chapter, section 2.2.2b)).

4.1.3 Identification of flavonoids

The identification and quantification of flavonoids in natural sources is a key point for the study of their biological activity (Marston and Hostettmann, 2006). A colourimetric method is commonly used for the quantification of the total flavonoid content, in which the total flavonoid content in methanolic or ethanolic extracts of plants was determined based on the formation of flavonoid-aluminum chloride ($AlCl_3$) complex (Naczk & Shahidi, 2006). Thin-layer chromatography (TLC) is another technique widely used for the qualitative analysis of flavonoids, and it may be appropriate for the quick screening of these compounds in plant and food extracts (Stalikas, 2007). Methods such as high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and capillary zone electrophoresis (CZE), are commonly used techniques due to their high efficiency in separation and characterisation of flavonoids (Gullon et al., 2017).

The commonly used colourimetric assays for quantification of flavonoids is the colourimetric Folin-Ciocalteu (F-C) assay. The F-C assay is simple and widely used to measure the total phenolics in vegetables and fruits (He et al., 2008). However, the F-C assay is unspecific for flavonoids and can detect compounds with an active hydroxyl group(s) such as flavonoids, phenolic acids, reducing sugars, ascorbic acid, some amino acids, aromatic amines, and proteins. Another method commonly used for quantification of flavonoids in reversed-phase high-performance liquid chromatography (RP-HPLC), this chromatographic method is sensitive and quantitative. The method has limitations due to the limited numbers of pure standard compounds commercially available and the resolution of an RP-HPLC column (Block, et al., 1992and Willett, 2002).

4.2 Aims:

As the main aim of this research is screening for the natural inhibitors of TG2 activity as a potential treatment for coeliac disease (CD). In addition, the extraction of bioactive compounds from natural sources is a very important step. Therefore, the hypothesis was developing a low cost, simple, more specific purification method for the flavonoids. This would aid the extraction of these components to be used with food extracts in the screening of their effect on the TG2 activity in relation to coeliac disease. To test this hypothesis, it was aimed to;

- Develop a colourimetric method to quantify flavonoids in a solution using the bicinchoninic acid (BCA) assay (Smith et al., 1985)(see chapter 22.2.6a).
- Employ IMAC as a novel method for purification of flavonoids from different food samples (See chapter 2, section 2.2.2b).
- Compare the flavonoid yield and purity between IMAC and SPE purification methods by RP-HLPC.
- Study the effect of food extracts on transamidating and deamidating activity of TG2.

- **Methods and Materials:**

See Methods and materials chapter:

- Bicinchoninic acid protein assay (BCA) assay(section2.2.6a).
- Solid Phase Extraction (SPE)(section 2.2.2a)
- HiTrap Chelating HP(section 2.2.2b)
- Qualitative analysis using High-Performance Liquid Chromatography (HPLC)(section2.2.3).
- Mass spectrometry for Flavonoids(2.2.5a)
- Transglutaminase transamidation activity assays(2.2.6c)

4.3 Results

4.3.1 Optimization of quercetin as standard for flavonoids quantification

To quantify of flavonoids in different food extracts, a modified BCA assay (Smith et al., 1985) was optimised to quantify the flavonoids content by using quercetin as standard. The assay found to be sensitive to quercetin concentrations ranging from 40 to 400µM. (See Figure 4-1).

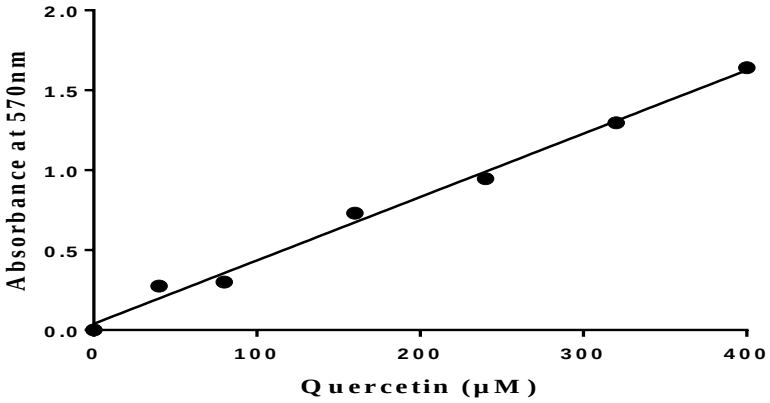


Figure 4-1: Optimization of quercetin as standard for flavonoids quantification.

Quercetin used as a standard to quantify the flavonoids in the food extracts. Different concentration of quercetin (0 -400µM) with BCA working reagent was applied to BCA assay (Smith et al., 1985)(see Materials and methods chapter, section2.2.6a).

The data in Figure 4-1display the sensitivity of BCA assay to quercetin, which is using in concentration arranged from 0 to 400µM.

4.3.2 Flavonoids isolation:

The isolation of flavonoids is carried out by refluxing fruit or vegetable samples in an acidified methanol solution in the presence of an antioxidant tetra butyl hydroquinone (TBHQ) (see section 2.2.1b). Then purified using a solid-phase extraction (SPE) method, and a novel method for purification of flavonoids, using immobilized metal ion affinity chromatography (IMAC). To compare two methods in term of purity and quantity obtained, samples from both extractions applied to RP-HPLC for qualitative analysis and quantitative assay by BCA method using quercetin as standard.

a) Solid Phase Extraction (SPE)

The solid-phase extraction (SPE) procedure allows retaining on of analytes that would interact with a C_{18} resin in the column, especially aromatic compounds, such as flavonoids, by the formation of hydrophobic interactions between the analytes and stationary phase that is styrene divenylbenzene C_{18}. The use of 80% (v/v) methanol as eluent and use of the BCA assay allowed for the quantification of analytes in the following concentrations expressed in mg/100g dry weight (DW)

Table 4-1: Quantity of flavonoids fractions from SPE

Food	Flavonoids (mg/100g DW Mean (±SEM)
apple	0.31 (±0.05)
green tea	1.16 (±0.23)
strawberry	0.46 (±0.08)
kale	0.49 (±0.08)
capper	0.38 (±0.06)
garlic	0.19 (±0.01)
onion	0.26 (±0.01)

b) IMAC method:

Flavonoids at alkali pH values will show increased deprotonation of hydroxyl groups, exposing electron-rich oxygen atoms allowing co-ordinate covalent interactions with

divalent metal ions bound to the IMAC resin. The isolation of flavonoid in this method was achieved using copper bound to the chelating resin to capture flavonoids prior to analysis. Quantification of flavonoids in these fractions was done by BCA assay in concentrations expressed (mg/100g) (Table 4-2).

Table 4-2: Quantity of flavonoids fractions from IMAC

Food name	flavonoids mg/100g DW Mean (±SEM)
apple	0.35 (±0.08)
green tea	1.02 (±0.34)
strawberry	0.70 (±0.18)
kale	0.47 (±0.11)
capper	0.42 (±0.11)
garlic	0.20 (±0.03)
onion	0.20 (±0.03)

The Table4-1 and Table4-2 show the quantity of flavonoids in different food sample by using two different methods of isolation, SPE and IMAC, the data suggest that no significant difference in quantity between the fractions using two methods.

4.3.3 Qualitative analysis using reversed-phase High-Performance Liquid Chromatography (RP-HPLC)

The IMAC method (See materials and methods chapter, section 2.2.2b) was used for purification of flavonoids fractions. To compare between SPE and the novel IMAC method in term of purity, the flavonoids extracted from different food samples were injected onto a C_{12} RP-HPLC (See Materials and methods chapter 2, section 2.2.3).

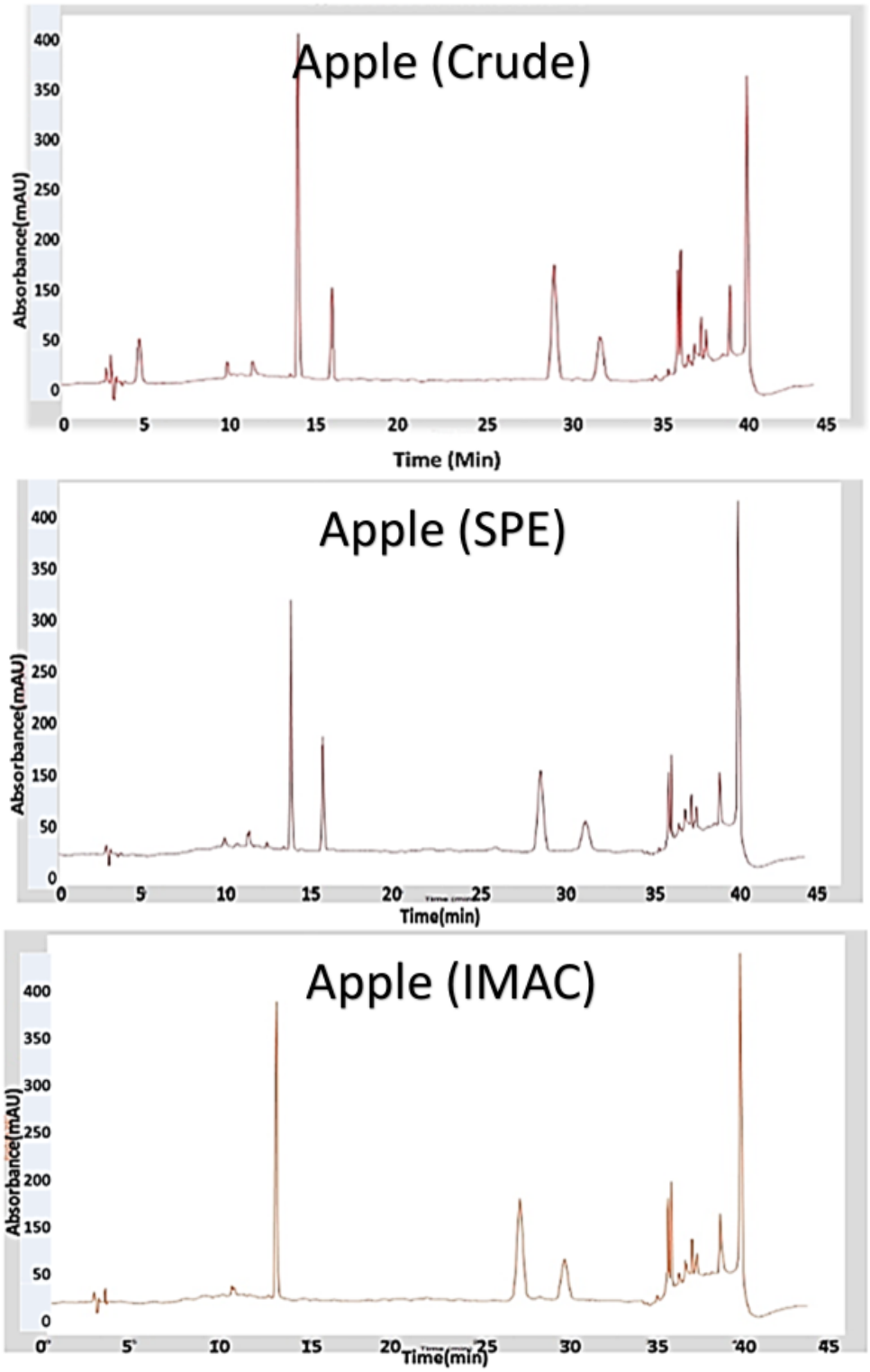


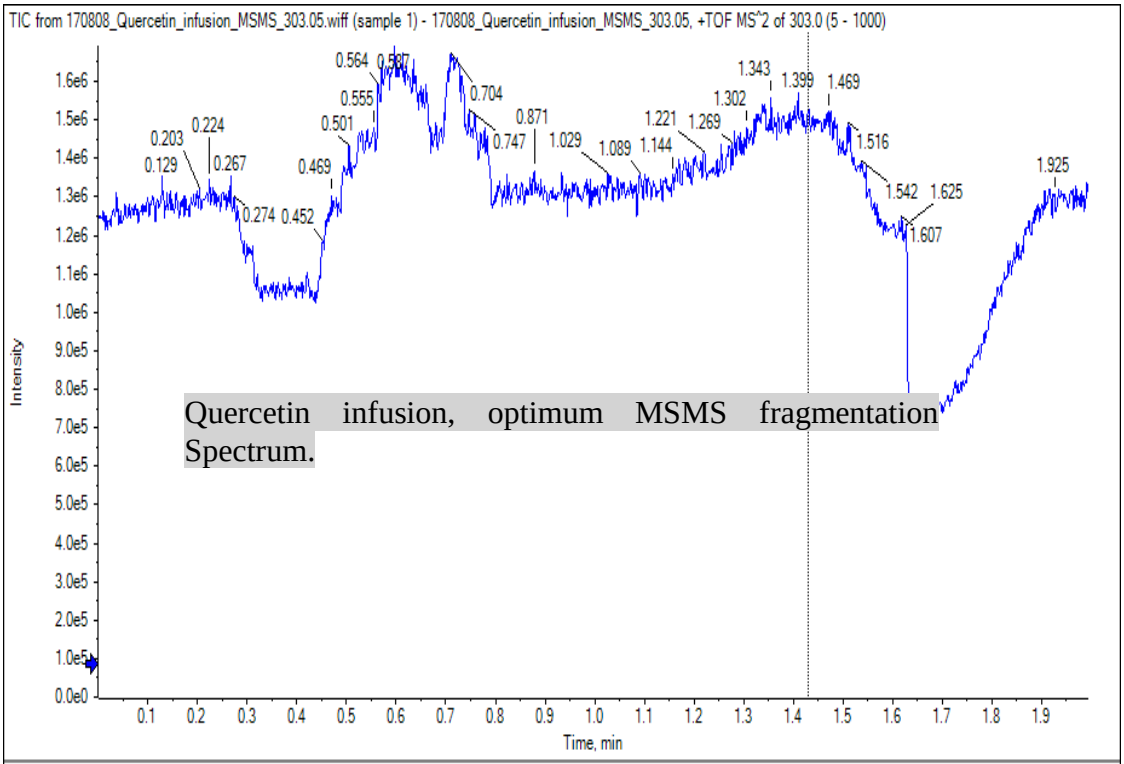
Figure 4-2: Chromatogram of apple flavonoids extracts.

Three different fractions from apple samples crude, SPE and IMAC were injected to RP-HPLC.

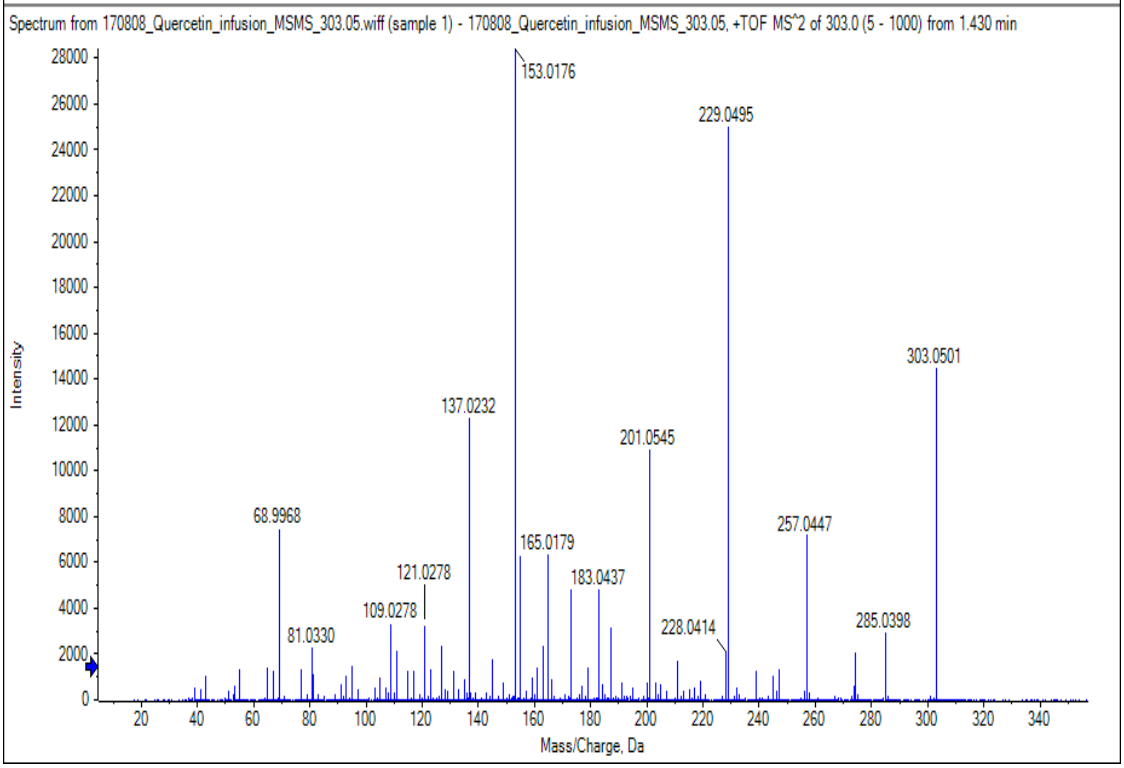
The data in Figure 4-2 show that apple IMAC extract chromatogram was more purified than crude and SPE extracts chromatograms. Other food samples chromatogram (presented in appendices chapter).

4.3.4 Qualitative analysis using LC/MS/MS

In order to confirm that IMAC extract food samples contain flavonoids, the food samples were injected to LC/MS/MS, the finding confirm that significant flavonoids were involved in some food samples.



Quercetin infusion, optimum MSMS fragmentation Spectrum.



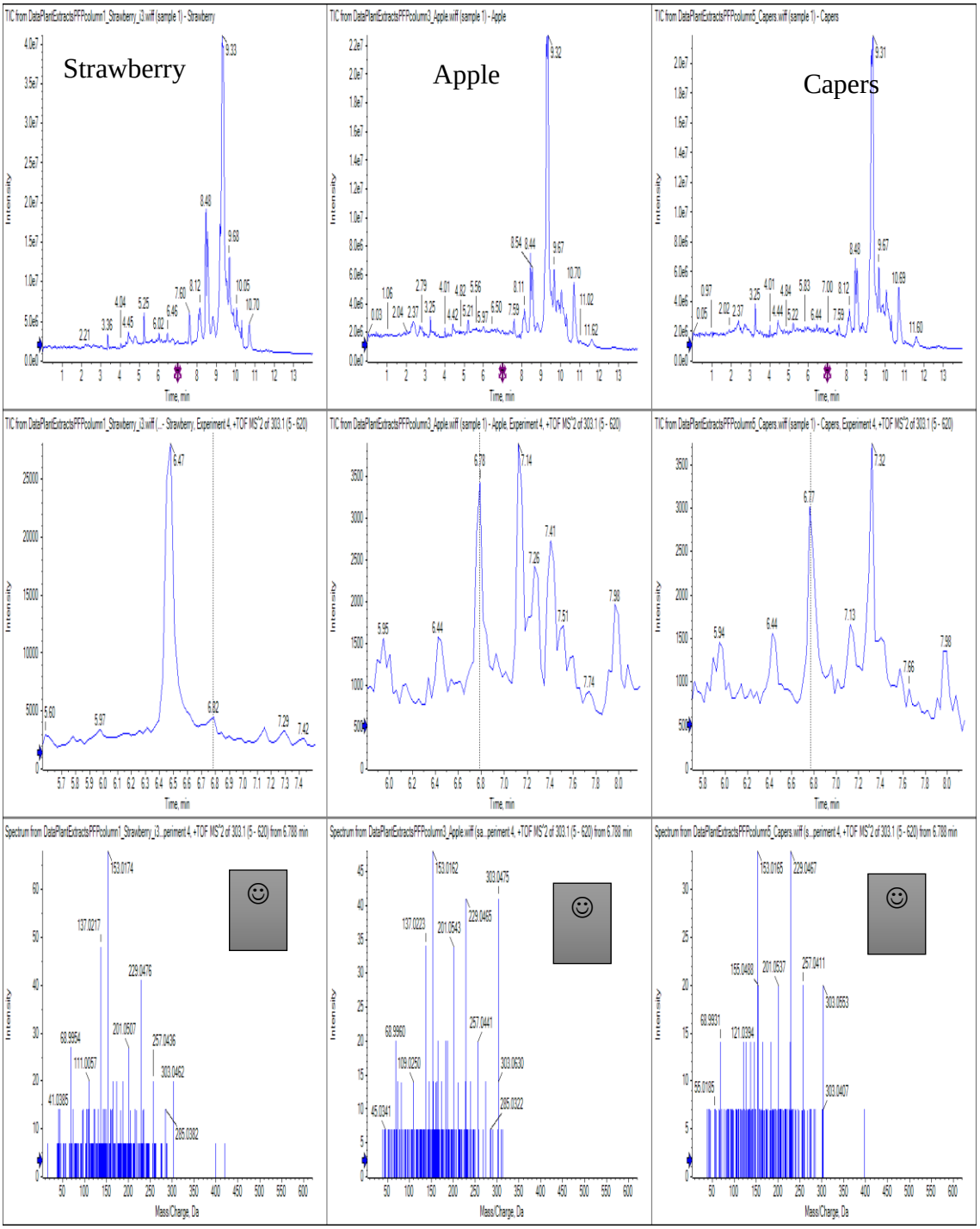


Figure 4-3: optimum MSMS fragmentation spectrum for the quercetin and food extracts which contain quercetin

The data in Figure 4-3 show that apple, strawberry and capers extracts spectrum show the same fragmentation of quercetin. Other food samples spectrum (presented in appendix chapter).

4.3.5 Application of flavonoids extracted from food to the TG2 biotin cadaverine assay:

The biological activities of pure flavonoids against hrTG2 (see chapter 3 flavonoids effect on TG2) provided the encouragement to screen flavonoid mixtures extracted from flavonoid-rich foods against TG2 activity as a potential treatment for coeliac disease. To observe the effect of food extracts on amine incorporation activity of TG2, were reconstitute with deoxycholate sodium in concentration (1mg ml^{-1}) then applied to the TG2 activity assay using hrTG2 and gplTG2.

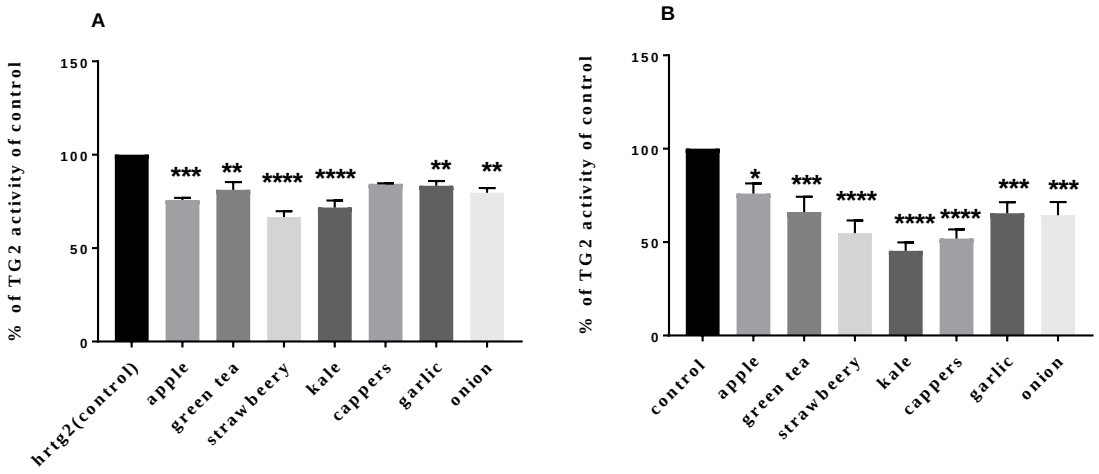


Figure 4-4: Effect of food extract on amine incorporation activity of TG2.

IMAC Food extracts dispersed in sodium deoxycholate 0.5 %(w/v) then incubated with A) 5µg ml^{-1} hrTG2. B) 10µg ml^{-1} gpl TG2 were subjected to the screen their effect on amine incorporation activity of both enzymes according to the method of Slaughter et al (1992) described in methods and materials chapter,(section 2.2.6c). The data points represent the mean± S.E.M from three independent experiments (n=3, P-value **** < 0.0001). All the values are as compared to the control.

All food extracts display a significant inhibitory effect on hrTG2 and gplTG2, (Figure 4-4 A and B)

4.3.6 Application of flavonoids extracted from food to the TG2 peptide/protein cross-linking assay:

To observe the effect of the food extracts on the cross-linking activity of TG2, the food extracts were dissolved in sodium deoxycholate 0.5 % (w/v). Then applied to the TG2 activity assay using hrTG2 and gplTG2.

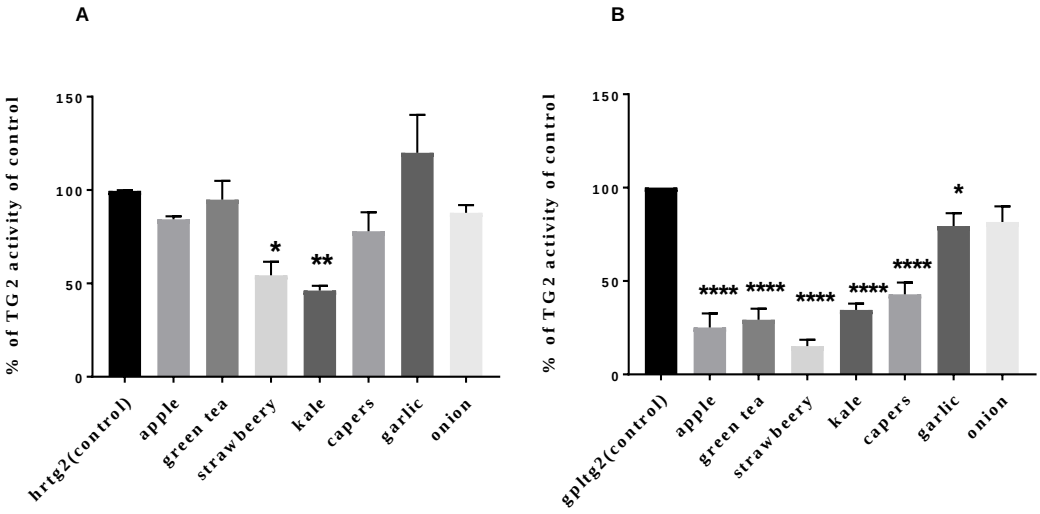


Figure 4-5: Effect of food on the cross-linking activity of TG2.

IMAC Food extracts dispersed in sodium deoxycholate 0.5 %(w/v) then incubated with A) 5µg ml-1 hrTG2. B) 10µg ml^{-1} gpl TG2 were subjected to the screen their effect on cross-linking activity of both enzymes according to the method of Trigwell et al (2004),(described in methods and materials chapter section 2.2.6c). The data points represent the mean± S.E.M from three independent experiments (n=3, P-value * < 0.05, **** < 0.0001). All the values are as compared to the control.

Most of the food extracts display a significant inhibitory effect on gplTG2, while, just kale and strawberry exert an inhibitory effect on hrTG2 (Figure 4-5 A and B). In addition, the data shows the variation in the response to the food containing flavonoids between gplTG2 and hrTG2.

4.3.7 Application of flavonoids extracted from food to the OPA TG2 deamidation activity assay

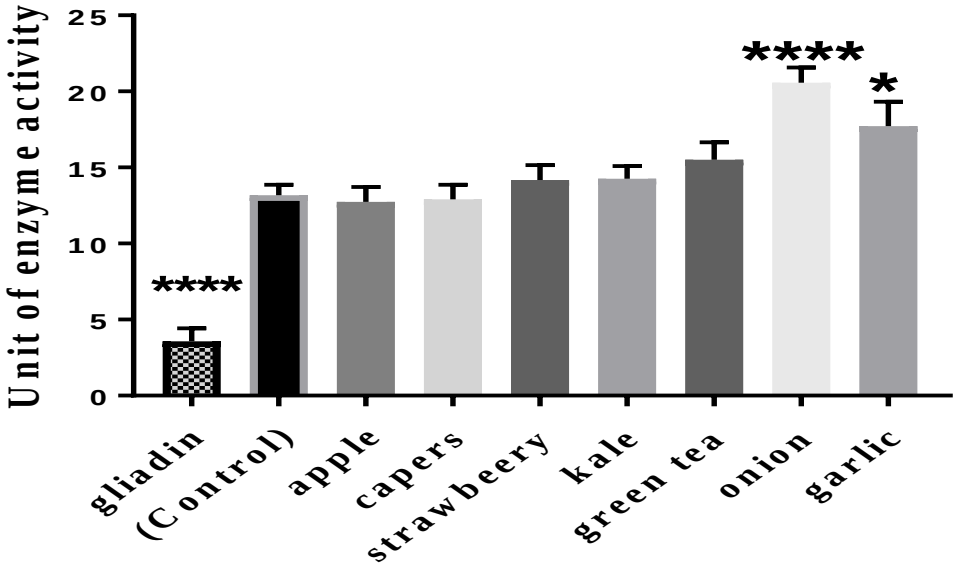


Figure 4-6: Effect of food on the deamidating activity of TG2.

IMAC Food extracts were subjected to the screen their effect on the deamidating activity of hrTG2(materials and methods chapter, section2.2.6b). The data points represent the mean± S.E.M from three independent experiments (n=three, P-value * < 0.05, **< 0.01). All the values are as compared to the control (Gliadin+Ca^{2+}+hrTG2).

The data (See Figure 4-6) show that there is no significant inhibition effect for all food extracts on the deamidating activity of hrTG2. Nevertheless, the two extracts from *Alliums* show significant activation of the deamidating activity compared to the control (Gliadin+Ca^{2+}+hrTG2).

4.4 Discussion

The biological activity of the dietary flavonoids motivated the study to screen flavonoid-rich foods and examine their effects against TG2 activity as a potential treatment for coeliac disease. Consumption of flavonoid-rich foods, such as fruits and vegetables, is associated with a lower prevalence of several diseases. Seven of 12 epidemiological studies assessing the risk of coronary heart disease reported protective effects of dietary flavonoids (Arts et al., 2005). Other studies also found inverse relation between flavonoid consumption and the risk of stroke, colorectal and lung cancers (Keli et al., 1996; Hirvonen et al., 2001; Knekt et al, 2002and Arts et al., 2002). It has been proposed that dietary flavonoids exert health benefits by an antioxidant mechanism, which elevated at

pH 7.0 values, the electron-rich oxygen atoms in the hydroxyl groups can donate electron pairs (co-ordinate covalent bonding to electron-deficient atoms such as divalent metal ions (Huyut et al., 2018). As these chronic diseases are accompanying with high oxidative stress and flavonoids are strong antioxidants *in vitro*, (Aviram and Fuhrman, 2002; Serafini, et al., 2002 and Rietveld, and Wiseman, 2003).

Because of the numbers of conjugated with target analytes, as the clean-up is usually necessary to remove coextracted matrix components that may interfere in subsequent steps (Obied et al., 2005), so more selective and sensitive analytical methods are required (de Rijke, et al. 2006). In this study, a novel method for purification of flavonoids from food extracts was used which is immobilized metal ion affinity chromatography (IMAC) (Cheung et al., 2012). This was then compared with a more conventional extraction method which is solid-phase extraction (SPE) method (Liu, Houme, et al., 2016).

The IMAC technique is a specialized form of affinity chromatography, proteins or peptides are separated according to their affinity for immobilized metal ions. At pH values above neutral, the amino acids cysteine, tryptophan, and histidine form co-ordinate covalent complexes with divalent metal ions (e.g., Cu^{2+}, Cd^{2+}, Hg^{2+}, Ni^{2+}, and Fe^{2+}). Then, can be eluted by either decreasing the pH to protonate an electron-rich atom, adding EDTA (0.05 M) to the mobile phase to chelate and strip the IMAC column of the metal ion, or increasing the mobile phase ionic strength (Otter 2003). Elution with imidazole is especially suitable for purifying recombinant proteins with poly-histidine fusions by competing with the polyhistidine on the his-tagged protein (Alves et al., 2017; Bonner 2018).

The IMAC technique has been used in this study to purify flavonoids from different food extracts. Based on the formation of coordinate covalent bonds between metal ions immobilized in the chromatography resin in the column, e.g. Cu^{2+} that accepts a pair of electrons directly from the deprotonated nitrogen in peptides or oxygen atoms on the flavonol. Electron rich oxygen atoms from the hydroxyl groups on the flavonoids as result of the alkaline pH that created by methanol with 0.2% (v/v) of 5M sodium hydroxide are now able to form coordinate covalent bonds with the copper atom held on the IMAC resin. The use of copper chelating resins to capture flavonoids prior to analysis allows the removal of contaminants including TBHQ and reducing sugars, which may subsequently interfere with colourimetric, assays e.g. BCA. Other material in the extract without the

electron-rich oxygen atoms will peculate through the column in the unbound fraction. (See method and material chapter).

Another more standard method used for flavonoid purification is SPE where alkyl-bonded silica or copolymer sorbents broadly used for analyte extraction and enrichment from sample extracts, in most cases the sorbent is C18-bonded styrene and the sample solution and solvents, are usually slightly acidified to prevent ionization of the flavonoids, which would reduce their retention (de Rijke, et al. 2006). The SPE retains analytes on the column, especially polar and aromatic compounds, such as flavonoids, by the formation of hydrophobic interactions between analytes and stationary phase that is styrene divinylbenzene. Polar material will not be retained and will be eluted with polar solvent in the unbound fraction.

For quantification of extracted flavonoids, the bicinchoninic acid (BCA) assay (Smith et al., 1985) was used to compare the quantity yielded between two methods (IMAC and SPE). This assay was used based on the ability of polyphenols (flavonoids) to bind with metal ions (Ivanov et al., 2001and Wei and Guo, 2014). This assay (BCA) is a colourimetric assay that is commonly used to estimate the concentration of protein in a sample, protein levels are measured through the formation of a purple, $Cu^{+1}(BCA)_2$ chromophore (λmax = 562 nm). BCA assay was optimised in a novel quantification method using quercetin as the standard. The use of BCA reagent allows the linear detection of quercetin in the standard assay in the range of 40 to 400µM (Figure 4-1). Based on a search in the literature no previous work used BCA for estimation of flavonoids in food samples. In this study, the BCA method used for quantification of flavonoids in different food extracts, data in Table4-1 and Table 4-2 show that the amount is varied between 0.20 to 1.02mg100^{-1}g DWin the IMAC fractions. Whereas, the amount in SPE fractions were arranged from 0.19 to 1.16 mg/100g. Green tea had the highest dry weight concentration of flavonoids followed by strawberry, kale, capper, and apple. The garlic and onion had the lowest flavonoids content. These data are inconsistent with Bhagwat, et al., (2014) study that found a higher flavonoid content in, for example, apples 0.51mg/100g dry weight. This may account for differences as well as different varieties (quercetin), garlic 1.9mg/100g (quercetin and kaempferol), green tea 0.54/100g (kaempferol and quercetin)(Bhagwat et al., 2014), this difference could be from using starting material, different extraction, purification, and quantification method. The

comparison between the fractions from two purification methods, in term of purity were conducted by RP-HPLC on a C12 resin.

The chromatograms for food fractions before using any purification method (crude extract) contains a lot of impurities as judged by the presence or absence of additional peaks in the elution profile, whereas, in chromatograms of SPE fractions less contaminating peaks are observed. However, the IMAC fractions have produced a clearer chromatogram as judged by the absence of contaminating peaks. Although, there is no significant differences between the quantities obtained from both methods, however, the HPLC chromatograms confirm that the IMAC fractions contain less contamination peaks, which are still observed in SPE chromatograms. These findings suggest that SPE extracted all the organic components, whereas IMAC extract the only the components that have two hydroxyl groups close together particularly those on the flavonoids. This might be due to the capturing of flavonoids using of copper chelating resins, allows the removal contaminants including TBHQ and sugars which may subsequently interfere with the assays. This finding present that IMAC method as a selective and appropriate technique to purify flavonoids in those food samples.

The application of chromatographic methods hyphenated with different detectors is necessary for identification of the flavonoid conjugates. LC-MS/MS is a powerful means to screen biologically active compounds from natural source extracts because of its high sensitivity and selectivity (Li, et al. 2009). In this study, the samples of IMAC flavonoids extracts were prepared and analysed by mass spectrometry lab in John van Geest Cancer Research Centre in Nottingham Trent University. In order to identify the flavonoids contents of IMAC food extracts, the samples were subjected to LC/MS/MS. The data (See Figure 4-3) display that there is a variation in the type of flavonoids among all the samples, although, quercetin occurs as an abundant type of flavonoid. The reason for this experiment was to confirm and verify that the IMAC technique is appropriate for purification of flavonoids from food samples.

In addition to pure flavonoids, flavonoid extracts were prepared from apples (*Malus Domestica*), Green tea (*Camellia sinensis*), Strawberry (*Fragaria vesca*), Kale (*Brassica oleracea*), Capers (*Capparis spinose*), Garlic (*Allium sativum*) and Onions (*Allium cepa*). The extracts were enriched for flavonoids using the novel IMAC method (See methods and materials) and quantified using the novel BCA method (Table4-1and Table 4-2). The

extracts were applied to the biotin cadaverine incorporation assay (Slaughter et al., 1992) and the peptide cross-linking assay (Trigwell et al., 2004) to examine their effect on TG2 transamidation activity. The data in Figure 4-4 A and B show that most of the food extract display a significant inhibition effect towards the amine incorporation activity of TG2. It is worth a mention, that all the food samples used contain the flavonols, which exert the inhibition action against the amine incorporation activity when used as pure (Figure3-8 and Figure3-18). For the effect on cross-linking activity of TG2, most of food extracts display inhibition effect on the gplTG2 cross-linking activity. Whereas, only the samples from kale and strawberry have inhibition effects on the hrTG2 activity (Figure 4-5 A and B).

There is difference in sensitivity towards the flavonoids extracts between gpl and hrTG2, as most of food extract display a significant inhibition effect towards the gplTG2, whereas, only strawberry and kale inhibit the cross linking activity of hrTG2. The subtle differences in homology between gpl and hr TG2, as mentioned previously (see chapter 3) could be the reason for these measured differences between human and guinea pig TG2 (Aeschlimann et al., 1994). These subtle differences could affect the topography of the enzyme's active site. Although the properties and structure of both gplTG2 and hrTG2 are similar, there is evidence from these plots (See Figure 4-5 A and B) that there is sufficient variation in their structure to demonstrate different responses to the flavonoids and the food extracts. As far as is known Guinea pigs do not suffer CD, Humans do, these data suggest there is a subtle difference between the two enzymes structures? There are indications from data that the search for agents to moderate TG2 in CD must be conducted with hrTG2, as the comparison the data of hrTG2 with gplTG2 display variations in the response between the two enzymes.

From the finding strawberry (33.3%) and kale (28.23%) have higher significant inhibition activity against hrTG2 comparing to other food samples. There are a naturally variations among the fruits and vegetables in; the overall flavonoid content of fruits and vegetables and the protection provided by fruits and vegetables against diseases may differ significantly from one kind of fruit or vegetable to another (Guo et al., 1997). These variations are the possible reason for the variation in the extent of the effect on TG2 activity. In addition, worth mention that the flavonols kaempferol and quercetin are essentially products of flavonoids in kale vegetables (Olsen et al., 2009). This agrees with the previous data when using pure kaempferol and quercetin against TG2 transamidating

activity (Figure3-8 and Figure3-13). Whereas, a study conducted by Tomohiro Itoh et al 2009, mentioned that kaempferol flavonoid extracts from strawberry suppressed the intracellular Ca^{2+} influx in rat basophilic leukemia RBL-2H3 cells. This may suggest a possible mechanism by which flavonoids inhibit TG2 (Tomohiro Itoh et al., 2009). As the bioavailability of a compound is a crucial factor for the assessment of any health-modulating functions in humans. The bioavailability of the flavonol aglycone influenced by the nature and degree of modification and substitution on the flavonol (Stevenson and Hurst 2007 and Itoh et al., 2009).

In order to screen the effects of food extracts on the deamidating activity of hrTG2, as this activity is crucial for the initiation of CD. The finding showed that no food extracts have an inhibition effect after using the optimised OPA assay, (see Figure 4-6). On the other hand, the two extracts from *alliums* (onion and garlic) show activation of the deamidating activity. It could be that people with CD could be activating TG in the *lamina propria* after digesting a meal containing onion and garlic (which is a very common combination for curry and Mediterranea food). The finding of this study presented that the dose produces 50% inhibition effect of the active flavonoids (kaempferol, morin, and quercetin) was 125µM. The question here is how much should be eaten from the food containing these flavonoids to achieve this 125uM concentration. Based on the literature that reported the quantity of flavonoids in food samples, to achieve that concentration the patient should eat more than one kilogram of fruit or vegetable which may be unpractical? Furthermore, to be effective a bioactive compound, not just should the intake be adequate high, however also the bioavailability (the absorption into the body) must be at a suitable level to gain active levels within the body (Van der Sluis et al., 2001).

4.5 Summary of key findings

- Immobilized Metal Ion Affinity Chromatography (IMAC) was used for the first time in the purification of flavonoids from food extracts.
- BCA assay was used in the quantification of flavonoids in food extracts. By using quercetin as standard; the use of the BCA reagent allows the linear detection of quercetin in the standard assay in the range of 1.25 to 400µM.
- The comparison between the fractions from SPE and IMAC purification methods in term of purity was done by HPLC, IMAC fractions have produced a clearer chromatogram.

- Most of the food extract used in this study display a significant inhibition effect towards the amine incorporation activity of TG2.
- Majority of food extracts display inhibition effect on the gpl TG2 cross-linking activity. On the other hand, only Kale and strawberry have an effect on hrTG2 activity.
- No food extracts were showing inhibition for the ammonia released (deamidation activity) effect after using the optimised fluorescence (OPA) assay.

Chapter 5 : Study the action of effective flavonoids on the *in situ* activity of TG2 and identification of TG2 substrates

5.1 INTRODUCTION

Transglutaminase 2 (TG2) possess many enzymatic functions'; transamidating, deamidating, GTP-binding/hydrolysing activity, intrinsic kinase and isopeptidase activities (Griffin et al., 2002; Chen, X et al., 2015). Therefore, it is active in many vital biological processes including proliferation, differentiation, and apoptosis (Griffin et al., 2002; Beninati et al., 2017). In addition, TG2 can incorporate polyamines into acyl-donor substrates, which leads to a change of the structure and function of specific proteins. For example, TG2 catalyses the polyamine incorporation of spermine and spermidine into the neurotransmitter peptide substance P, producing enriched resistance to proteases action *in vitro* (Esposito et al., 1999). In addition, TG2 has ability to catalysing the incorporation of polyamines into a variety of substrates including proteins, peptides, mono- and polyamines and nucleotides. To fully understand the cellular role of TG2 a knowledge of TG2 substrates and interacting proteins, which might be acting as novel drug targets is required. In this line, the development of selective TG2 inhibitors could regulate TG2 activity and therefore could act as novel therapeutic solutions (Badarau et al., 2013).

There are numerous biochemical and cellular assays, which help to characterise the potency of TG2 inhibitors. These assays involve assessing the transamidation activity of the target TG2 itself, measuring the TG2 selectivity among the TG isoforms and cross-reactivity to other catalytic cysteine-containing enzymes (e.g. caspase-3) as well as other side effects such as cytotoxicity.

5.1.1 Viability assays

In vitro viability assays are necessary tools to aid in the assessment of drug efficacy in combination with *in vivo* analyses. The general concept of *in vitro* viability assays requires the incubation of cells with a particular compound(s) and then measuring for cell viability to quantify drug-induced cell toxicity (Single et al., 2015). A cell-based method is required for the evaluation the drug induced cell cytotoxicity including measuring cellular metabolic activity. A widely used method is the MTT assay based on the ability

of viable cells to convert a soluble tetrazolium salt [3– (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) into an insoluble formazan precipitate (Supino et al., 1995). The MTT assay is a rapid colorimetric quantification assay for the growth and survival of cells (Mosmann 1983)Freimoser et al., 1999). In contrast to endpoint methodologies, a real-time cell-based assay approach permits following cellular growth over the whole time course of an experiment. Real-time assays are classically accomplished with equipment capable of capturing cell images at regular time intervals and quantifying cellular surface area coverage as a measure of proliferation (Single et al., 2015). In this study, human large intestine carcinoma cell lines (HT29) was used with MTT and real-time IncuCyte assays to assess the effects of three flavonoids (kaempferol, morin, and quercetin) were shown to moderate TG2 activity (Chapter 3) using *in vitro* assays on the viability and the growth of colon carcinoma cells.

5.1.2 Transglutaminase 2 substrates

The list of TG2 protein substrates is continuing to grow with the increase in TG2 enzymatic functions. However, the description of substrates for all these functions, how they influence the role of TG2 within the cell is proving difficult. Table 5.1 presents some of the major substrates for each chemical reaction catalysed by TG2.

Table 5-1: TG2 substrates and their possible involvement in human diseases

Substrate	Involvement in
Acetylcholine esterase	Neurological diseases (Hand, Dias and Haynes 2000).
Actin	Cytoskeleton regulation. (Nemes, et al. 1997)
Aldolase	Metabolic and endocrinology diseases, genetic disease and other autoimmune, inflammatory and related diseases.(Lee, et al. 1992)
Amines (monoamines, diamines, polyamines): cadaverine, histamine, putrescine, serotonin, spermidine, spermine	Autoimmune, inflammatory and related diseases.
Androgen receptor	Endocrine and metabolic diseases (Mandrusiak, et al. 2003).
Annexin I (lipocortin I)	Autoimmune and inflammatory diseases, Cytoskeleton regulation.(Ando, et al. 1991)
Beta amyloid peptide	neurological diseases
Calgizzarin-S100C protein-MLN70—S100A11	Endocrine and metabolic diseases, dermatological diseases
Cell adhesion molecule C-CAM	extracellular matrix-cell interaction and stabilisation (ECM-S)
Collagen alpha 1(III)	stabilization, autoimmune and inflammatory diseases(Orban, et al. 2004)
beta A3,B3 and BP crystalline	cytoskeleton regulation, and protein stabilisation
Gluten proteins (alpha/beta-, gamma-gliadin, and low molecular weight glutenin)	Coeliac disease(Kim, Jeitner and Steinert 2002)
Huntingtin	neurological diseases
Fibronectin	Protein stabilisation, extracellular matrix interaction and stabilisation [6
Insulin (A and B chain)	metabolic and endocrinology diseases
Keratin, type II cytoskeletal 2 epidermal	dermatologic diseases, cytoskeleton regulation, and membrane traffic and membrane structure/function
Osteocalcin	Autoimmune and inflammatory diseases.
alpha-Synuclein	neurological diseases
Tubulin	cytoskeleton regulation

Adopted from: Odii and Coussons (2014)

5.1.3 Involvement of TG2 substrates in cell life and death

Many reports have shown that TG2 could act as a pro-apoptotic protein (Fesus, et al., 1991; Falasca, et al., 2005). Some of the identified TG2 substrates, which have a covalent modification, may be related to the induction of the cell death, such as, cytoskeletal proteins (e.g. actin and beta-tubulin) and nuclear histone proteins (Ballestar, et al, 1996). Transglutaminase 2 catalyses covalent modifications to proteins, that play a role in the programmed cell death cascade (Huppertz, Frank and Kaufmann 1999, Tabolacci, et al. 2019). In addition, TG2 has been shown to cross-link key modulators of cell survival and death, such as retinoblastoma protein (Oliverio, S et al., 1997). TG2 can also modify a member of Ras superfamily (RhoA) *in vivo* (Singh et al., 2001). Retonic acid-induced activation of transglutaminase leads to the transamidation of the RhoA protein. (Singh et al., 2003).

5.1.4 The involvement of TG2 substrates in coeliac disease

The upregulation of TG2 expression and/or function has been shown to be involved in a several human diseases, such as coeliac disease, diabetes mellitus, some cancers (e.g. breast) and a number of neurological diseases e.g. Alzheimer's and Parkinson disease (Lai et al., 2017). The role of TG2 in coeliac disease is related to deamidation of glutamine side chains in a 33mer gliadin peptide, the deamidation reaction is preferred to transamidation when the pH decreases (Fleckenstein, B et al., 2002; Fleckenstein, B et al., 2004). Thus, gluten proteins and their derived peptides represent substrates of different TG2-catalysed reactions.

5.1.5 Enzymes activity in living intestinal cells

In order to study the activity of enzymes such as TG2 in the digestive tract, human intestinal cell lines demonstrate a valuable model. Transglutaminase 2 functions appear to depend on its particular location. The occurrence of different available proteins which able to act as a substrate for TG2 in specific cell determines the activity of TG2 (Oliverio et al., 1997). The main aim of this study has been focussed on TG2 deamidating activity in relation to coeliac disease. Therefore, to better understand the function of the TG2 in the initiation of CD it is crucial to identify TG2 substrates in the intestinal cell lines such as CaCo2 and HT29 cell lines. Although both of these cell lines are derived from a colonic

neoplasm, they display features of small intestinal differentiation in the post-confluent state (Pinto, 1983). These cells (HT29 and CaCo2) have been used widely for studying TG2 enzymatic activity *in situ* and for identification of TG2 substrates, more than 25 TG2 substrates were identified in CaCo-2 cell lines using top-down proteomic methods. Whereas, fructose-l, 6-bisphosphate aldolase A was identified as a TG2 substrate in HT29 cells (Lee et al., 1992; Orrù et al., 2003)

However, the main impediment to this type of investigation is that there are no animal or cell models for CD. With this in mind, this study has focussed on the effect of the flavonoids on TG2 activity by using intestinal epithelial cell models. Therefore, knowledge on the actions of flavonoids at the GI tract is essential to design dietary and pharmacological approaches for the prevention and/or treatment of disease. The later sections will explain the interactions of flavonoids in the intestinal tracts.

5.1.6 Host Metabolism of Flavonoids

After ingestion, flavonoid glycosides in food are deglycosylated and further conjugated in the small intestine where some are absorbed, while the rest pass into the colon where they metabolized by the gut microbiota (Manach et al., 1997; Hein, et al., 2008). Two enzymes in the human small intestine have been identified to deglycosylate flavonoids (Day, Andrea J., et al., 2000; K Németh et al., 2003). The brush border enzyme lactase-phlorizin hydrolase a was reported to hydrolyse O-glucosides of quercetin, genistein, and daidzein *in vitro* (Day et al., 2000). Whereas, cytosolic beta-glucosidase in the enterocytes was stated to hydrolyse O-glucosides of quercetin and genistein, in cell-free extracts from human intestine (Day, et al., 1998). Generally, the physicochemical properties such as molecular size and configuration, lipophilicity, solubility, and pKa are the most important factors influence the absorption of the flavonoid (Hollman 2004).

5.1.7 Flavonoids actions on the gastrointestinal tract:

Flavonoids can target the lumen content and/or the different cell types that are involved in sustaining gastrointestinal tract (GI tract) physiology. The intestinal monolayer is composed of: i) intestinal epithelial cells that engage in nutrient absorption, and the preservation of barrier integrity ii) goblet cells that responsible for the production of mucins that act as a lubricant and a barrier against bacteria and toxin incursion and iii) Paneth cells that secrete bactericidal peptides (Figure 5-1).Immune cells located at the

lamina propria work in combination with the above mucosal cells to integrate the GI tract immune system (Brown et al., 2013). Additionally, ingested flavonoids have beneficial actions on the GI tract such as i) protection of the intestinal wall against pharmacological and food toxins; ii) sustaining the intestinal barrier integrity; iii) modulation of the secretion of gut hormones; iv) modulation the GI tract immune system; v) have possible anti-colorectal cancer activity; and vi) shape microbiota profiles(Oteiza, et al. 2018)

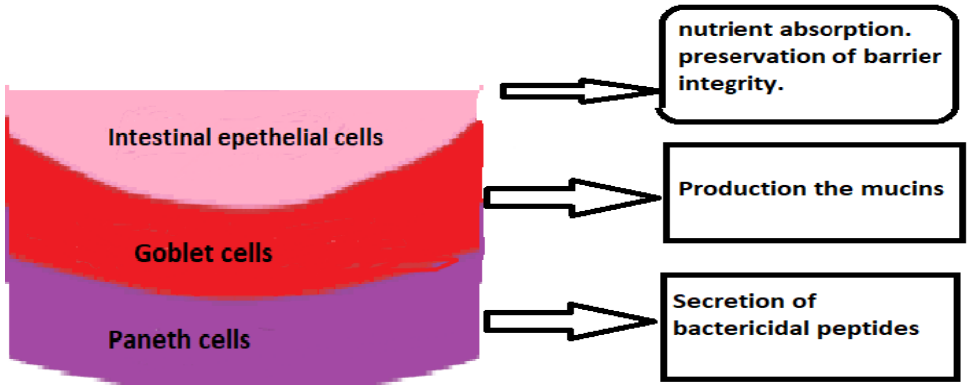


Figure 5-1 Schematic diagram for intestinal monolayer

Human carcinoma large intestine epithelial cell lines such as HT29 and CaCo2 have been used in the study the different biological actions of different flavonoids. Kameoka et al., (1999) used the CaCo2 cell line to study the effect of flavonoids (genistein, biochanin A, quercetin, and kaempferol) on the expression of antioxidant proteins. Salucci and co-worker (2002) used epicatechin, epigallocatechin gallate, gallic acid and quercetin with CaCo2 cells to investigate their antioxidant activity, cellular uptake and cytotoxicity effects at different concentrations of flavonoids. The CaCo2 cell lines also have been used to study the structure and permeability relationship of flavonoids to this type of cells (Fang Yajing et al., 2017). Whereas, HT29 cells were used in the study of the different effects of flavonoids such as investigate the antitumor activity of taxifolin in colorectal cancer cell lines (Razak, et al, 2018). Based on own search of the literature there are no published studies which deal with the effect of flavonoids on the *in situ* activity of TG2. The aim of this chapter was to study the cytotoxicity of the flavonoids (kaempferol, morin, and quercetin) which have been shown to moderate the activity of TG2 *in vitro* (chapter 3). In addition, using HT29 cells to study the effects of kaempferol, morin, and quercetin on the *in situ* activity of TG2 and determine some of the cellular substrates TG2 targeted

5.2 Hypothesis

The flavonoids that have TG2 inhibitory actions are non-toxic to use with the human cell lines (HT29) and are cell-permeable. In addition, the human cell lines (HT29) contain TG2 protein substrates. When cellular TG2 activity is moderated by flavonoids that have TG2 inhibitory actions. These protein substrates may be identified.

To test the hypothesis, it was aimed to;

- Study the effect of flavonoids on cell proliferation and cell viability using MTT reduction assay and a cell proliferation assay (IncuCyte FLR; Essen BioScience, DE).
- Evaluate the effect of flavonoids on cellular TG2 activity using HT29 cell lines through Western blotting and fluorescent microscopy.
- Identify target TG2 substrates using SDS-PAGE and RP-HPLC/MS/MS.

- **Methods and Materials:**

See Methods and material chapter:

- MTT reduction assay (section2.2.9e).
- Live cell imaging: (section2.2.9e).
- Visualisation of *in situ* TG2 transamidase activity (section2.2.6c)iii.
- *In situ* biotinylation of proteins in HT29 cell lines(2.2.9d)

5.3 Results

5.3.1 The effect of three flavonoids on cell viability

A widely used method to determine the cytotoxicity behaviour of drugs includes endpoint measuring cellular metabolic activity and real-time assay systems that allow for the tracking of cellular growth over the entire time course of an experiment. In order to observe the cytotoxic effect of three flavonoids (kaempferol, morin, and quercetin) which moderated TG2 *in vitro* activity (see chapter 3), it was deemed important to observe any potential toxic effects, which could influence cell viability. This was done by two methods: an (MTT) assay (Vyas et al., 2016)and a cultured cell proliferation assay (incuCyte by ESSEN Bioscience DE).

a) The effect of flavonoids TG2 inhibitors on cell viability using MTT assay

TheHT29 cell extracts were treated with different concentrations of kaempferol, morin and quercetin flavonoids, any changes in the cell viability were monitored via MTT reduction assay (section 2.2.9e)i.

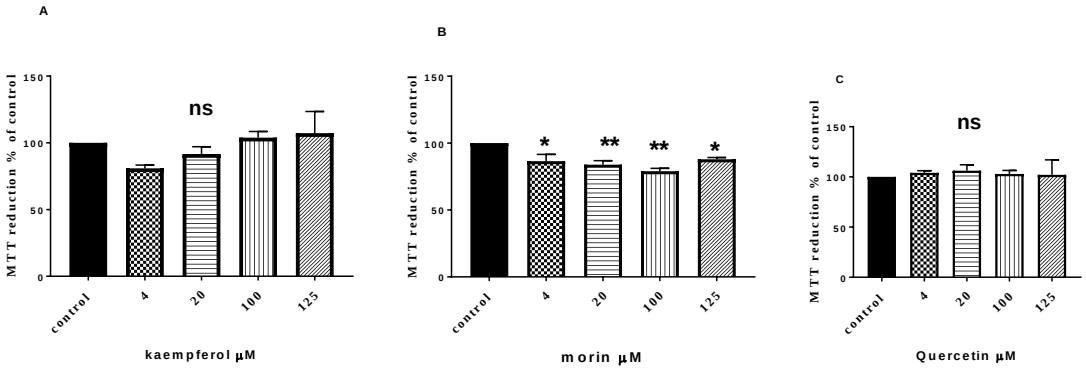


Figure 5-2: MTT reduction assay to measure cell viability in HT29 cells following treatment with kaempferol, morin, and quercetin.

HT29 cells were treated with different concentrations (4-125 µM) of kaempferol, morin, and quercetin. and incubated for 1.0 h at 37°C. Cell viability was determined by MTT reduction assay (section 2.2.9). The data points represent the mean ± S.E.M from 3 independent experiments. All the values are as compared to the control (n=3, P-value * < 0.05, **** < 0.0001).

The large intestine epithelial HT29 cell line showed no loss of cell viability (as measured by MTT reduction assay) when treated with different concentrations of kaempferol and quercetin, whereas, morin displayed a moderate but statistically relevant effect on the viability of the cells as measured by MTT assay.

b) The effect of flavonoids TG2 inhibitors on cell growth using a real-time proliferation assay (incuCyte).

HT29 cells were treated with kaempferol, morin and quercetin flavonoids TG2 inhibitors to visualise and quantify any effect on the cell proliferation.

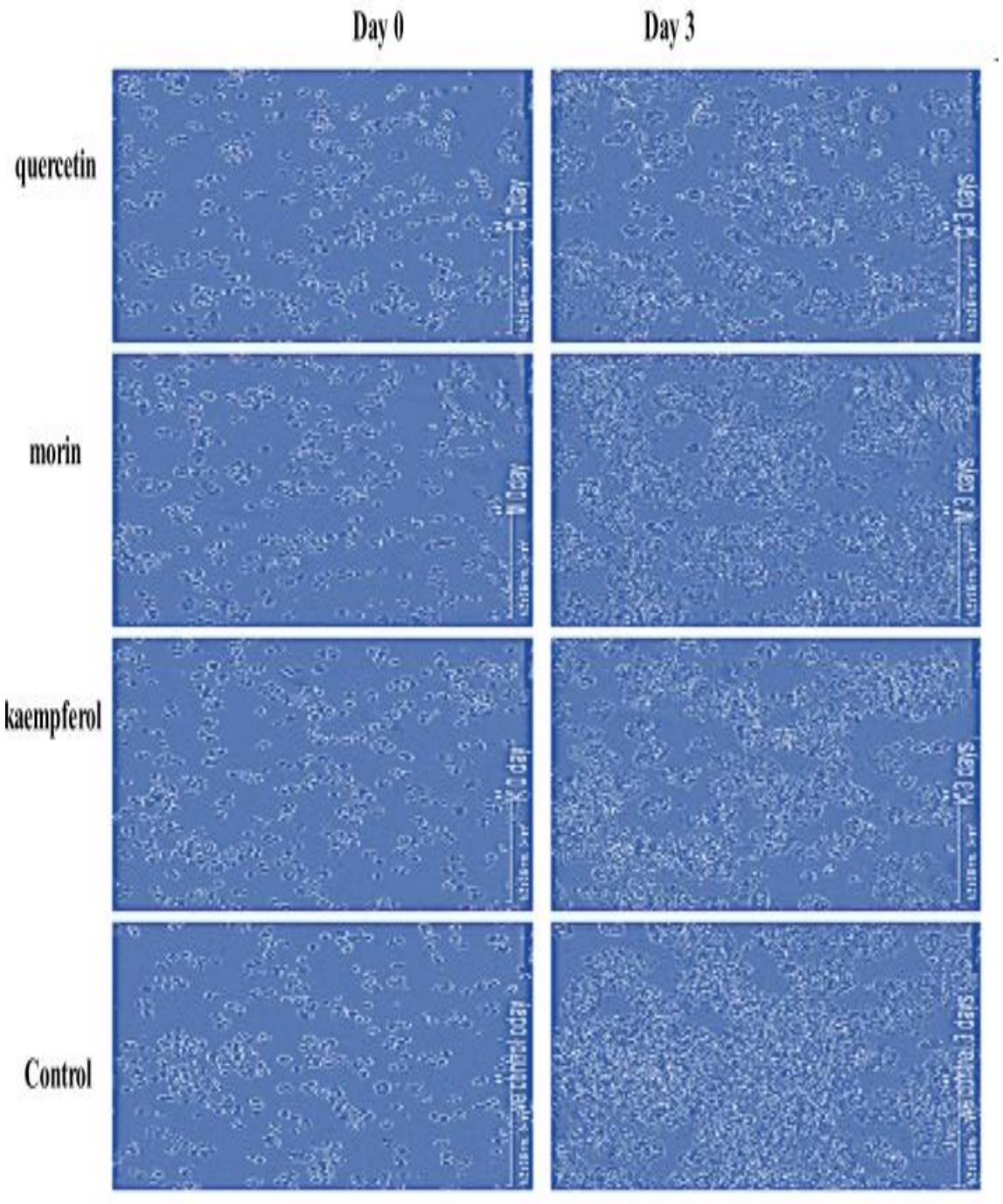


Figure 5-3: Images show the effect of significant flavonoids TG2 inhibitors on HT29cell growth

Cells were treated with kaempferol, morin, and quercetin at 125µM. Then allowed to proliferate for three days. Images were taken using Incucyte S3 live-cell imaging system (10× objective). Control: cells without flavonoids treatment.

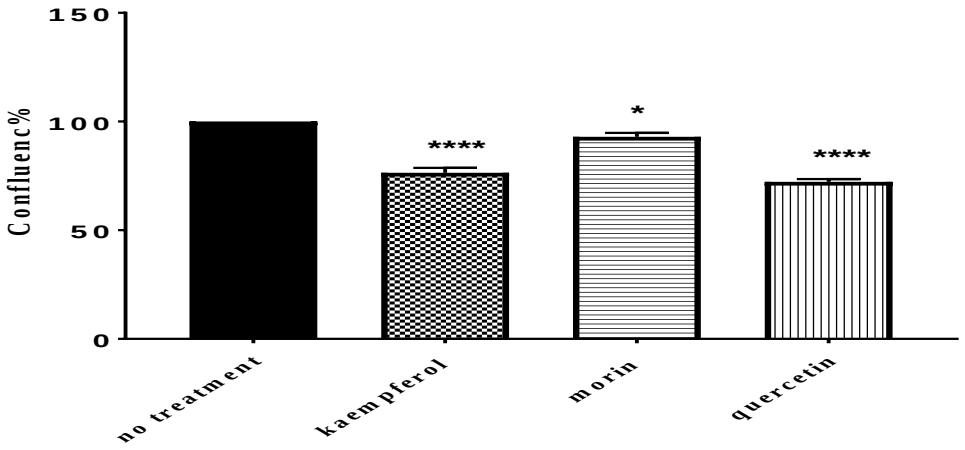


Figure 5-4: The effect of flavonoids TG2 inhibitors on HT29cell Confluence.

HT29 cells were treated with (125 µM) of kaempferol, morin, and quercetin. Then allowed to proliferate for three days. Their effect on the growth of the cell was determined using Incucyte S3 live-cell imaging system (10× objective). (Materials and methods chapter, Section 2.2.9e)ii). The data points represent the mean ± S.E.M from 3 independent experiments. All the values are as compared to the control (n=3, P-value * < 0.05, **** < 0.0001).

The data in Figures 5-3 and5-4 shows the inhibition effect of kaempferol, morin, and quercetin at 125µM on the HT29 cell growth, the effect of kaempferol and quercetin was significantly more than the morin effect.

5.3.2 Fluorescence microscopy of HT29 cells incubated with biotin-X-cadaverine

HT29 cells were treated with biotin-X-cadaverine followed by incubation with 125µM flavonoids (kaempferol, morin, and quercetin) followed by probing with FITC ExtrAvidin.The intensity of green colour indicates intracellular TG2 activity (see section 2.2.9d). ZDON used as negative control.

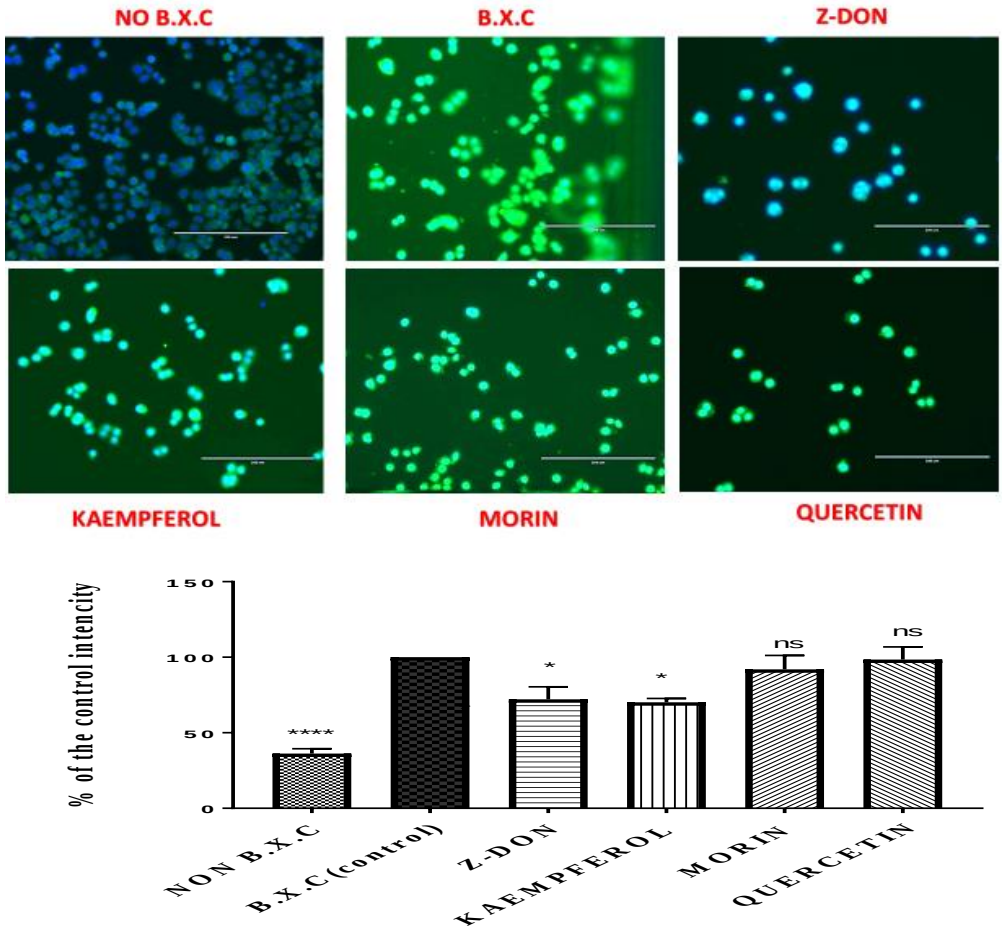


Figure 5-5: Fluorescence microscopy of HT29 cells.

Fluorescence image of the HT29 cell lines incubated with biotin-X-cadaverine (B.X.C) (Materials and methods chapter, section2.2.6c)iii). No B.X.C: untreated HT29 cells as a negative control. B.X.C: HT29 cells treated with 1.4 mM biotin-X-cadaverine as a positive control. kaempferol, morin, quercetin: HT29 cells treated with 1.4 mM biotin-X-cadaverine and then 125 µM flavonoids for 1.0 h. Z-DON 125 µM (TG2 inhibitor): HT29 cells treated with 1.4 mM biotin-X-cadaverine and then with 125 µM 1.0 h. After treatments, cells were fixed and permeabilised before the TG2 activity was visualised using ExtrAvidin - FITC (green) and DAPI was used for nuclear staining (blue). The images were viewed using an EVOS FL imaging system (ThermoFisher).

The fluorescence assay confirmed the TG2 inhibitory characteristic of the kaempferol because the treatment of HT29 cell lines with this flavonoid displayed significant inhibition of *in vivo* TG2 activity (green colour). Whereas, morin and quercetin showed no statistically significant inhibition of *in vivo* TG2 activity(Figure 5-5).

5.3.3 Identification of biotin cadaverine labeled proteins in HT29 cells that show reduced labeling with flavonoids TG2 inhibitors

To identify potential protein targets of TG2 activity following treatment by flavonoids, cells were incubated with biotin-X-cadaverine and flavonoids. The proteins labeled with biotin in the lysates from cells were subjected to SDS-PAGE on a 4-15 % polyacrylamide

gradient gel, Western blotting and probed with extravidin HRP. Protein labeled with biotin-X-cadaverine were visualised using ECL reagents (2.2.9d)

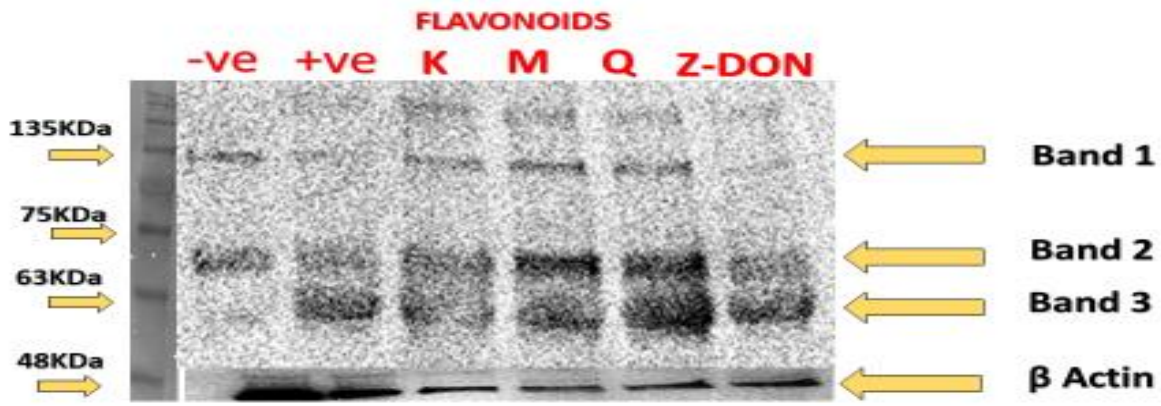


Figure 5-6: Western blotting analysis of the inhibition of TG2 substrate labeling in HT29 cells by three significant flavonoids.

HT29 cells were incubated for 4.0 h with 1.4 mM biotin-X cadaverine (a cell-penetrating substrate of TG2) (section 2.2.9d). The biotinylated proteins were extracted and 20 µg separated by SDS-PAGE, transferred to nitrocellulose membrane filters and probed with Extra Vidin Peroxidase (Materials and methods chapter, section 2.2.9d). HT29 cell lines treated with 1.4 mM biotin-X-cadaverine as a positive control (+ve), HT29 cell lines treated with 1.4 mM biotin-X-cadaverine for 4.0 h and then treated with 125 µM of flavonoids (K, kaempferol, M, morin, Q, quercetin) and 125µM Z-DON (TG2 inhibitor). Untreated HT29 cell lines (-ve) 1.0 h. The staining of β Actin on the same blots confirmed equal protein loading.

Two bands were visualized in the negative control of non-biotin cadaverine treated HT29 cells. In contrast, the positive control cells show three biotin cadaverine labelled protein bands, corresponding to approximate molecular weight of 135, 75 and 63 kDa. The flavonoids at these concentrations displayed different effects on the *in situ* activity of TG2. Kaempferol showed slight inhibition of the band labelling, more than the effect of morin and quercetin. (Figure 5.6).

5.3.4 Biotinylation and fractionation of TG2 substrates

The biotinylated target proteins in treated cells were subjected to SDS-PAGE on a 4-15 % polyacrylamide gradient gel, Western blotting and probed with extravidin HRP. Protein labelled with biotin-X-cadaverine were visualised using ECL reagents. The protein bands which showed decreased labelling following kaempferol treatment were excised from the gels and subjected to tryptic digestion (see materials and methods chapter 2 section ??). Peptide fragments were then directly analysed by RP-HPLC -MS/MS. By this method, multiple proteins were identified within a single protein band (Table 5-2).

Table 5-2: Identification of TG2 proteins substrates in HT29 cells.

Protein	Mass (Da)	functions
BAG family molecular chaperone regulator 3	61,595	Adenyl-nucleotide exchange factor activity(Rauch Gestwicki, 2014) protein-containing complex binding() protein transporter activity
RAF proto-oncogene serine/threonine-protein kinase	73,052	Enzyme binding (Brown et al., 2013) metal ion binding, protein kinase activity and protein serine/threonine kinase activity (Crespo et al.,1994; Polzien etal., 2009).
Heat shock factor protein 4	53,011	DNA-binding transcription factor activity (Gaudet et al., 2011) protein phosphatase binding (Hu Y and Mivech,2006)

5.4 Discussion:

In order to test the therapeutic efficacy and safety of the flavonoids (kaempferol, morin, and quercetin) that displayed *in vitro* inhibition against hrTG2 transamidation activity as explained in chapter 3. It is important to characterise these proposed flavonoids TG2 inhibitors in more detail. Obstacles impeded the evaluating of the therapeutic potential behaviour of TG2 inhibitors in relation to CD, such as the non-availability of animal or cell model for the coeliac disease to test the CD correlated histopathological, immunological and serological abnormalities (Auricchio, S et al., 1984 and Papista et al., 2012). Although HT29 cells are not ideal for studying CD, however, the cells provide an indication of the effects of different reagents on disease relevant to a human cell type. The HT29 cell line was derived from large intestine carcinomas but are used worldwide by the researchers as the model of human intestinal epithelial cells. As TG2 is active in the *lamina propria* of human small intestines and believed to be exported from epithelial cells, it would be an advantage to use a small intestinal epithelial cell line (Papista et al., 2012). However, the human small intestine carcinoma cell line FHs 74 Int are slow to grow, difficult to culture and maintain. In this study, HT29 was used and the results must be viewed with the limitation that they are not the best cell model but they will provide some insight into the role of TG2 in intestinal epithelial cells.

Kaempferol and quercetin (4 to 125 µM) have no inhibition effect on the large intestine epithelial HT29 cell viability (as measured by MTT reduction assay) (Figure2-3)). Whereas, morin displayed a moderate inhibition effect on the viability of the cells. Morin

shows slight differences in structure compared with the other two flavonoids, as it contains hydroxyl group in ring B at carbon 2, while, kaempferol and quercetin contain OH groups in other locations (Figure2-3). These findings are in line with the notion that was reported by Pradhan et al., (2018), that the presence and location of hydroxyl groups on the B-ring of flavonoids impacts on its activity. Additionally, these findings agree with the study done by Fernández-Blanco and co-worker (2016), which reported that quercetin, has no effect on the viability of HT29 and Caco2 cell lines. One of the proposed mechanism of cell protective effect of quercetin is the ability to penetrate into the polar medium inside the cells and to protect them against the toxins (Rezaei-Sadabady et al., 2016). As mentioned by Kuntz and co-workers (1999), the cellular protective effects of flavonoids in colon cancer development should be accompanied with the inhibition of cell proliferation and/or initiation of the apoptotic pathway to delete cells carrying mutations and to maintain a normal cell population. This directed the research to study the effect of kaempferol, morin, and quercetin on the proliferation of the large intestine epithelial HT29 using the IncuCyte® Live-Cell Analysis Essen Bioscience System. The data in Figure 5-3 and Figure 5-4 show a statistically significant inhibitory effect of the three flavonoids on cell proliferation. These findings are consistent with the study done by Kuntz et al (1999), which reported that flavonoids display growth inhibition effect in the absence of cytotoxicity. Furthermore, the anti-proliferative effect the flavonoids are in agreement with number of studies that present the anti-proliferative behaviour of flavonoids against a number of cancers such as gastric, colon cancer cells and leukemia T-cells (Yoshida et al., 1990).

Flavonoids are as known have antioxidant actions, so the reduction of cell growth might, therefore, depend on the ability of these compounds to serve as free radical scavengers. The accumulation of dietary flavonoids was higher in the intestinal epithelium than cells in other tissues (Kuo, 1998). That indicates that the dietary polyphenols could have a significant role in the prevention of colon cancer by blocking hyperproliferation of the epithelium. There is a high expression of TG2 in stroma of cancer cells (Eckert et al., 2015), which suggests this TG2 expression in tumour stroma is required for metastatic progression of colon cancer. TG2 was found to induce chemo resistance through inhibition of apoptosis and autophagy in human breast cancer cells (Antonyak, M.A., 2004; Kumar et al., 2010 and Calcagno, et al., 2010). It has been mentioned, in different studies that TG2 levels are lower in colon cancer cells comparing to normal cells, and this

increase leads to apoptosis and cell growth inhibition (Takaku, et al. 1995 and Kosa, et al. 1997). Nevertheless, other studies have proposed that TG2 levels raised in colon cancer and may be a useful indicator for the prognosis of colorectal cancer (D'argenio, et al. 1995 and Miyoshi, et al. 2010). The development of molecular inhibitors selectively targeting TG2 may provide promising results in the treatment of inflammatory disorders (Badarau et al., 2013). Published knowledge has displayed the importance of the inhibition of TG2 activity as a potential therapeutic intervention in various cancer types (Carbone, et al. 2017). Based on these reports and by comparing the significant inhibition for the transamidation activity of hrTG2 via the flavonoids used in this study and the anti-proliferative data of the same flavonoids, all these data could be providing an indication that there may be a link in the mechanisms of different actions of these flavonoids.

As there is little information available on TG2 substrates in HT29 cells, hence the identification of the TG2 substrates can be considered an achievement. Moreover, because of the association of TG2 with CD, the identification of its substrates in human intestinal cell lines may help to identify potentially important drug targets (Caputo et al. , 2004). To detect the effect of the kaempferol, morin, and quercetin, on *in situ* and to identify the TG2 target substrate biotin-Xcadaverine (a cell-penetrating substrate of TG2) was used to label intracellular proteins in HT29 cells via intracellular TG2 activity. When viewed with a fluorescence microscope using ExtrAvidin®−FITC to spot biotin labelled proteins (Figure 5-5). The data showed that the intensity of green colour in fluorescence assay, which reflects the intracellular TG2 activity, was reduced by 125µM kaempferol treatment confirming the TG2 inhibitory characteristic of the kaempferol. Whereas morin and quercetin their inhibition of TG2 activity in HT29 cells was statistically insignificant, these findings are inconsistent with *in vitro* TG2 assays (Figure3-8 and Figure3-13).

To understand the reasons for this variation, factors must take into account, such as the fact that inhibition of TG2 by the flavonoids was assessed using an *in vitro* assay, where the TG2 and substrates are pure, and the environment of the assay was different. In addition, the flavonoids in the cells could be exposed to pharmacokinetics reactions such as metabolism that lead to change in flavonoids structure (Manach, and Donovan, 2004), which may consequently impact on their effect towards the TG2 in the cell lines. Moreover, the presence of other members of TG family in cell lines could provide overlapping biological functions that will affect the results of the *in vivo* assays (Facchiano et al., 2006). Additionally, these findings are consistent with the results by

González-Gallego et al (2018), which state the flavonoid metabolites have different antioxidant and biological properties than their parent compounds.Further studies are needed to examine the mechanisms of the actions of the flavonoids on the cellular activity of the TG2.

In order, to determine the specific intracellular TG2 protein targets in the HT29 cells. The biotin cadaverine labelled proteins were extracted and analysed by SDS-PAGE, and Western blotting. The Western blotting image for HT29 cell lines (Figure 5-6) showed that certain bands had partially disappeared in cell treated with the Z-DON. However, the intensity of bands in the cells lysates subjected to treatment with kaempferol decreased significantly in HT29 cell lines. The morin and quercetin treatments show a non-significant decrease for the bands chemiluminescent intensity. These findings confirm the finding of the fluorescence microscope experiments and agreed with study done by Walle and colleagues (1999), which mentioned that effective metabolism by the cancer cells (Caco2) might limit the oral bioavailability of flavonoid (chrysin) in humans. However they do disagree with the *in vitro* TG2 assay (Figure3-8 and 3-13).which show that the kaempferol, morin, and quercetin have inhibition effect on the TG2 activity. This may be partially explained by a different environment and different substrate that the TG2 was assayed in.

Because of the fact, that different physiological activity of TG2 is based on the diversity of the protein substrates of the transamidation activity (Ichikawa et al., 2008). Hence, it is necessary to have a better knowledge of its substrates, target sites and interacting proteins that may act as novel drug targets or new diagnostic markers. Therefore, the attempt to identify the protein substrates of TG2 was conducted in order to understand the physiological roles of TG2 at the molecular level. Various proteins were identified by using SDS-PAGE linked to RP-HPLC-MS/MS, this approach was used previously by (Orrù et al., 2003and Robinson et al., 2007) to detect TG2 protein substrates. The advantage of the current method was to use the biotin-X-cadaverine, which is the cell-penetrating synthetic TG substrate as a probe to identify TG2 protein substrates. This amine substrate does not need the addition of a calcium activation buffer and does not interfere with normal cell processes (Esposito and Caputo, 2005). The proteins were extracted from gel by first dehydration in acetonitrile and hydration in 50.0 mM ammonium bicarbonate, followed with trypsin digestion as described in Material and Methods (see chapter 2 section2.2.4). The tryptic peptide samples were then directly

analysed by RP-HPLC-MS/MS in which multiple proteins were identified within a single protein band (~70 kDa). Identified proteins following peptide mapping using software were listed in Table5-2.

As this band was extracted from 1D Gel, many proteins have been detected, however, the proteins that show high sequence coverage and corresponding to the band size were selected from the data. The peptide sequences obtained from MS/MS were searched for homologies in the databases; (https://www.uniprot.org/). The identified TG2 substrates in this study indicate the diversity of proteins with biological functions might be used as substrates of TG2.

According to the TRANSDAB database

http://genomics.dote.hu/wiki/index.php/Category:Tissue_transglutaminase

Some of the identified proteins have not been previously identified as TG substrates in either HT29 cells or in other cell lines. These include the BAG family molecular chaperone regulator 3, and RAF proto-oncogene serine/threonine-protein kinase. According to their biological functions, the identified target proteins were categorised into several groups. These include chaperone proteins, kinases, serine/threonine-protein kinase transferase, activator, DNA-binding and actin binding. The BAG family is a multifunctional group of proteins which have several functions ranging from apoptosis to tumorigenesis. Six BAG family members were identified in humans, which have been proposed to play a role in HIV infection, carcinogenesis, and Parkinson's disease (Kabbage and Dickman 2008). Therefore, these proteins are considered as potential therapeutic targets, and their expression in cells may act as a predictive tool for those diseases.

BAG-3 (CAIR-1/Bis) has a unique domain arrangement: it contains a WW domain followed by a proline-rich region with PXXP motifs (Figure 5-7) (Kabbage and Dickman 2008). The CAIR-1 annotation happened when calcium entry blocker-treated cells showed increased BAG3/CAIR-1 expression levels (Doong, et al. 2000). Overexpression of BAG-3 resulted in the inhibition of the degradation of poly-ubiquitinated Hsp70 client proteins (Doong, et al. 2003). BAG-3 has a potential role in both cancer and HIV therapies. In cancer cells, BAG-3 was found damaging the response to therapy and sustaining cell survival, hence, they are considered as a probable target for anti-neoplastic therapies

(Rosati, et al., 2007). In HIV-1, BAG-3 blocks the function of p65, providing a potential tool for suppressing HIV-1 gene expression (Rosati, Leone, et al. 2007).

Figure 5-7: Structure of the human BAG3

Structure of the human BAG family proteins, showing the conserved BAG domain, TXSEEX and PXXP repeats, nuclear localization signal (NLS), and WW domain. The number of amino acids is shown on the right of BAG protein.

Another protein was identified as serine/threonine-protein kinase, which works as a regulatory link between the MAPK/ERK cascade and the membrane-associated Ras GTPases. This regulatory link has crucial roles in determining cell fate processes including proliferation, differentiation, apoptosis, survival and oncogenic transformation (Brown, et al. 2013). Upon activation, Raf-1 phosphorylates mitogen-activated protein kinase kinase (MEK), which in turn activates mitogen-activated protein kinase/extracellular signal-regulated kinases (MAPK/ERKs), leading to the propagation of signals (Chen, et al., 2001). Additionally, it plays a function in the oncogenic transformation of epithelial cells through repression of the TJ protein, occludin (OCLN) by activating the up-regulation of a transcriptional repressor SNAI2/SLUG that stimulates down-regulation of OCLN (Brown, et al. 2013). It is noteworthy, that TG2 has kinase activity since it was indicated that in breast cancer cells, TG2 phosphorylates insulin-like growth factor-binding protein-3 (IGFBP-3), thereby minimising its pro-apoptotic activity (Mishra & Murphy, 2004). Therefore, it might be interacting with this protein as substrate to perform the protein kinase activity.

Heat shock factor protein 4, is the other protein identified in this study, which has already been reported as substrates for TG2, but not in HT29 cells. Cells from most organisms respond to a diversity of stresses through the rapid synthesis of a highly conserved series of polypeptides known as heat shock proteins (HSPs). There are considerable indications that these stress proteins are critical for survival at both normal and high temperatures. HSPs also appear to play a crucial role in the development of thermotolerance and protection from cellular damage associated with stresses such as cytokines, ischemia, and energy depletion (Kregel et al., 2002). Heat shock factor protein 4 belongs to the Hsp70 group of the protein and has been linked with different functions in the human body

(Tavaria et al., 1996and Morano, 2007). Proteins in the Hsp70 group have common protein sequences, however, they synthesized in response to different stimuli. Several studies have reported, that the activation of these proteins was accompanying with the increase of tolerance to a diversity of stresses. Such as, hypoxia (Guttman, et al. 1980and Hahn and Li 1982), ischemia (Marber, et al. 1995), energy depletion (Schläfli, et al. 2011), cytokines such as tumor necrosis factor-α (TNF-α) (Jaattela and Wissing 1993) , acidosis (Weitzel, Pilatus and Rensing 1985), and ultraviolet radiation (Barbe, et al. 1988). Benjamin and co-workers (1998) reported that the heat shock proteins are crucial for the wound healing process through protection of the tissues against injury. These actions were performed by repairing any damaged proteins. As TG2 is also involved in the wound healing process, hence it is likely that TG2 might be interacting with Hsp 70 to protect the intestinal cells from harmful components as one of its biological function. The accurate mechanisms by which TG2 interacts with this substrates are not clear and additional studies are reasonable to elucidate the interaction of TG2 with these proteins in human intestinal cell lines and its implications in the physiological functions.

The noticeable finding from this part of the work is the presence of numerous number of proteins identified in gel extracts. To deconvolute the results it could be necessary to use Captavidin-agarose (García-Aljaro, Muñoz and Baldrich 2009). These avidin proteins have the ability to form tight complexes with one or more biotinylated compounds (substrates) (Haugland and Bhalgat 2009). It could be utilised to bind and release biotinylated bio components thus reducing the background contamination. An additional experiment is needed in future using captavidin in order to capture specific TG2 biotinylated substrates.

Generally, in relation to coeliac disease as mentioned in literature flavonoids such as quercetin exert their cellular protective effect in CD by different mechanisms, such as restoration of intestinal barrier tight junction impairment that induced by oxidative stress and inflammatory cytokines (Suzuki and Hara 2011). Flavonoids have been assessed in normal and cancer cultured cells, and studies have shown that these bio compounds exerted their effects in a selective way, which mean by using the same concentrations, same compounds, and same assay induced. For example, the major catechin in tea, (EGCG) has been reported to activate the apoptosis of different types of cancer, such as gastric cancer (Facchiano 2006, Horie, et al. 2005), colon cancer (Chen, et al. 2003), lung cancer (Yang, et al. 1998). However, study by Hsu et al (2003) has reported a differential

effect of EGCG on cell proliferation in normal human primary epidermal keratinocytes after 24 h of exposure (Hsu, et al. 2003). These differences expose the problems with different research groups working with different cell lines.

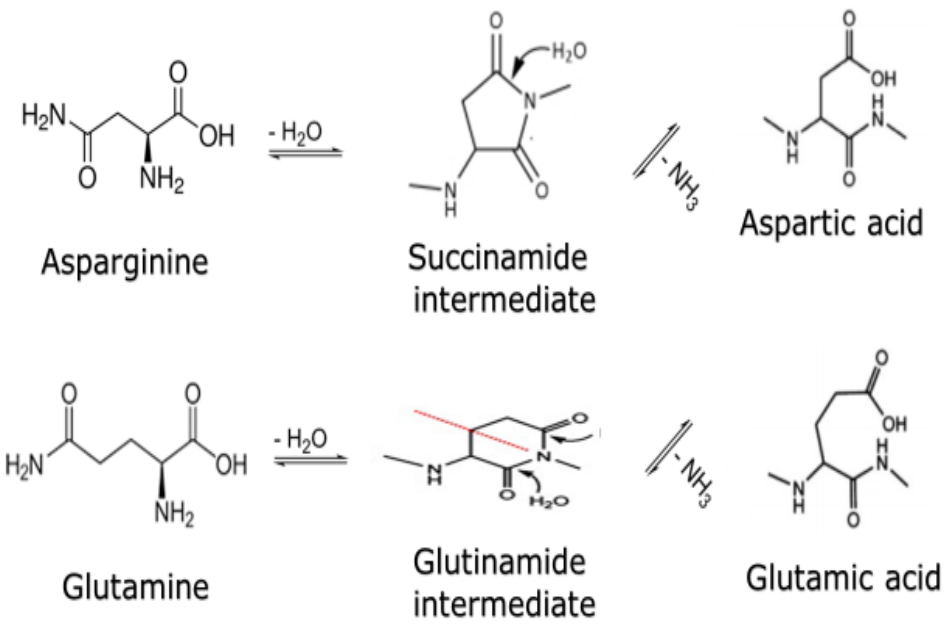
5.5 Summary of key findings

- Quercetin and kaempferol did not influence on Ht29 cell viability, whereas morin has slight effect.
- The fluorescence assay confirmed the TG2 inhibitory characteristic of the kaempferol on Ht29 cell lines.
- BAG family molecular chaperone regulator 3, and RAF proto-oncogene serine/threonine-protein kinase are proteins were identified as TG substrates in HT29.

Chapter 6 : Optimisation of Deamidation Assay

6.1 INTRODUCTION

The most abundant *in-vivo* and *in-vitro* processes involving proteins are post-translational modifications, such as oxidation, phosphorylation, glycosylation, and deamidation (Gervais 2016). Deamidation and oxidation could be damaging to all proteins; for example, oxidation has been reported to be associated with several pathological diseases, such as ischemia perfusion, rheumatoid arthritis, and atherosclerosis (Stadtman 1990). Whereas, deamidation is associated with a group of age-related disorders, neurological diseases, cataracts and autoimmune diseases such as rheumatoid arthritis and coeliac disease (Riggs, Gomez and Julian 2017). On the other hand, glycosylation can play a key role in improving proteins stability (Solá and Griebenow 2009). Deamidation affects asparagine (Asn or N) and glutamine residues of proteins (Gln or Q) (Robinson and Rudd 1974; Hasan, et al., 2006; Schroeter and Cleland 2016). Asparagine deamidation proceeds by a succinamide intermediate and that of glutamine by a glutanimide intermediate producing aspartic and glutamic acid, respectively (See Figure 6-1). *In-vitro* deamidation is undesirable, but perhaps unavoidable because deamidation proceeds none enzymatically independent of the pH value (Patel et al., 1990). It may lead to changes in the secondary, tertiary or quaternary structure of proteins, thus compromising their function or promoting undesired protein-protein aggregation (Gupta and Srivastava 2004; Dutta, et al., 2012).



Adapted from Cournoyer et al., 2008

Figure 6-1: Schematic demonstration for non-enzymatic asparginine and glutamine deamidation reactions.

6.1.1 Non-enzymatic deamidation:

There are two non-enzymatic post-translational modifications of proteins – deamidation of glutamine and asparagine, and racemisation of aspartic acid, which are essential reactions in the aging of cells and organisms (Truscott and Friedrich 2016). The *in vivo* release of ammonia from protein-bound amides results in alterations to proteins such as, conformational changes and the loss of biological activity. For example, the decrease in the potency of human growth releasing factor results from the deamidation of asparagine residues (Huang, et al., 2005). Another non-enzymatic, post-translation is racemisation, the major amino acids involved in racemisation are Asp and Asn. Racemisation leads to the accumulation of abnormal proteins in numerous human tissues. It has an important impact in the pathogenesis of diseases in old age such as the formation of cataracts, atherosclerosis, and cerebral age-related dysfunctions (Ritz-Timme and Collins 2002).

Acid or alkali pH values are responsible for the non-enzymatic deamidation (Robinson 1974) Deamidation catalysed by acid is less important at physiological pH. In an acidic environment, the carbonyl side chain of protein-bound glutamine becomes protonated to a hydroxyl, which leads to an increase its susceptibility to nucleophilic attack by water (Cournoyer and O'Connor 2008). Base-catalysed deamidation starts by deprotonation of

the backbone amide of protein-bound glutamine, leading to the formation of a negatively charged amide that reacts with the carbonyl side-chain generating a glutinamide intermediate. This reaction is enhanced in basic conditions and is principally prohibited when the C-terminal amino acid is proline (Cournoyer and O'Connor 2008). Similar reaction mechanisms can be drawn for asparagine.

6.1.2 Enzymatic deamidation:

Several enzymes that have been reported to deamidate the amide groups of proteins; transglutaminases, peptidases, and peptidoglutaminases (Hamada and Swanson 1994; Zhao, et al., 2016). Modifications of proteins mediated by enzymes are more effective than non-enzymatic deamidation (chemical), due to their ability to work at under milder conditions and their high specificity (Yong, Yamaguchi and Matsumura 2006). The deamidation rate of protein-bound amide residues is dependent on the properties of solution such as temperature, ionic strength, pH, buffer ions, the primary sequence of the target proteins and their three-dimensional structure. (Maeda, 1977; Robinson 2002). A negative charge forms when a deamidation occurs at neutral pH, which occasionally, also leads to β- isomerization. These modifications in structure may influence the biological properties of peptides and proteins (Robinson 2002a). It has been recorded that small levels of deamidation (e.g. 2-6 %) may significantly enhance protein functions. For example, the deamidation of glutamine in wheat proteins improves their solubility in mildly acidic conditions by lowering their isoelectric point (Zhang, Lee and Ho 1993).

6.1.3 Biological impact of deamidation

It has been proposed that *in vivo* deamidation of proteins acts as a molecular timer of biological actions and as a mechanism for post-synthetic production of proteins of cellular significance (Robinson 2002b). The lack of reliable experimental and theoretical information about the deamidation of proteins leads to an insufficient understanding of deamidation and its biological impacts. There are various implications of the deamidation of protein glutamine residues such as in degenerative proteinopathies, including Alzheimer's disease (AD), Parkinson's disease (PD), cataracts, vascular dementia (VaD) and coeliac disease(CD) (Serra 2016).

In coeliac disease, the immunologic peptides such as 33-mer arrive at the *lamina propria* where they are deamidated by TG2, which is crucial for the peptides to act as HLA-DQ-

restricted T-cell antigens that are responsible for initiating the disease (Lauret and Rodrigo 2013). Production of negative charges in gluten-derived peptides due to deamidation catalysed by TG2 promotes their binding affinity to the autoimmune disease-associated HLA molecules. (See chapter 1 section 1-8) (Hasan, et al., 2006; Stamnaes and Sollid 2015 and Serra, et al., 2016). As the most published methods for measuring the deamidating activity of TG2, use mass spectrometry technique. Hence, there is necessity to establish a simple low-cost method to confirm deamidation by TG2. These methods would enable investigation of naturally occurring inhibitors of TG2 and their potential use as moderators of coeliac disease.

6.1.4 Seed storage proteins as a tool for deamidation studies

The amount of storage proteins in seeds has been found to be 10% dry weight (DW) (in cereals) to 40% DW (in certain legumes and oilseeds) (Shimada, et al., 2003; Liu, et al., 2017). One of the oldest cultivated legumes is the faba bean (*Vicia faba*). The major proteins in *Vicia faba* are albumins (20%) and globulins (60%) as convicilin, vicilin and legumin (Liu, et al., 2017). The albumin includes trypsin inhibitors and contains more sulfur-containing amino acids than the globulins (Mandal 2000). These dicotyledonous seed storage proteins accumulate in high quantities in seed throughout mid-maturation period of seed growth and are utilised during germination, and they have been shown to become more electronegative after the initiation of germination (Manda, 2000). It has been proposed that this is a prerequisite to degradation by *de novo* synthesised peptidase enzymes. It has been reported that transglutaminase enzymes were not involved in dicotyledonous seed storage protein deamidation(Shutov et al., 1987). Previous work at NTU (Durose et al., 2003) has shown that an enzyme purified from 14-day old germinating *Vicia faba* seeds could incorporate biotin cadaverine into N`, N`, dimethyl casein in a calcium-dependent manner. This enriched enzyme preparation was able to deamidate day 1 after germination *Vicia faba* storage globulins. This was demonstrated by increased electronegativity of the storage proteins on a non-dissociating polyacrylamide gel after treatment with the enriched *Vicia faba* TG2. The deamidation was inhibited by EDTA showing that this protein deamidation was calcium ion-dependent, inhibited by a cystamine and partially inhibited by ZDON a specific active site inhibitor of mammalian TG2 (Badarau, et al., 2013). The use of these legumin storage proteins and the increased electronegativity on a non-dissociating PAGE as a measure of

deamidation provided an initial attempt to measure the deamidating activity of human recombinant TG2.

Storage proteins could be used as a tool to study the deamidation catalysed by TG2. The important group in constituents of cereal grains is the proteins, which is, represent in a range of 8-12%. In particular, storage proteins, which in some cereals considered as initiating factors for coeliac disease (CD) (Koehler, Wieser and Konitzer 2014). In this research, storage proteins used as a substrate for TG2 to optimised a fluorimetric assay to measure the deamidation of glutamine in the cereal peptides. Storage proteins represent about 70–80% of total grain proteins. Their main role is to afford the grain embryo with nitrogen and amino acids during germination (Cournoyer and O'Connor 2008). It consists of prolamins (gliadins) and glutenins (glutelins). Glutenins are insoluble in weak acids such as acetic acid. Whereas, prolamins are insoluble in water, but soluble in aqueous alcohol solutions (e.g. 60–70% (v/v) ethanol). Prolamins characterised by a high content of proline and glutamine (35–37% glutamine and 17–23% proline) (Cournoyer and O'Connor 2008; Koehler, Wieser and Konitzer 2014). The high content of proline has two implications; first, they make gluten proteins resistant to completely proteolytic degradation in the gastrointestinal tract by protease enzymes. This leads to the production of peptide fragments in the stomach and small intestine. Second, proline affects the specificity of the TG2 toward glutamine and, therefore, only specific glutamine residues in the gliadin peptides are substrates for deamidation, which are initiators of CD. Peptides with high glutamine are good substrates for the TG2 (Cournoyer and O'Connor 2008).

6.1.5 Measurement of deamidation:

The deamidation of proteins leads to a charge and mass shift in the overall structures of proteins, which produces a mixture of isomers that impede accurate measurement the deamidation, due to the difficulty in differentiation those mixtures (Cournoyer and O'Connor 2008). Quantification of the deamidation in peptides or proteins generally precedes a series of techniques to separate the mixture of isomers by different methods, such as chromatography, electrophoresis, chemical derivatives, isoelectric focusing, enzymatic digests, or mass spectrometric methods (Lampi 1998). These processes often result in differential responses or losses of the amidated and deamidated peptides, which complicit the quantitation. (Robinson et al. 2005).

Numerous methods have been used for quantification of protein/peptide deamidation. Robinson, et al (2005), utilised direct electrospray injection and fragmentation in a Fourier transform mass spectrometer, to measure the deamidation of lens crystalline that is associated with cataracts and aging. Another published work by Jia and Sun (2017) reported that using structure-based descriptors for deamidation prediction provides better understanding of the molecular basis of deamidation processes.

Other published methods for measurement the deamidation are:

- **Proteolytic digestion**, Kameoka, and Imoto (2003) used a peptidase that cleaves on the N-terminal side of an l-aspartyl residue. Mass spectrometry used to detect deamidation of asparagine and isomerisation of aspartyl residues in hen egg lysozyme as a model protein (Kameoka, Ueda and Imoto 2003).
- **Reversed-phase HPLC**, generally, chromatography is an attractive method for separation and can be used in combination with other methods that help in identifying the separated species. For example, RP-HPLC used to measure the deamidation rates of peptides and the enzyme kinetics correlated with the protein isoaspartyl methyltransferase (Murray and Clarke 1984).
- **Ion exchange chromatography**, useful for separating species depending on their ionic charge. Could be used to separate the deamidated form the native a protein/peptide, as for example, asparagine/glutamine are converted to their ionizable acid homologues (Huang, et al. 2005).
- **Electrophoresis**, Gel electrophoresis could be used to visualise proteins affected by deamidation, as both the shape and isoelectric point are altered. For example, non-denaturing PAGE has proved a useful technique for the analysis of the three forms of partially deamidated calmodulin (N97 to isoD/D97), where the isoD97 form migrated the slowest followed by the D97 and then N97 form (Johnson, Langmack and Aswad 1987). Staminas et al (2008), measured peptide deamidation and transamidation simultaneously through capillary electrophoresis (CE) to detect factors that affect the relative tendency for the two possible products.
- **Nuclear magnetic resonance** (NMR), 2D NMR has been utilised for differentiation of the aspartyl from isoaspartyl residues in peptides. This technique provides additional structural information, however, the disadvantage is that each experiment needs a large amount of sample, which may make this technique is

inappropriate to biological experiments deamidation (Chazin, et al. 1989, Mamula, et al. 1999, Cournoyer and O'Connor 2008).

However, this current research concentrates on the deamidation mediated by hrTG2; to date, most of published assays are for quantifying the level of deamidation catalyzed by TG2 are used either capillary electrophoresis or mass spectrometry techniques. Fleckenstein et al (2002) studied the affinity of different peptides to TG2, characterized the specificity of the enzyme, and examined the tendency of the enzyme to catalyse transamidation and deamidation reactions through using capillary electrophoresis with fluorescence-labeled gliadin peptides (Fleckenstein et al., 2002). Dørum et al (2009) used mass spectrometry method to measure the differences in the degree of deamidation for the different gliadin epitopes (Dørum, et al., 2009b).The mass spectrometry methods have been successfully utilised to characterize peptides in complex mixtures, these results so far have largely been qualitative (Ong and Mann 2005) with the associated high costs. Therefore, an effort was made to optimise a low-cost assay that could accurately measure the TG2 catalysed deamidation.

The measurement of ammonium ions in solution has been conducted by a variety of methods, include the Berthelot colour reaction (Weatherburn 1967), titration, electrode-based, enzymatic and colourimetric/ fluorimetric methods (Barsoliti 2001). The fluorimetric method based on the reaction of ammonia with a reagent to form a colour complex that is measured by spectrometry or fluorometry. The first colour reaction used was the indophenol reaction, described by Berthelot in 1859; the bluish colour was formed as a result the reaction of ammonia with phenol and hypochlorite (Proelss and Wright 1973). Another colourimetric reaction is the Nessler reaction, in which a brown-orange colour produced due to reaction of ammonia with potassium or mercury iodide in an alkaline solution (Avan 1980). Furthermore, ammonia assay kits from Sigma was used by Berti and his coworkers (2007) to measure the quantity of ammonia released after treatment of gliadins (extracted from bread wheat flour) with TGase from *Strepto Verticillium sp.* This assay based on the reaction of ammonia with reduced nicotinamide adenine dinucleotide phosphate (NADPH) and α-ketoglutaric acid (KGA) in the presence of L-glutamate dehydrogenase (GlDH) leads to formation of L-glutamate and oxidized nicotinamide adenine dinucleotide phosphate (NADP+). The reduction of the absorbance due to the oxidation of NADPH will proportional to the ammonia concentration.

There are two fluorimetric reagents that have been used successfully for amino acid analysis: fluorescamine and o-phthaldialdehyde (OPA). The main advantages to these methods are rapidity, simplicity, specificity and high sensitivity (Barsoliti 2001). The OPA reagent is particularly suited to measuring pico mole to-nano mole amounts of ammonia in samples (Schuster 1988; Roth 1971). Roth (1971) showed that OPA in aqueous alkaline medium reacts with amino acids in the presence of 2-mercaptoethanol (2-ME) leading to the formation of fluorescent compounds. Ortho-phthaldialdehyde and 2-mercaptoethanol react with primary amines including the number of amino acids (Lee and Drescher 1978) (See Figure 6-2).

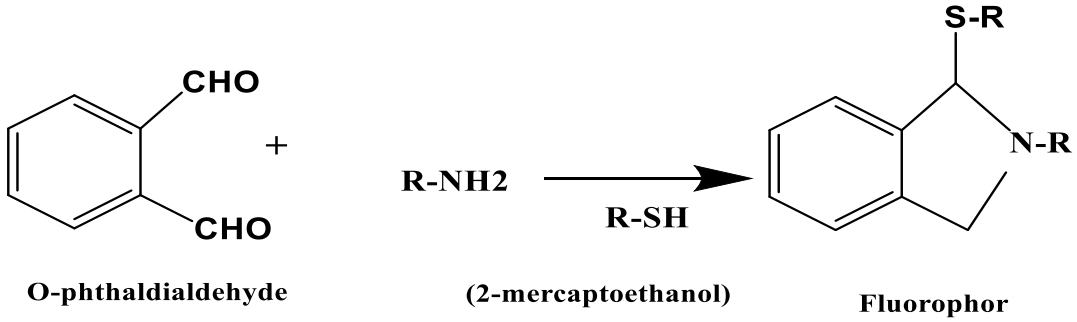


Figure 6-2: schematic representation for the reaction of OPA and primary amino groups.

Adapted from Held, 2001

The reaction can be used to assay ammonium ions (NH_4^+) in specialised situations (Taylor, et al. 1974 and Corbin 1984). Because of the high sensitivity and low detection limit of the OPA-sulfite-NH_3 reaction, the OPA was used in a number of different methods (Kulla and Zuman 2008). Therefore, the aim of this research is to develop an assay to measure ammonia-based upon a reaction with OPA resulting in a fluorescent adduct which measured 340nm ex and 430nm em. The method was developed as a microplate assay at NTU (Aldubyan 2014).

6.2 Hypothesis

A simple assay for measurement the TG2 deamidation activity would be essential for testing the effect of a biologically active compound on the TG2 activity as a potential treatment of coeliac disease. To test the hypothesis, it was aimed to;

- Optimise the OPA assay for gliadin protein and 33-mer peptides as a substrate to TG2 to measure the release of ammonia.

- **Methods and Materials:**

See Methods and material chapter:

- Transglutaminase deamidation activity assay(section2.2.6b).

6.3 Result

Because of the high content of glutamine in *Vicia faba* storage proteins, these proteins were isolated from *Vicia faba* cotyledons one day after the initiation of imbibition. They were then used as the deamidation substrates to follow hrTG2 deamidation assays. Previous work is done in NTU (Aldubayan 2014) showed that the rate of natural deamidation of the isolated storage proteins from *Vicia faba* cotyledons increased (as judged by increased electronegativity) between 7th to 14th days after seed germination (See Figure 6-3). It is not yet been established the nature of the enzyme responsible for the deamidation of the storage proteins in the dicotyledonous seeds, but it has been hypothesised that the deamidation precedes proteolytic degradation (Müntz 1996). Previous work at NTU suggests that a *Vicia faba* transglutaminase is responsible for the deamidation of the storage proteins.

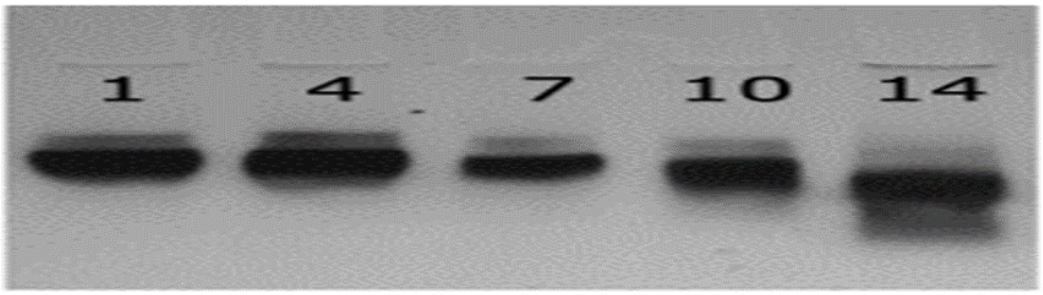


Figure 6-3: The non-denaturing PAGE of *Vicia faba* storage proteins isolated from the cotyledons of seeds at different days following germination.

Adopted from (Aldubayan 2014).

It has been hypothesised that legume storage proteins isolated early after imbibition of water can provide a useful tool to measure the calcium-dependent deamidating activity of human TG2 the enzyme central to the initiation of CD.

6.3.1 Non-denaturing PAGE (Qualitative assay for TG2 deamidation activity)

a) non-denaturing PAGE of seed storage proteins

Non-denaturing PAGE of seed storage proteins (qualitative assay), used to visualise the effect of EDTA and Ca^{2+} on deamidation activity of hrTG2. Seed storage proteins from

Vicia faba were incubated with hrTG2, as described in (materials and methods chapter), and then subjected to non-denaturing PAGE. An increase in the electronegativity of proteins samples was observed (See Figure 6-4).

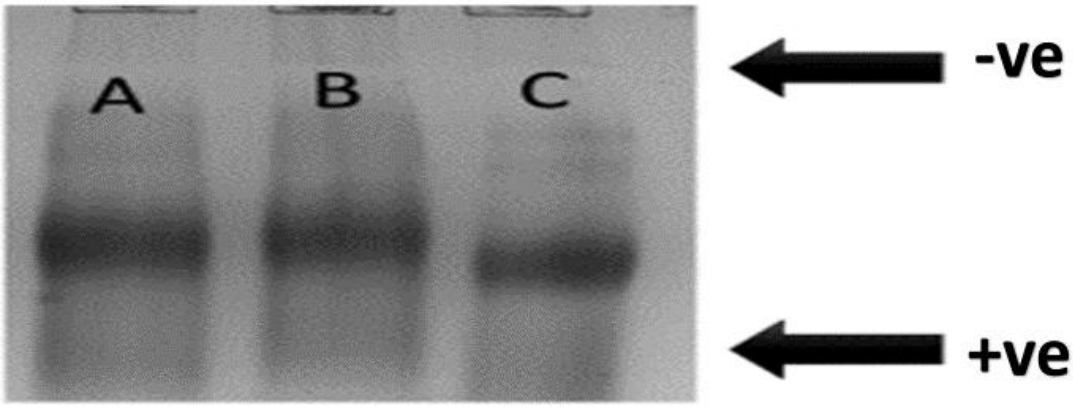


Figure 6-4: non-denaturing PAGE of *Vicia faba* storage proteins.

50.0µl Storage proteins (10 mg ml^{-1}) isolated from *Vicia faba* seeds were incubated with 50.0µl hrTG2 (5µg/ml^{-1}) in the presence of 50.0µl Ca^{2+}or EDTA (2mM). After overnight incubation at 37°C, the proteins (10 µl) were loaded onto a 5% (w/v) polyacrylamide gel and subjected to non-denaturing PAGE (materials and methods chapters,Section 2.2.7b)). **A** (water +storage protein + hrTG2).), **B** (EDTA +storage protein + hrTG2), **C** (Ca^{2+} +storage protein + hrTG2). The gel bands were revealed using Instant blue stain (Expedeon, UK).

The *Vicia faba* storage proteins incubated with hrTG2 in the presence of 2.0 mM $CaCl_2$ showed increased electronegativity compared to samples incubated with EDTA and the water.

b) non-denaturing PAGE of seed storage protein treated with hrTG2 at different $CaCl_2$ concentrations

In order to observe the effect of increasing Ca^{2+} concentration on deamidation activity, seed storage proteins from *Vicia faba* were subjected to non-denaturing PAGE with different $CaCl_2$ concentrations and change in the electronegativity (increased electronegativity) was observed (See Figure 6-5).

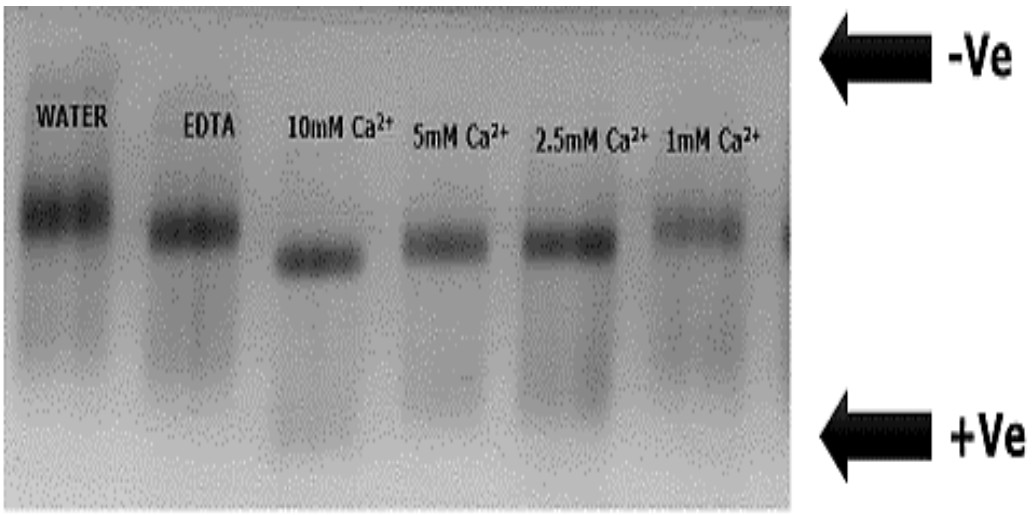


Figure 6-5: Effect of different concentrations of Ca^{2+} on the deamidation of *Vicia faba,* storage proteins.

50.0µl *Vicia faba,* storage proteins (10 mg ml^{-1}) incubated with 50.0µl hr TG2 (5µg/ml^{-1}) in the presence of 50.0µl EDTA (2mM) or 50.0µl $CaCl_2$ at different concentrations. After overnight incubation at 37°C, the proteins (10 µl) were loaded onto a 5% (w/v) polyacrylamide gel and subjected to non-denaturing PAGE and instant blue staining (materials and methods chapter, section 2.2.7b).

Figure 6-5 shows that the increasing of electronegativity of the samples with Calcium increased by increasing the calcium concentration.

6.3.2 OPA assay (quantitative assay for TG2 deamidating activity)

To validate the qualitative data shown in Figure 6-4 and Figure 6-5 there is a necessity to develop and optimise quantitative assay to quantify the deamidation by measuring the ammonia released during the interaction.

a) Optimization of ammonium chloride as standard for deamidation

To develop an assay for measureing TG2 deamidating activity, a fluorescent microplate ammonium assay (Poulin and Pelletier 2007) was optimised to quantify the release of ammonia catalysed by the deamidating activity of TG2. Previous work at NTU (Aldubyan 2014) showed that this assay was very sensitive to ammonia in the micromolar concentration range.

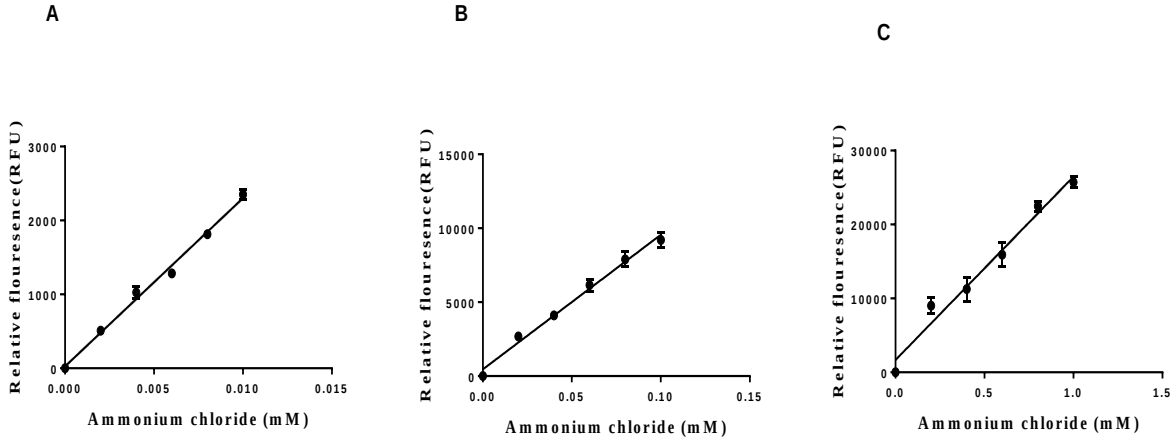


Figure 6-6: Optimization of ammonium chloride as standard for deamidation.

Ammonium chloride (0-1mM) was used as a standard to quantify the ammonia released in the deamidation assay. Different concentration of ammonium chloride A; 0-0.01mM, B; 0-0.1mM, C; 0-1mM, with OPA were applied to a fluorescence microplate and the interaction between ammonium ions and OPA resulted in a fluorescent adduct, which was measured at λex = 360 nm and λem = 430 nm using a Fluostar Optima 96 well plate reader(Materials and methods chapter, section2.2.6b).

The sensitivity range of the assay was determined, and the data displayed that the assay was sensitive to ammonium in concentrations between 0.0025 to 1mM (See Figure 6-6).

b) Deamidation assay for storage proteins (*Vicia faba*) (Quantitative assay)

The fluorescence microplate assay was used to measure the release of ammonia from the seed storage proteins after treatment with gpl or hrTG2 (± Ca^{2+}). *Vicia faba* storage proteins incubated with either human recombinant or guinea pig liver TG2 in the presence of 2mM CaCl2 showed a significant increase in the release of ammonia compared to other treatments (see Figure 6-7).

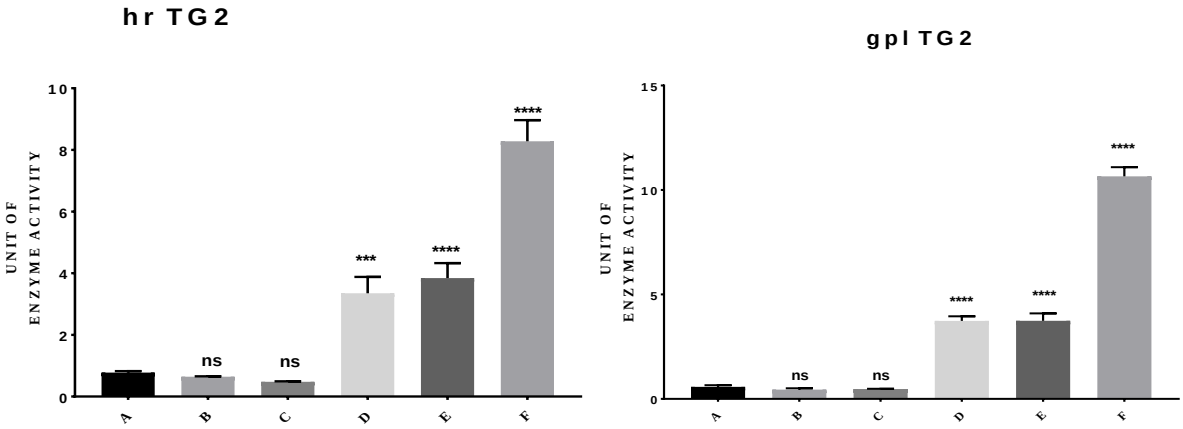


Figure 6-7: Deamidation of storage proteins using TG2.

Seed storage proteins (SSP) (10.0 mg ml^{-1}) isolated from *Vicia faba* seeds were incubated with gpl TG2 and hrTG2 (1 mg ml^{-1}) in (+/-) of Ca^{2+} for 16 h at 37°C. The supernatant was assayed for ammonia by OPA assay. (Materials and methods chapter,section2.2.6b).The data point A = SSP, B = SSP+EDTA, C = SSP+ Ca^{2+}, D = WATER+SSP+TG2, E = EDTA+ SSP + TG2, F = Ca^{2+} + SSP + TG2. The data points represent the mean ± S.E.M from 3 independent experiments (n=3, ****P-value < 0.0001). All the values are as compared to the control (Column A). The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one hour (1nmol hr^{-1}).

The Figures show that the highest ammonia quantity was from the sample (F) that contain TG2 with calcium.

c) Deamidation assay for *Vicia faba* storage proteins by TG2 at different calcium concentrations

The release of ammonia from the seed storage proteins when treated with gpl or hr TG2 in the presence of different calcium concentrations was measured by the fluorescence microplate assay as shown in Figure 6-8.

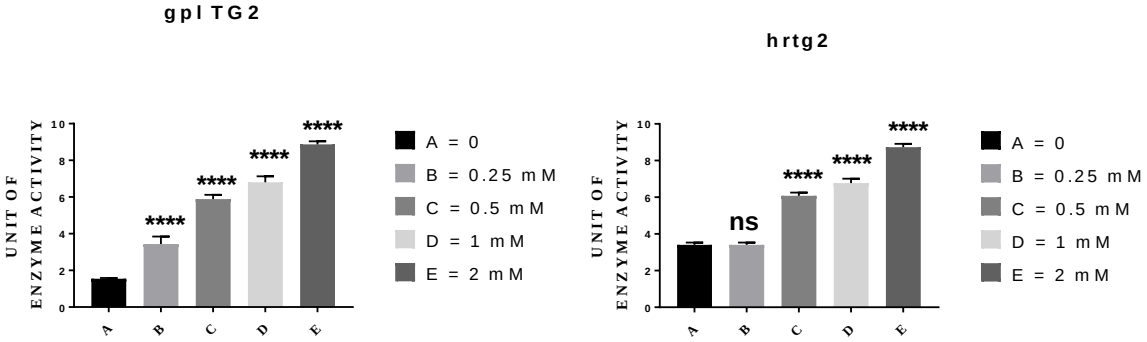


Figure 6-8: Effect of different concentrations of Ca^{2+} on Deamidation of storage proteins.

Seed storage proteins (SSP) (10.0 mg ml^{-1}) isolated from *Vicia faba* seeds were incubated with gpl TG2 (10.0µg/ml^{-1}) and hrTG2 (5.0µg/ ml^{-1}) in presence of increased concentrations of $Cacl_2$for 16 h at 37ºC. The supernatant was assayed for ammonia (Materials and Methods chapter, section 2.2.6b). The data points represent the mean ± S.E.M from 3 independent experiments (n=3, ****P-value < 0.0001). All the values are as compared to the control (column A). The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one hour (1nmol hr^{-1}).

The *Vicia faba* storage proteins incubated with gpl or hr TG2 in the presence of different $CaCl_2$ concentrations showed increased ammonia quantity with increasing concentrations of calcium (See Figure 6-8).

d) Deamidation assay of *Vicia faba* storage proteins in the presence of TG2, $CaCl_2$, and variable cystamine concentrations.

Cystamine is known to be an inhibitor of TG2 (Jeitner et al., 2018), in order to observe how cystamine affected the deamidating activity of TG2, the seed storage proteins from *Vicia faba* were incubated with hrTG2 and $CaCl_2$ with variable concentrations of cystamine.

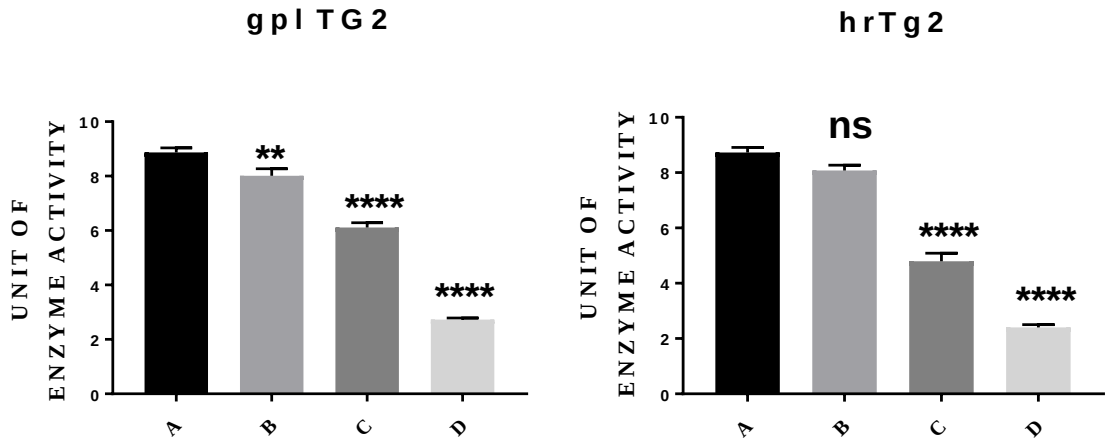


Figure 6-9: Deamidation of storage proteins using hr and gpl TG2 in presence of different concentrations of Cystamine.

Seed storage proteins (SSP) (10.0 mg ml^{-1}) isolated from *Vicia faba* seeds were incubated with hrTG2 (5.0µg/ ml^{-1}) or gplTG2 (10.0µg/ml^{-1}) in presence of $Cacl_2$ and cystamine as inhibitor with increasing concentrations, (0.025, 0.5 and 2.5mM) for 16 h at 37°C. The supernatant assayed for ammonia (see materials and methods chapter, section b)2.2.6b) . **A** = Ca^{2+} + SSP + TG2, **B** = cystamine 0.025mM, **C** = cystamine 0.5mM, **D** = cystamine 2.5mM. The data points represent the mean ± S.E.M from three independent experiments (n=three**P-value < 0.001, ****P-value < 0.0001). All the values are as compared to the control (Column A). The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one hour (1nmol hr^{-1}).

The *Vicia faba* storage proteins incubated with $CaCl_2$ and TG2 in the presence of increasing cystamine concentrations showed a decrease in measurable ammonia released compared to the Ca^{2+} control. (See Figure 6-9).

6.3.3 Gliadins

Having established a method to quantify the ammonia released by TG2 from *Vicia faba* storage proteins, it was decided to change the substrate to gliadins peptides mixture, as it has a crucial role in the initiation of CD.

a) Deamidation assay for gliadin protein

As the main aim of this research is measuring the deamidating activity of hrTG2 against gliadin peptides in relation to coeliac disease. The microplate deamidation assay developed for *Vicia faba* storage proteins was transferred to the assay of the deamidation of wheat (*Triticum*) gliadin peptides (Sigma). Because the deamidation of gluten peptides in gliadins by TG2 is crucial for the peptides to act as HLA-DQ-restricted T-cell antigens that are responsible for initiating the celiac disease (Garber et al., 2017).

A fluorescence microplate assay was optimised to measure the release of ammonia from a crude preparation of wheat (*Triticum*) gliadin peptides when treated with hrTG2 (± Ca^{2+}) (See Figure 6-10)

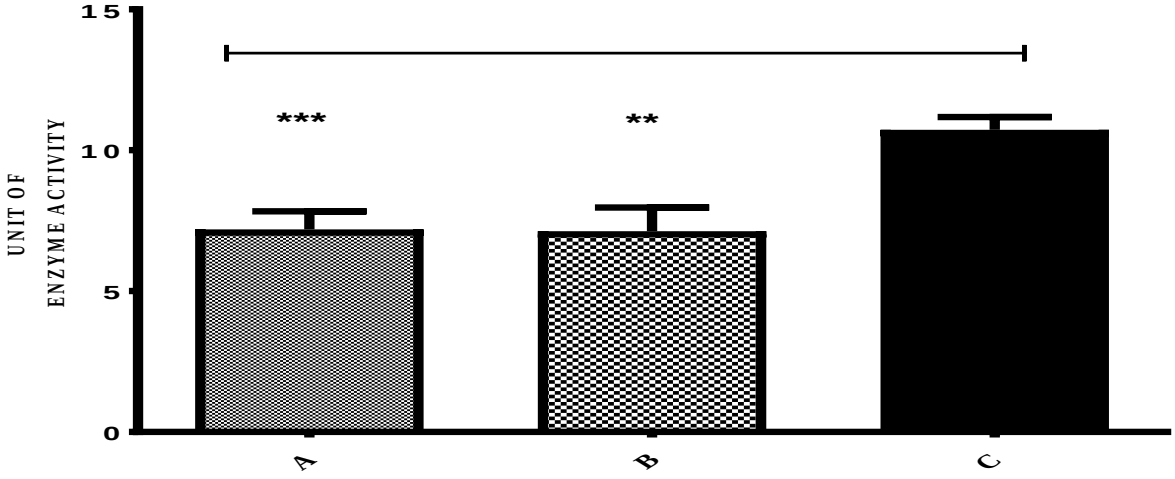


Figure 6-10: Deamidation assay for gliadin.

Gliadin (2.0 mg ml^{-1}) incubated with 5.0 µg ml^{-1} hrTG2 in presence and absence of $Cacl_2$ (2mM) for 10 min at RT. **A** = gliadin+water+hrTG2, **B** + gliadin+EDTA+hrTG2, **C** = gliadin+Ca^{2+} +hrTG2.(Materials and methods chapter,section 2.2.6b). The data points represent the mean ± S.E.M from 3 independent experiments (n=3, ***P-value < 0.001). All the values are as compared to the control (column C). The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one min (1nmol min^{-1}).

The treatment of wheat gliadin peptides with hrTG2 in presence of Ca^{2+} resulted in the significant release of ammonia (Figure 6-10). There was also release of ammonia from the gliadin peptides treated with water and EDTA.

b) Deamidation of crude gliadin protein by hr TG2 with increasing incubation times

To optimise the appropriate time for measuring the ammonia released in the assay, the gliadin protein was incubated with an increasing time period (1.0- 60min) with hrTG2 (See Figure 6-11).

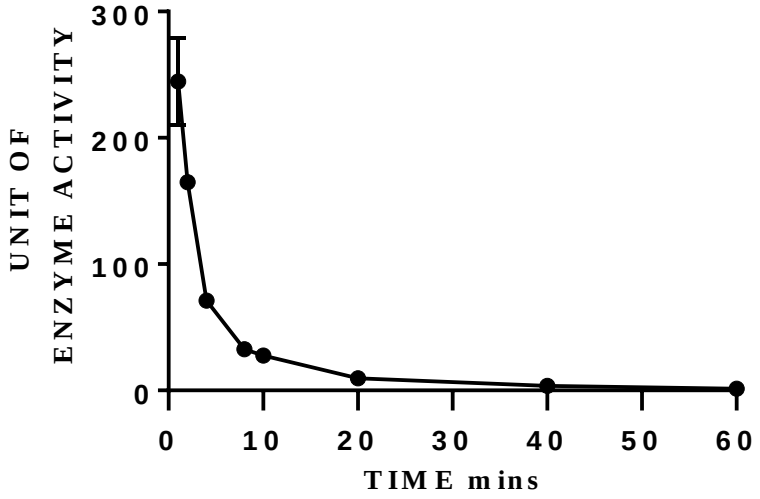


Figure 6-11: Deamidation assay for gliadin peptide mixture in increasing time of incubation.

Gliadin (2.0 mg ml^{-1}) incubated with 5 µg ml^{-1} hrTG2 in presence of $Cacl_2$ (2mM) in increasing the time of incubation at RT.(Materials and methods chapter,section 2.2.6b).The data points represent the mean ± S.E.M from 3 independent experiments. The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one min (1nmol min^{-1}).

The optimum incubation time for the hrTG2 with gliadin protein was found to be between 2 to 10 min at RT.

c) Comparison between the deamidation activity of hrTG2 and denatured hrTG2 using gliadin protein as substrate.

In order to confirm the ammonia released in this assay was hrTG2 dependent, active hrTG2 and denatured (boiled) hrTG2were incubated with -gliadin in the presence of $CaCl_2$ (See Figure 6-12).

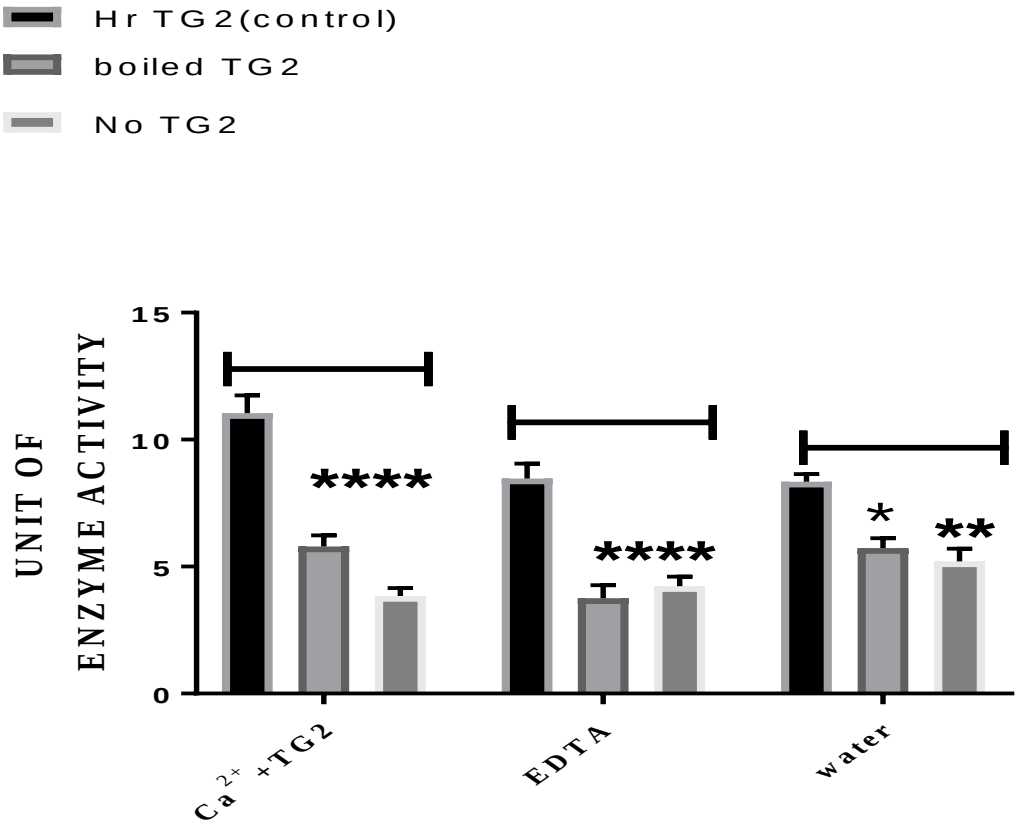


Figure 6-12: Deamidation assay for gliadin protein in a different form of TG2.

Gliadin (2.0 mg ml^{-1}) incubated with active hrTG2 5 µg ml^{-1} and denatured hrTG2 in +/- $Cacl_2$ (2mM) for 10 min at RT. (Materials and methods chapter,section 2.2.6b)The data points represent the mean ± S.E.M from three independent experiments (n=3, ****P-value < 0.0001). All the values are as compared to the control (black columns). The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one min (1nmol min^{-1}).

The treatment of wheat (*Triticum*) gliadin protein with different forms of hrTG2 in presence of Ca^{2+} resulted in a significant difference in the release of ammonia between the active form denatured and no hrTG2 samples (See Figure 6-12).

d) Deamidation activity with increasing human recombinant TG2 concentrations using gliadin protein as substrate.

A fluorescence microplate assay used to measure the release of ammonia from the wheat (*Triticum*) gliadin protein when treated with increasing concentrations of hrTG2 in the presence of calcium See Figure 6-13.

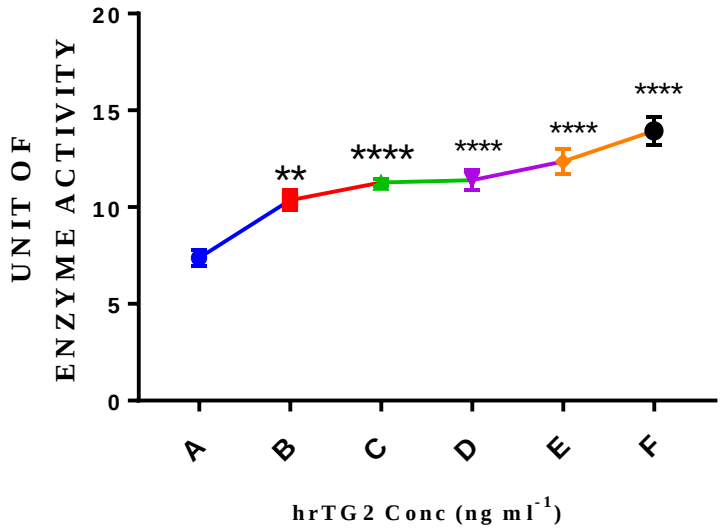


Figure 6-13: Deamidation assay for gliadin protein in increasing concentration of hrTG2.

Gliadin peptides (2.0 mg ml^{-1}) incubated with different concentrations of hrTG2 (0.05 - 5000 $ngml^{-1)}$ in presence of $Cacl_2$ (2mM) for 10 min at RT.**A** = 0, **B** = 0.05, **C** = 0.50, **D** = 5.0, E = 50, **F** =5000.0 $ngml^{-1}$. The ammonia released was measured (seematerials and methods chapter, section 2.2.6b)ii). the data points represent the mean ± S.E.M from 3 independent experiments (n=3, ****P-value < 0.0001). All the values are as compared to the control (A). The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one min (1nmol min^{-1}).

The wheat *(Triticum aestivum*) gliadin protein incubated with increasing hrTG2 concentrations in the presence of $CaCl_2$ showing the increased release of ammonia with increasing concentrations of hrTG2. The result indicates that the TG2 dependent ammonia released can be measured using the ammonia detection microplate method.

e) Deamidation activity of hrTG2 with increasing calcium concentrations using gliadin protein (substrate)

The fluorescence microplate assay used to measure the release of ammonia from gliadin protein when treated with hrTG2 in the presence of increasing calcium concentration as shown in Figure 6-14.

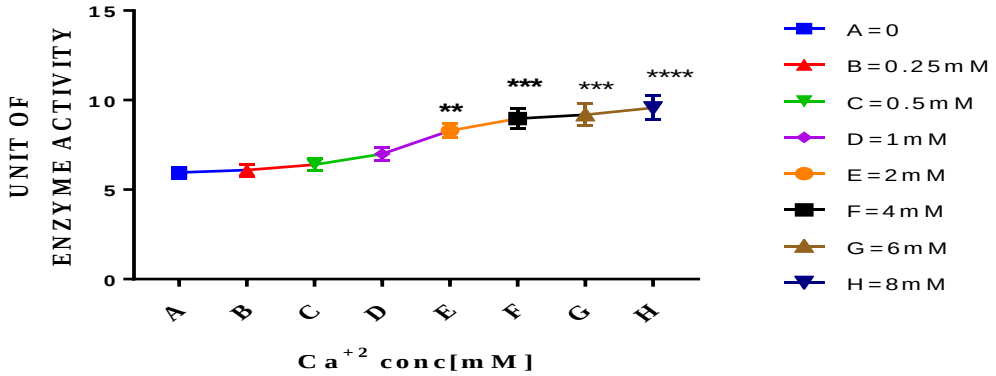


Figure 6-14: Deamidation of Gliadin protein using different Concentration of $CaCl_2$.

Gliadin (2.0 mg ml^{-1}) incubated with 5µg ml^{-1} hrTG2 in presence of increased concentrations (0.25 – 6mM) of $Cacl_2$ at RT. (Materials and methods chapter,section 2.2.6b).The data points represent the mean ± S.E.M from 3 independent experiments (n=3, ****P-value < 0.0001). All the values are as compared to the control (A). The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one min (1nmol min^{-1}).

The gliadin protein incubated with hrTG2 in the presence of different $CaCl_2$ concentrations showed the increased release of ammonia with increasing concentrations of calcium. The highest concentrations of calcium that give highest quantity of ammonia were from 2-6mM.

f) Deamidation activity of hrTG2 with other proteins (Albumin and amylase) instead of using gliadin.

To examine the TG2 specificity of the deamidation assay, albumin and α-amylase were used instead of hrTG2 (Figure 6-15),

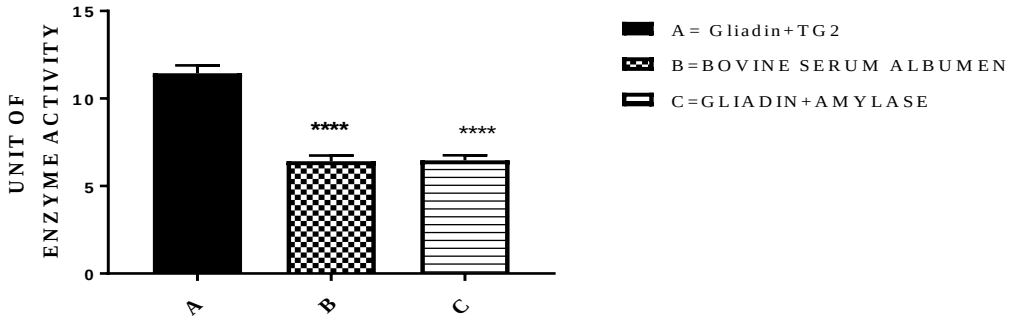


Figure 6-15: Deamidation assay for hrTG2 in prescience of Albumin and α-amylase.

Gliadin protein (2.0 mg ml^{-1}) incubated with 5 µg ml^{-1} hrTG2 compared with other samples containing albumin or α-amylase (5 µg ml^{-1}). (materials and methods chapter, section 2.2.6b).The data points represent the mean ± S.E.M from 3 independent experiments (n=3, ****P-value < 0.0001). All the values are as compared to the control (Column A). The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one min (1nmol min^{-1}).

The finding displays a significant reduction in ammonia released in albumin and α-amylase containing samples (Figure 6 -15).

g) Effect of TG inhibitors on deamidation of gliadin protein by active hr TG2

In order to compare the effect of known TG2 inhibitors on the deamidating activity of TG2, two selected TG2 inhibitors (cystamine and ZDON) were incubated with hrTG2 in the presence of $CaCl_2$ (See Figure 6-16).

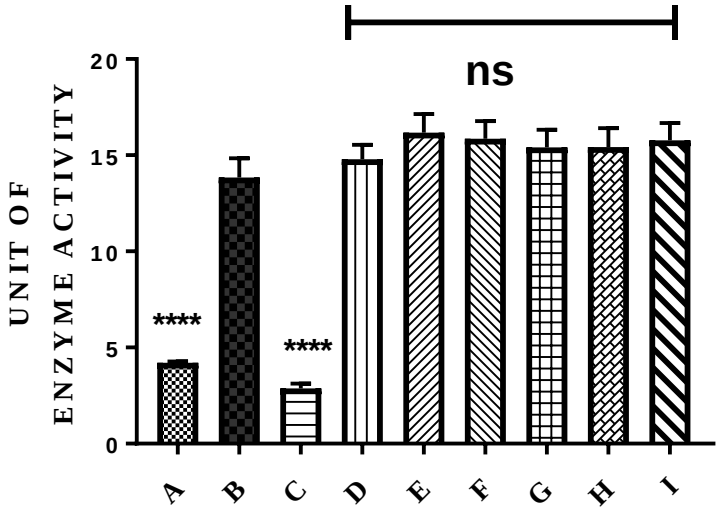


Figure 6-16: Deamidation assay for gliadin protein by active TG2 in the prescience of known TG inhibitors.

Gliadin protein (2.0 mg ml^{-1}) incubated with 5 µg ml^{-1} thrTG2 in presence of different concentrations of cystamine and ZDON in 10 min at RT. **A** = gliadin+ water, **B** = gliadin+ TG2+$Ca2^{+,}$ **C**= cystamine 10.0mM, **D**= cystamine 1.0mM, **E** = cystamine 0.20mM, **F** = cystamine 0.02mM, **G** = ZDON1mM, **G**= ZDON 0.1mM, h = ZDON 0.01mM. (materials and methods chapter,section 2.2.6b)The data points represent the mean ± S.E.M from 3 independent experiments (n=3, ****P-value < 0.0001). All the values are as compared to the control (Column B). The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one min (1nmol min^{-1}).

The gliadin protein (2.0mg ml^{-1}) incubated with hrTG2 (5 µg ml^{-1}) in presence of $CaCl_2$ (2mM) cystamine (0.02 – 10.0mM) and ZDON (0.01 -1.0mM), no significant inhibition of deamidation of gliadin protein observed, significant inhibition was at higher concentration 10mM. This result indicated that cystamine and ZDON at this level of concentrations were unable to inhibit the deamidating activity of hrTG2 using gliadin protein as substrates (See Figure 6-16).

h) Deamidation of gliadin protein using inactive hrTG2

To compare the deamidation activity between active TG2 and modified active site (Cys277, Cysteine replaced by serine) (inactive TG2; Zedira, De), the modified active site TG2 was incubated with gliadin protein in the ±of $CaCl_2$ (See Figure 6-17).

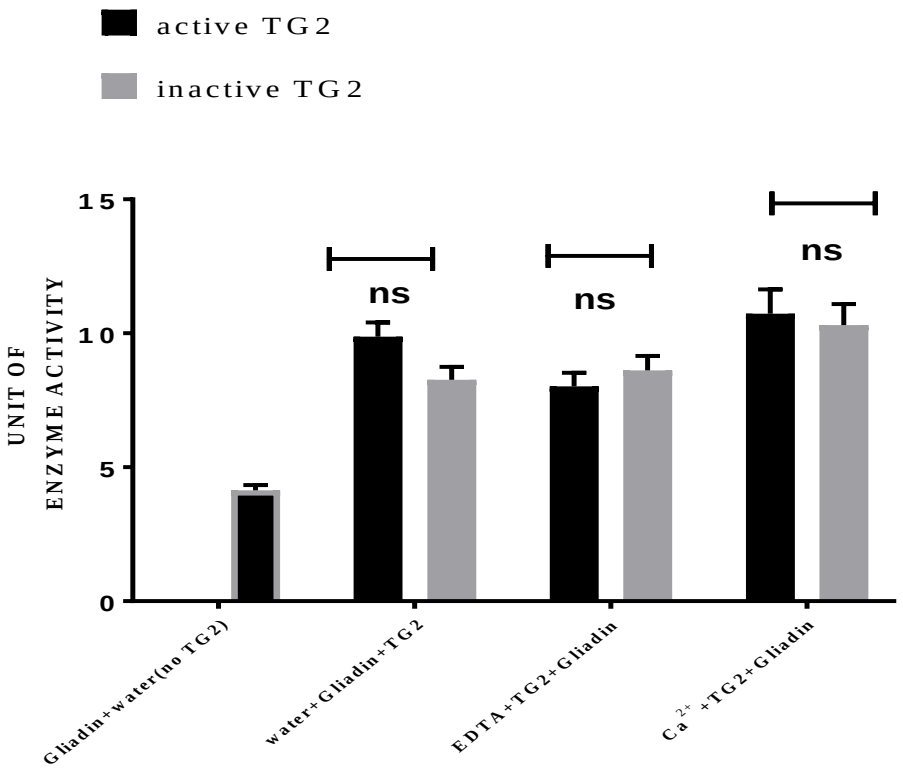


Figure 6-17: Deamidation assay for gliadin by inactive TG 2.

Gliadin (2.0 mg ml^{-1}) incubated with 5 µg $ml^{-1}$of inactive hrTG2 or active hrTG2 in presence of (± Ca^{2+}) (2mM) for 10 min at RT. (materials and methods chapter,section 2.2.6b)The data points represent the mean ± S.E.M from three independent experiments (n=three, **P-value < 0.005). All the values are as compared with each other. The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one min (1nmol min^{-1}).

The treatment of wheat gliadin protein with the inactive form of hrTG2 in +/- Ca^{2+} resulted in the similar release of ammonia amount comparing to the active form of hrTG2 (See Figure 6-17).

i) Effect of TG inhibitors on deamidation of gliadin protein by inactive hr TG2

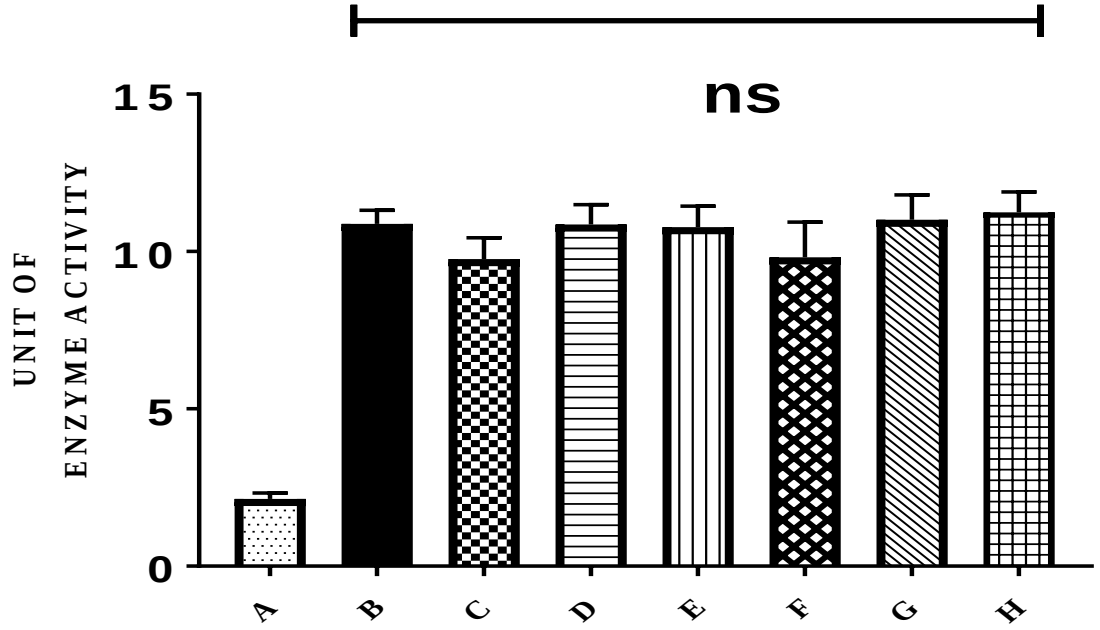


Figure 6-18: Deamidation assay for gliadin by inactive TG2 in the prescience of known TG inhibitors.

Gliadin protein (2.0 mg ml^{-1}) incubated with 0.5 µg ml^{-1} inactive hr TG 2 in presence of different concentrations of cystamine and ZDON in 10 min at RT. **A** = gliadin+ water, **B** = gliadin+ TG2+$Ca2^{+,}$ **C**= ZDON1mM, **D**= ZDON 0.1mM, **E** ZDON 0.01mM =, **F** = cystamine 1mM, **G**= cystamine 0.2mM, H = cystamine 0.01mM. (materials and methods chapter,section 2.2.6b)The data points represent the mean ± S.E.M from 3 independent experiments (n=3, ****P-value < 0.0001). All the values are as compared to the control (Column B). The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one min (1nmol min^{-1}).

The gliadin protein incubated with $CaCl_2$ and inactiveTG2 in presence of cystamine (0.02 – 1.0mM) and ZDON (0.01 – 1mM),both inhibitors were unable to show inhibition of deamidation at these concentrations. This result indicated that cystamine and ZDON at this level of concentrations were unable to inhibit the deamidating activity of inactive hrTG2 using gliadin protein as substrates (See Figure 6-18).

To validate that ammonia released represents the deamidation of gliadin protein by TG2 and to mimic the celiac disease condition, 33-mer gliadin peptide fragment identified as the gliadin fragment, which initiates CD, was applied to the assay. This peptide fragment is produced by normal gastrointestinal proteolysis, which becomes a potent T cell stimulator after deamidation by tissue transglutaminase (TG2). (Herrira et al., 2018)

6.3.4 Deamidation of 33 mer gliadin peptides by hrTg2:

The fluorescence microplate assay used to measure the release of ammonia from the 33-mer gliadin peptide when treated with hrTG2 in the presence of calcium and/or TG2 inhibitors as shown in Figure 6-19.

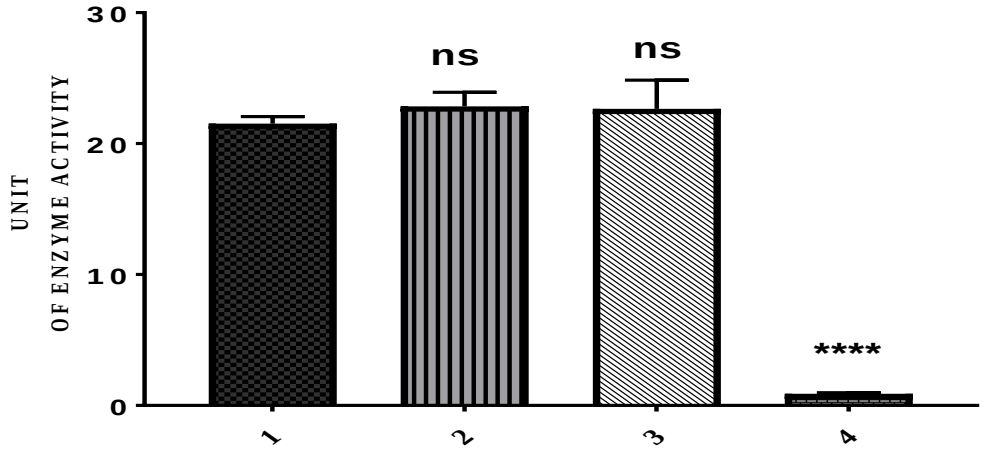


Figure 6-19: deamidation assay for33-mer gliadin peptide using hr TG2.

33-mer gliadin peptide (1mM) incubated with 5 µg ml^{-t} hrTG2 in presence of $CaCl_2$ (2mM), EDTA(2mM), ZDON (1mM) and cystamine (2mM) in 10 min at RT. **1**= 33-mer gliadin peptide + Ca^{2+} , **2** = 33-mer gliadin peptide + EDTA , **3** = 33-mer gliadin peptide +ZDON , **4** = 33-mer gliadin peptide +cystamine. (materials and methods chapter, section 2.2.6b). The data points represent the mean ± S.E.M from three independent experiments (n=three, ****P-value < 0.0001, **P-value < 0.01,). All the values are as compared to the control (Column 1). The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one min (1nmol min^{-1}).

a) Comparison between 33mer gliadin peptides and gliadin protein protein using active human recombinant TG2.

The fluorescence microplate assay used to measure the release of ammonia from the 33-mer gliadin peptide when treated with hrTG2 in the presence of calcium and compared with ammonia released from gliadin protein as shown in

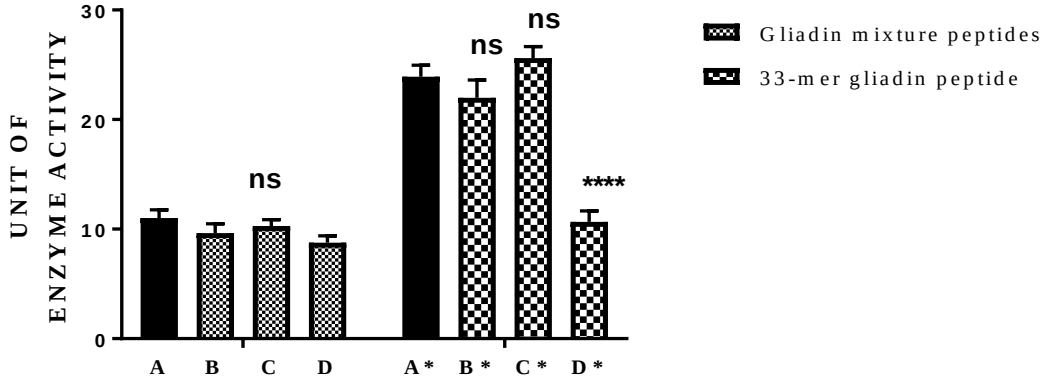


Figure 6-20: comparison between 33-mer gliadin peptide and gliadin protein using hrTG2.

Gliadin (2.0 mg ml^{-1}) and 33-mer gliadin peptide (1mM) incubated with 5 µg ml^{-1} hrTG2 in presence of $CaCl_2$ (2mM), EDTA (2mM), ZDON (1mM) and cystamine (2mM) in 10 min at RT. (materials and methods chapter,section 2.2.6b)A: gliadin mixture peptides+Ca^{2+} +hrTG2. B: gliadin mixture peptides+EDTA +hrTG2. C: gliadin mixture peptides+Z-don +hrTG2. D: gliadin mixture peptides+cystamine +hrTG2. A*: 33-mer gliadin peptides+Ca^{2+} +hrTG2. B*: 33-mer gliadin peptides+EDTA +hrTG2. C*:33-mer gliadin peptides+Z-DON +hrTG2. D*:33-mer gliadin peptides+Cystamine+hrTG2. The data points represent the mean ± S.E.M from three independent experiments (n=three, ****P-value < 0.0001, **P-value < 0.01,). All the values are as compared to the control (Column A and A*).). The unit of the TG2 activity in different samples was defined as follow; 1 unit of activity is 1nanomole product formed per one min (1nmol min^{-1}).

The 33-mer gliadin peptide incubated with hrTG2 in +/- Ca^{2+} and known TG inhibitors (cystamine and ZDON) then compared with same treatments using gliadin protein. No difference for ammonia released between gliadin protein and 33-mer peptides, the difference was in effect of the cystamine on the ammonia quantity. An unexpected finding was that cystamine inhibited the ammonia released by hrTG2 when the 33-mer gliadin peptide was used in the assay, (Figure 6-20)

b) Comparison between 33mer gliadin peptide and gliadin protein using inactive hr TG2.

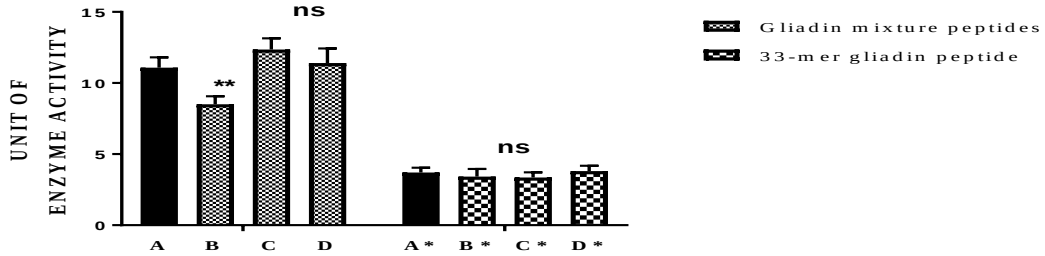


Figure 6-21: comparison between 33-mer gliadin peptide and gliadin proteins using inactive human recombinant TG2.

Gliadin (2.0 mg ml^{-1}) and 33-mer gliadin peptide (1mM) incubated with 5 µg ml^{-1} inactive hrTG2 in presence of $CaCl_2$ (2mM), EDTA (2mM), ZDON (1mM) and cystamine (2mM) in 10 min at RT. (Materials and methods,section 2.2.6b). The data points represent the mean ± S.E.M from three independent experiments (n=three, ****P-value < 0.0001) All the values are as compared to the control using graph pad7.

The 33-mer gliadin peptide incubated with an inactive form of hrTG2 in +/- Ca^{2+} and known TG inhibitors then compared with same treatments using gliadin protein. The data shows there is a significant difference between gliadin protein and 33-mer gliadin peptide in releasing of ammonia and effect of ZDON and cystamine.

6.3.5 Deamidated Peptide Sequencing by Mass Spectrometry:

In order to validate the data of fluorescence assay, samples were prepared to run by a mass spectrometry technique to detect the deamidation of gliadin peptides. The samples containing 33- mer peptides with either EDTA or Calcium ions were incubated with active hr TG2, then applied to mass spectrometry.

The mass spectrum data did not show a significant difference in deamidation level between all the samples comparing with undeamidated peptides (control)(Summary of Data are presented in appendices chapter).

6.4 Discussion:

Deamidation of gliadin by TG2 is critical for the initiation of the immune response by allowing binding of gliadin peptides to two variants of type II human leukocyte antigen (HLA) called HLA-DQ2 and DQ8, which in turn induce the inflammation and villous atrophy (coeliac disease). To measure the deamidation activity of TG2, there is a necessity to establish a simple assay for the quantification of the TG2 deamidation activity, which could be used in studying of proposed TG2 inhibitor as a potential intervention on the CD.

The seed`s storage proteins of leguminous plants (legumin and vicilin) undergo deamidation prior to processing by proteolytic enzymes ultimately aiding the development of the emerging plant (Müntz 1996). The deamidation of the legume storage proteins in the seeds will provide vital nitrogen for the emerging plant's protein synthesis before the peptidase enzymes degrade the storage proteins. The deamidation of the storage proteins after the onset of germination can be viewed using non-denaturing PAGE (See Figure 6-3). They have become more electronegative due to deamidation in the protein bodies within the cells of the cotyledons by a deamidating enzyme (plant transglutaminase?) as germination proceeds. The storage proteins could be developed into a useful tool to measure the calcium-dependent deamidating activity of gpl and hrTG2, the enzyme that important to the initiation of CD (Mehta and Eckert 2005, Sollid and Jabri 2011).

Previous work at NTU (Aldubayan 2014) presents a preliminary attempt to optimise the *Vicia faba* storage proteins for a TG2 deamidation assay. That study reported the highest natural deamidation rate was between 7th to 14th days of seed germination in *Vicia faba* globulins (See Figure 6-3). Furthermore, by using non-denaturing PAGE reported the increased electronegativity as result of incubation of *Vicia faba* storage proteins with gplTG2 in presence of 2mM $CaCl_2$, this increase was calcium-dependent compared to samples incubated with EDTA and the blank, those findings were tentatively validated using microplate OPA assay to measure ammonia. However, to date, no effective assay has been produced to quantify the deamidation activity of hrTG2 or the release of ammonia from the target proteins/peptides. As mentioned previously, the deamidation of gluten peptides by TG2 is crucial for the peptides to act as HLA-DQ-restricted T-cell antigens in coeliac disease (CD) (Stamnaes and Sollid 2015). Therefore, in this research,

the aim was to optimise an assay for quantification of the ammonia released from seed storage proteins, a mixture of gliadin peptides and a 33mer peptide (deamidation) by modifying a previously published assay (Poulin et al., 2007).

In order to mimic the environment of coeliac disease, human recombinant TG2 was used to visualise TG2 deamidation activity using *Vicia faba* storage proteins. The increased electronegativity of the *Vicia faba* storage proteins observed in non-denaturing PAGE, by incubation with hrTG2 in presence of calcium ions, means an increase in the protein's electronegativity and migration towards the anode comparing with samples incubated with EDTA or control (See Figure 6-4). To examine the effect of increasing calcium concentration, the *Vicia faba* storage proteins were incubated with hrTG2 in the presence of different $CaCl_2$ concentrations, the finding show increased electronegativity dependent on the concentration of calcium (See Figure 6-5) (Tian, et al., 2014; De Re, Magris and Cannizzaro 2017). These results confirm deamidation of amide nitrogen from the *Vicia faba* storage proteins by hrTG2 is a calcium-dependent deamidation (See Figure 6-5). Moreover, these results support a previous NTU study (Aldubayan 2014) which used gplTG2 while the current study hrTG2 was used.

As mentioned before, the aim was to quantify the deamidation activity of TG2. Therefore, there was a necessity to translate the qualitative deamidation assay of *Vicia faba* proteins (non-denaturing PAGE assay) into a quantitative assay. By using an OPA reagent, the flouremetric microplate assay was optimized. The assay is based on the reaction of OPA with NH_4^+ released in the *Vicia faba* storage protein by the enzyme action of TG2, resulting in a florescence signal. The intensity of the signal would be proportional to the amount of ammonia in solution and consequently level of deamidation induced by TG2 in the presence of calcium ions (see introduction section). Through developing this microplate deamidation assay, many problems encountered, due to the high background of the assay, which could be resulting from the reaction of OPA with amines that occur in a number of ambient environments such as air, water, hair and foods (Del castello 1990). To help in resolve these problems distilled water from different sources were used in preparation of the assay reagents, to reduce the source of background signal (data not presented), ultrapure water was used throughout in the preparation of all reagents to give consistent results. Another source of the variations in the results was using plastic microfuge tubes in the assay. These tubes were not gas sealed enough to prevent of the released ammonia from leaking at 37^{o}C. To solve this problem, glass sealed vials

(chromocol) were used with crimp sealed gas-tight lids, which proved to provide consistent results. In this study, different buffers were used such as HEPES, Tricine and Tris all of them at two concentration 100 and 50 mM, and pH 8.3. They produced inconsistent results (Data not presented). Finally, lowering the Tris/HCl buffer concentration to 10mM (pH 8.3) produced consistent results required for a robust assay. This buffer concentration was low enough not to interfere in the development of the OPA ammonium assay. The data in Figure 6-6 shows that a microplate assay can be used to quantify the ammonia in the concentration range from 0.0025 to 1mM. This data supports the previous study that mentioned the OPA reagent exhibits greater sensitivity to amines rendering it suitable for measurement of picomole to-nano mole range of ammonia and amino acids in a sample (Peterson 1983).

Using *Vicia faba* storage proteins as substrates for gplTG2 or hrTG2, the release of ammonia was measured after overnight incubation at 37^{o}C (See Figure 6-7). The results indicate that there is a high quantity of ammonia released in samples with $CaCl_2$ (8.28nmol/hr ±0.68), comparing to other samples which suggest that the ammonia released is Ca^{2+} dependent. However, results indicate that there is ammonia released in EDTA (3.3nmol/hr ± 0.45) and blank samples by either gpl or hrTG2; it is a small but a detectable change. The possible reasons for detected ammonia (deamidation) in EDTA and blank samples are the exogenous calcium was not completely removed by EDTA or perhaps by nonenzymic deamidation (Raki, et al. 2007). In order to examine whether the ammonia released from the storage proteins was calcium-dependent or not. Different concentrations of $CaCl_2$ were used in the assay for both gpl and hr TG2 samples (See Figure 6-8 a and b), the finding shows the ammonia releasing (deamidation) was enhanced by increasing calcium ions concentration 0.025 to 10 mM Ca^{2+}. The deamidation of the seed storage proteins was reduced in the presence of known transglutaminase 2 inhibitors e.g. cystamine (See Figure 6-9), cystamine inhibition was dose-dependent, showing the highest inhibition at 2.5mM. The deamidation assay indicates that deamidation of seed storage proteins of *Vicia faba* was dependent on the presence of TG2 and Ca^{2+} ions. As the main aim of this research is, develop assay to measure the deamidating activity of hrTG2 against gliadin peptides because of its relevance to celiac disease. The microplate deamidation assay developed for *Vicia faba* storage proteins was transferred to the measurement of the deamidation of wheat gliadin peptides.

The ingestion of gluten is the critical factor in the initiation of celiac disease (Siegel and Khosla 2007). Gluten found in cereals such as wheat, barley, and rye, it consists largely of monomeric and polymeric proteins called gliadins and glutenins. They have a complex composition, rich in proline (15%), glutamine (35%) and hydrophobic amino acids (19%) collectively known as prolamins (Barro, et al. 2016). The gliadin protein (2.0mgml^{-1}) was incubated with 5.0 µgml^{-1} of hr TG2 for 10 min in presence of 2mM of Ca^{2+} ammonia released was recorded, 10.74 nmol min^{-1} (±0.45). This finding is in line with the amount of ammonia measured in study by Berti et al., (2007) after microbial TGase treatment of gliadins. By comparing the amount of ammonia in sample contains calcium ions with samples containing EDTA or blank, Figure 6-10 shows there was a significant increase in ammonia measured in samples containing hrTG2 and Ca^{+2} ions. Although, detectable ammonia was also measured in absence of Ca^{2+} suggesting that the deamidation of gliadin protein was enhanced but not dependent on presence of calcium ions (see Figure 6-10).

The amount of ammonia released using gliadin protein was observed at different incubation times. The data (See Figure 6-11) shows that ammonia released peaked and was constant after 2.0min. This result is in agreement with the previous NTU work by Aldubyan 2014, which mentioned that deamidation and release of ammonia from gliadin peptides proceeded at a faster rate than the deamidation of *Vicia faba* storage proteins (Aldubyan 2014).

To confirm, an enzyme-dependent activity, instead of using active hrTG2, boiled hr TG2 was applied to the assay. The findings show a significant decrease (>50%) in the ammonia released in both boiled and non-hrTG2 samples, this finding confirm that the ammonia released from incubation of hrTG2 with gliadin protein was catalysed by a biologically active hrTG2 (See Figure 6-12). In addition, the data in Figure 6-13 show a significant increase in the ammonia released (deamidation) by increasing the concentration of hr TG2,however, there is detectable ammonia in absence of TG2, this might be due to spontaneous deamidation of protein which could occur at all pH values. This modification may be able to arise spontaneously in 'ageing' proteins (Anderton 2004).

The amount of TG2 that give highest ammonia quantity ranged from 0.25-50 ng. Additionally, the results in Figure 6-14 indicated that the ammonia released (deamidation) after incubation of gliadin mixture peptides with hr TG2 was enhanced by increasing calcium ions comparing with no Ca^{2+}. In order to examine the specificity of this

deamidation to TG2, albumin and α-amylase proteins were used instead of gliadin peptides in the assay. These findings display that the ammonia released was reduced to about 50% when other proteins used in the assay (see Figure 6-15), this suggest that ammonia released was specific to hrTG2.

To observe the effect of the TG2 inhibitors on the deamidation activity of TG2, two commercially available TG inhibitors, were used cystamine and Z-DON, in this assay. Cystamine is a nonspecific reversible non-competitive TG inhibitor which can reversibly inhibit TG by binding to the catalytic site, (Keillor et al., 2015; Szondy et al., 2017; Jeitner et al., 2018). It exerts the TG2 inhibitory action by the formation of disulphide mixture with the active site cys277 that leads to preventing TG2 catalytic activity (Jefiner, 2018). It is considered as a nonspecific TG2 inhibitor because it will form disulphide mixtures wth surface as well as active site sulphydryl groups on proteins, but is still used by biologists (As of early 2018 there have been more than 200 publications citing this compound as a TG2 inhibitor) (Palanski and Khosla 2018). Z-DON has been designed to irreversibly inhibit glutamine-dependent amidotransferases (Hausch et al., 2003). It targets the active site cys277 residue of the TG2 (IC50 of >1μM). It has been designed to react covalently with the highly nucleophilic sulphur atom from the catalytic site cys residue in TG2, leading to completely inactivation of the enzyme (Badarau et al., 2013). These inhibitors were unable to prevent the release of ammonia from gliadin mixture peptides in the presence of (250 ng) of hrTG2, cystamine (0.02 – 10.0mM) and ZDON (0.01 - 1.0mM). Cystamine was found to have an inhibitory effect at high concentrations >10mM (See Figure 6-16). Based on this data the active site Cys^{277} might be not involved in all the deamidation of the gliadin mixture peptides. This motivated the study to expand this experiment to use inactive human recombinant TG2 (Cys^{277}, cysteine replaced by serine) instead of active TG2. This form of TG2 (250ng) was incubated with gliadin protein 2.0 mg ml^{-1}.

A striking observation was that the release of ammonia was similar when using active hrTG2. The high value (10.34±0.78nmol min^{-1}) compared to the control sample with no hrTG2 (4.13±0.19nmol min^{-1}(see Figure 6-17) suggesting that either the cysteine ^{277} at the active site of hrTG2 is not exclusively involved in the deamidation reaction of TG2 or that the assay is in some way compromised. Although, this finding disagrees with Staminas et al., (2010), which hypothesized that TG2 mutants impede the calcium triggered conformational change that is required for deamidation activity (Stamnaes, et

al. 2010). However, it is consistent with previous finding, (See Figure 6-10) shows that the ammonia releasing (deamidation) of gliadin protein by TG2 is calcium enhanced not dependent. Cystamine and ZDON as TG2 inhibitors applied to examine their effect on ammonia released from inactive TG2. The data in Figure 6-18_shows no significant inhibitory action at the concentrations used cystamine (0.02 – 1.0mM) and ZDON (0.01 – 1mM). To help in clarifying these finding, there was a necessity to use the 33-mer gliadin peptide that is deamidated in initiation CD with this form of TG2 (see section1.8.4).

There could be some discussion about these findings, particularly when ammonia also released after incubation of gliadin proteins with TG2 may not be from deamidation only, but could be produced by transamidation (see section 1.5.3). Because the gliadin peptide mixture contains both lysine and glutamine in the peptide mixture it could be argued that the ammonia released was derived from transamidation as well as deamidation. The general acceptance in the literature is that deamidation has been shown to occur parallel to transamidation proceeding via the same catalytic pathway. The ratio of transamidation versus deamidation is high in the presence of amine (Fleckenstein, B. et al 2002). It has been assumed that the transamidation assays of biotin cadaverine incorporation (Slaughter et al., (1992) and peptide incorporation (Trigwell et al., 2004) fairly reflect the deamidation activity of TG2. They both proceed via the same catalytic mechanism but it should be remembered that there will be little or no deamidation in the presence of an amine. It is difficult to rule the possibility that deamidation is different to transamidation without an assay to measure the two activities. Moreover, deamidation by TG2 could be a substrate-dependent (Stamnaes 2008).

To validate that the ammonia released does represent the deamidation of gliadin protein by hrTG2 and to mimic the celiac disease condition, 100µM of 33-mer gliadin peptide fragment were incubated with two different forms of transglutaminase 2, the active and inactive forms. A peptide fragment of (α2-gliadin56–88) 33mer (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF), produced by normal gastrointestinal proteolysis, becomes a potent T cell stimulator after deamidation by tissue transglutaminase (TG2) (Schalk et al 2017). This fragment contains high proline content (13 of 33 residues are proline), which is responsible for its high resistance to breakdown by intestinal brush-border and luminal proteases enzymes (Qiao et al., 2004). Crucially this peptide does not contain any lysine and as such, there can be no transamidation. The

only amine residue is on the first amino acid in peptides structure and this represents an alpha-amino not an epsilon amino present in the lysine side chain. TG2 uses the epsilon amino on the side chain of lysine for the transamidating reaction. The data in Figure 6-19 and Figure 6-20 show there is no significant difference between the quantity of ammonia released between gliadin peptide mixtures and 33-mer peptides after incubation with active hrTG2. These findings suggest that the ammonia released in the assay is due to the deamidation catalytic action of hrTG2. By applying inhibitors (cystamine and ZDON) to the assay, cystamine displays a significant inhibition on the ammonia released from 33-mer gliadin, whereas there is no clear inhibition with gliadin protein (Only at high concentration). On the other hand, ZDON treatment samples did not show any inhibition on both gliadin protein and 33-mer peptides (Figure 6-20).

By incubation, the inactive form of TG2 with the 33-mer peptide, finding display that there is a significant reduction in releasing of ammonia comparing to gliadin peptide mixture sample (See Figure 6-8). This result suggests that ammonia released when the inactive TG2 incubated with gliadin peptide mixture is due to other peptides other than 33-mer gliadin peptide. Moreover, neither cystamine nor ZDON gives any inhibition. The data presented in this study agrees with the previous research saying that this sequence specificity of TG2 clarified the variation between peptides as TG2 substrates (Fleckenstein et al., 2002). In order to confirm the data of fluorescence assay, samples were prepared to run by mass spectrometry technique to detect the deamidation of gliadin peptides. The samples containing 33- mer peptides with either EDTA or calcium were incubated with active hr TG2, and undeamidated peptides as control were applied to mass spectrometry. The non and deamidated peptides eluted at the same point on the reversed-phase gradient and the mass difference of 1Da between deamidated and undeamidated peptides, making it difficult for MS to recognise the deamidated peptide. Further experiments are needed for purification and identification of deamidated through specific chromatographic method before applying to high sensitive mass spectrometer.

6.5 Summary of key findings

- Modified ammonia assay developed to quantify the ammonia released in deamidation activity for hrTG2
- Deamidation of storage proteins by hrTG2 was calcium-dependent and was verified by the OPA assay.

- Deamidation of gliadin protein by hr TG2 was enhanced by calcium, but there is detectable ammonia released in the EDTA and control samples.
- Cystamine and ZDON did not inhibit the ammonia released from incubation of gliadin protein with hrTG2.
- Inactive TG2 (Cys^{277}, cysteine replaced by serin shows deamidating activity using the crude gliadins peptides mixture. The release of ammonia was, similar to that produced by active hrTG2, this finding suggests that deamidation might occur in another part of the hrTG2 molecule other than the active site.
- To confirm that ammonia released was because of deamidation not transamidation the33-mer gliadin peptide that initiates CD was used. This peptide does not contain lysine excluding the possibility of a transamidation reaction. This peptide fragment produced quantifiable ammonia when incubated with active hrTG2. This activity was inhibited by cystamine. This finding shows that hrTG2 has a quantifiable deamidating activity toward the 33mer gliadin peptide sequence and other gliadin peptide sequences

Chapter 7 : General discussion and future work:

7.1 Discussion and Future work

Tissue transglutaminase (TG2) is a universally expressed enzyme of the mammalian transglutaminase (TG) family that catalyses the formation of an intra- or inter-molecular isopeptide bond between glutamine and a lysine side chains, leading to the post-translational modification of proteins. The isodipeptide bonds formed between cross-linked proteins are peptidase resistant (Kanshan et al., 2015). In addition to protein-protein cross-linking, TG2 mediates the incorporation of primary amines into proteins and, in the absence of amine nucleophiles, water acts as the nucleophile resulting in the deamidation of protein/peptide glutamine residues to glutamate (Figure 1-1; Kárpáti et al., 2018). The biological functions of TG2 include tissue repair, inflammation, and the development of some cancer, fibrosis and coeliac disease (Kobayashi et al., 1998; Bergamini et al., 1999 and Verederio et al., 2005). Transglutaminase 2 upregulation has been involved in many different diseases processes. For example, neurodegenerative disorders such as Huntington's and Parkinson's diseases (Hoffner and Djian, 2005), different cancers such as breast, ovarian and pancreatic (Mangala and Mehta, 2005). The deamidating action of TG2 is involved in the development of age-related cataract and in the initiation of the coeliac disease (see chapter 1). In coeliac disease, dietary gluten in wheat, rye, and barley leads to an abnormal immune response in genetically liable individuals carrying the human leukocyte antigen (HLA)-DQ2 or -DQ8 haplotypes(Hietikko, et al. 2018). Coeliac disease, characterized by small-bowel mucosal damage, which develops gradually from normal villous morphology to inflammation, and finally to villous atrophy (Lebwohl, Sanders and Green 2018).

The adaptive immune reaction occurs in coeliac disease individuals is dependent on deamidation of undigested gliadin (33mer) peptide found in the intestinal *lamina propra* by the TG2 (Dahlbom, et al., 2005; Lionetti and Catassi, 2011; Cebolla et al., 2018). Deamidation magnifies the immunogenicity of the 33mer gliadin peptide, facilitating binding to the HLA-DQ2 or HLA-DQ8 molecules on antigen-presenting cells, the only currently available treatment, is removal of gluten from the diet. Although, in recent years the high quality of gluten-free products has improved; however, the adherence to these

products depends on different individuals and environmental factors (Hall et al., 2013), such as, not easily available in for all suffers or it is expensive (Kelly et al., 2015).

The critical role of TG2 in CD makes inhibition of TG2 activity a potential therapeutic target. A number of TG2 inhibitors have been developed, including reversible inhibitors such as cinnamoyl compounds, thienopyrimidines, and acylidineoxindoles and irreversible inhibitors such as thiadiazoles, epoxides, and dihydroisoxazoles (Plugis and Khosla 2015). However, as TG2 is present in all cells, chemical inhibitors of the enzyme may have an unfavorable effect on cellular processes other than CD. Natural substances such as garlic and milk present less of a toxicity problem, as many people without adverse side effects ingest them. Milk and garlic have been shown to contain TG2-mediated transamidation inhibitors, (Williams et al., 2005; Lee et al., 2007;Aldobyan,2014).

Flavonoids, a group of natural substances with changeable phenolic structures, are found in vegetables, fruits, roots, stems, grains, flowers, and tea. These natural products possess beneficial effects on health such as anti-oxidative, anti-mutagenic, anti-inflammatory and anti-carcinogenic effects. In addition, their ability to modulate key cellular enzyme function (Heller and Forkmann 2017)(See Chapter 3). Some studies have shown that flavonoids could have an impact on the role of TG2 in some diseases. These effects include the influence of flavonoids extracts on the role of TG2 in the wound healing process, the role of dietary quercetin in the reduction of reproductive capacity in female mice, and the proposed role of TG2 in the regulation of the ovarian aging process. (Beazley and Nurminskaya 2016, Malla, et al. 2016). From the literature, there are no studies describing the effects of flavonoids on transamidating or deamidating TG2 activities. As mentioned in publication, more than 80% of drug substances were directly from natural products or were the molecules derived from natural sources. Therefore, the main aim of this study was to screen the effect of dietary flavonoids on the amine incorporating and protein cross-linking activity of TG2 as well as the deamidating activity (see chapter 3) as a potential method to moderate the initiation of coeliac disease (CD). In addition, trying to optimise a simple assay for measurement the TG2 deamidation activity that would be essential for testing the effect of biological active compound on the TG2 activity as potential treatment of coeliac disease.

Thirteen pure flavonoids were used in this study, including; quercetin, kaempferol, morin, myricetin, apigenin, luteolin, hesperetin, hesperidin, naringin, cyanidin, catechin,

epicatechin and taxifolin. Three flavonoids (kaempferol, morin and quercetin) in either sodium deoxycholate or DMSO, demonstrated inhibitory behaviour against hrTG2 and to a lesser extent on gplTG2 transamidating amine incorporation activity, at 125µM, (Figure3-13 and Figure 3-14). Those three flavonoids have a similar chemical structure (Figure2-3). The major structural difference between flavonoids was in the position of hydroxyl groups. In general the B-ring hydroxyl configuration is the most significant determinant of the biological effects of the flavonoids (Burda and Oleszek, 2001 and Pannala et al., 2001). Therefore, it was hypothesised that the position of the hydroxyl group within the flavonoids was related to the affinity of binding between flavonoids and transglutaminase. In the presence of calcium, TG2 exposes hydrophobic pockets (Pinkas, et al. 2007). This suggests that the interaction between the flavonol and Tg2 may rely on the presence of calcium ions. If the presence of the flavonol disturbs the binding of calcium to TG2 it may prevent the enzyme from completely opening up, this, in turn, could alter the substrate from binding to the active site cys377. The major exception to TG2 enzyme inhibition was that there was less inhibitory effect on amine incorporation activity of gplTG2 suggesting that although the two enzymes have similar properties and homology that there must be subtle differences between the two enzymes in terms of their interaction with these natural products, perhaps around the calcium-binding sites or hydrophobic pocket.

The effect of flavonoids on the TG2 protein cross-linking activity *in vitro* was assessed according to the method of Trigwell et al (2004), the 13 flavonoids (see above) dissolved in DMSO and sodium deoxycholate were applied to the assay using gpl and hrTG2. Most of flavonoids display – (particularly that dissolved in DMSO) - a significant level inhibition ($p<0.05$) for the hrTG2 protein cross-linking activity at concentration 1.25, 12.5 125µM. The hrTG2 was more sensitive to the effect of flavonoids than gpl TG2. The finding of the cross-linking assay was in agreement with studies that reported intensive inhibition effect of flavonoids on cross-linking of proteins (Wu and Yen 2005, Urios, Grigorova-Borsos and Sternberg 2007). In addition, to the flavonoids binding to the enzyme or substrate it is also known that they bind divalent metal ions including Ca^{2+}, which play a role in the activity of TG2 (Urios et al., 2007).

In an attempt to understand the mechanism of the inhibition of TG2 by the flavonoids kinetic analysis was performed. The Michaelis-Menten kinetic constant (Km) and Vmax were graphically determined to measure the kinetic properties of the flavonol on TG2.

The data showed variation in the mechanism of inhibition among the flavonoids used. In the inhibition of amine incorporation activity of hrTG2 (Table3-1,Table 3-2 and Figure3-12, Figure 3-22), the data displayed the competitive inhibition mechanism with the biotin amine substrate by kaempferol and quercetin. (The Km value of the biotin substrate with hrTG2 was increased the presence of the flavonoids) (Boyer, 2011). Whereas, morin exhibited uncompetitive inhibition of hrTG2 transamidating activity with the biotin cadaverine substrate. In contrast the inhibition of the cross-linking activity (See Table 3-3,Table 3-4 and,Figure 3-27 and Figure 3-17) of kaempferol, morin, and quercetin was uncompetitive inhibiton.The differences between that mechanism is in the competitive mechanism, the inhibitor may link to the free enzyme (Amine et al., 2016), the inhibition here refers to binding of inhibitor at or near to the active site on the enzyme, While, the uncompetitive inhibitors specifically targets the enzyme-substrate (ES) complex without competing with the substrate for binding (Palmer and Bonner,2007; Dougall and Unitt 2015). These findings describe the situation of the inhibition of the TG2 enzyme by flavonoids using this substrate. Although, there are no published reports about the effect of flavonoids on activity of TG2 or their mechanism, whoever, Many studies have confirmed that the flavonoid inhibition effect is related to their structures, and the nature of the enzyme and substrates, as from the data, the difference in the mechanism of inhibition is might be related to that factors. (Xie et al., 2003; Phan et al., 2013, Ribeiro, Daniela et al., 2014).

It was decided to study the effect of the combinations of flavonoids on hrTG2 and gplTG2 transamidation and cross–linking activity to possibly mimic the mixtures that could be present in food. The data in Figure 3-10,Figure 3-11,Figure 3-15and Figure 3-16 display that the combination of three flavonoids have a higher inhibitory action on the amine incorporation activity ofTG2 than when they are presented individually. Another curious finding is that the combination of flavonoids has significant inhibitory action at a lower concentration (12.5µM). For gpl activity, even mixtures of the three flavonoids improve the action of individual flavonoids effect on amine incorporation activity of gplTG2. In contrast, Kaempferol, morin, and quercetin were mixed, and applied in cross-linking activity assay, according to the method of Trigwell et al (2004). Data in Figure 3 20, Figure 3 21, Figure 3 25 and Figure 3 26 show no synergism effects between three flavonoids, individually the inhibitory effect was more pronounced than in a mixture.This finding agrees with the literature, which reports that the individual antioxidants do not

appear to have consistent preventive effects with the mixture of food contain antioxidant (Liu, 2003).

As the deamidating activity of TG2 is crucial in initiation of CD and because one of the main aims of this research was optimising a simple assay for measurement the deamidating activity of TG2 (See Chapter 6). The pure flavonoids were applied in the OPA assay to measure their effect on the deamidating activity of hrTG2). The data in Figure 3-28shows that none of the tested flavonoids have a significant reduction in amount of ammonia released when a mixture of gliadin peptides was used as the substrate. The ammonia released with kaempferol, morin and quercetin at 125µM were consequently (9.7nmol min^{-1} ± 0.47; 9.2nmol min^{-1} ± 0.46; 9.8nomol min^{-1} ± 0.44) this means that these flavonoids inhibit ~20% of ammonia released compared to the control. It is worth mentioning, that kaempferol, morin, and quercetin are also active against amine incorporation activity of TG2 (Figure3-13)

This finding suggests that those flavonoids are active against TG2 activity in general, so the food containing morin and quercetin such as kale and strawberry could help the CD patient and offer new trend in helping the CD sufferer.

The biological activity of the dietary flavonoids motivated this research to screen flavonoid-rich foods and examine their effects against TG2 activity as a potential treatment for coeliac disease. As it's known, the analyses of flavonoids in plants, medicine, and food products are more complicated. Therefore, the novel method for purification of flavonoids from food extracts was suggested, which is Immobilized Metal Ion Affinity Chromatography (IMAC) (Cheung et al., 2012). The yield and purity of this method then compared with a more conventional Solid Phase Extraction (SPE) method (Liu, Houme et al., 2016). For the quantification of extracted flavonoids, the bicinchoninic acid (BCA) assay (Smith et al., 1985) was used to compare the quantity yielded between two methods (IMAC and SPE). The BCA assay relies on the Biuret reaction of the peptide bond reducing Cu^{2+} to Cu^{+}, which then chelated by BCA to produce a purple colour. Other compounds with a reducing centre can be quantified using BCA e.g. reducing sugars (Anthon and Barrett 2002).With this reason that flavonoids were quantified using BCA reagent because they also possess the ability to reduce divalent metal (Ivanov et al., 2001and Wei and Guo, 2014).

Data in Table4-1 and Table 4-2 show that the amount of flavonoids varied between 0.20 to 1.12mg 100^{-1}g DW. Whereas, the amount in SPE fractions were arranged from 0.16 to 0.26 mg/100^{-1}gDW. The comparison between the fractions from two purification methods in term of purity was determined by RP-HPLC. The chromatograms for food IMAC fractions have produced a clearer chromatogram as judged by a reduction in background peaks (Figure 4-2). Although there are no significant differences between quantities obtained from both methods, the HPLC chromatograms confirm that the IMAC fractions contain fewer contamination peaks, which are observed in SPE chromatograms (Figure 4-2). These findings confirm that SPE extracted all the organic components with the ability to bind to a C18 , whereas IMAC extracts the components that have at least two hydroxyl groups close together on an organic structure particularly those on the flavonoids. This finding confirms that IMAC method provides a more selective and appropriate technique to purify flavonoids in those food samples. Additionally, IMAC fractions were subjected to LC/MS/MS in to confirm that fractions contain flavonoids; the data (Figure 4-3) were reported that all food fraction contains flavonoids, although, they differ in type of flavonoid available in each fraction.

In addition to pure flavonoids, flavonoid extracts were prepared from apples (*Malus Domestica*), green tea (*Camellia sinensis*), strawberry (*Fragaria vesca*), kale (*Brassica oleracea*), capers (*Capparis spinose),* garlic (*Allium sativum*) and onions (*Allium cepa*). The extracts were applied to the biotin cadaverine incorporation assay (Slaughter et al., 1992) and the peptide cross-linking assay (Trigwell et al., 2004) to examine their effect on TG2 transamidation activity. The data in Figure 4-4 A and B display the inhibition behaviour of most of the food extracts used towards the amine incorporation activity of TG2. Whereas, their effect on cross-linking activity of TG2, was not the same on two forms of TG2, as most of food extracts display inhibition effect on the gplTG2 cross-linking activity. Whereas, only the samples with kale and strawberry have inhibition effects on the hrTG2 activity (See Figure 4-5 A and B). The finding display the difference in the sensitivity towards the flavonoids extracts between gpl and hrTG2 activity. The subtle differences between hrTG2 and gplTG2 could affect the topography of the enzymes active site. In order to screen the effects of food extracts on deamidating activity of hrTG2, as mentioned previously, this activity is crucial for initiation of CD. No food extract showed inhibition of hrTG2 deamidating activity using the optimised fluorescence assay, (See Figure 4-6). However, the two extracts from alliums (onion and garlic) show

stimulation of the deamidating activity (high ammonia released). In light of the finding of this study, the people with coeliac disease should perhaps try to avoid containing food containing too much onion and garlic. The study of the relationship between consumption of the dietary flavonoids containing food and the CD prevalence might give another indication about the effects of dietary flavonoids on the CD suffers.

The main impediment in the evaluating of the therapeutic potential behavior of TG2 inhibitors in relation to CD, is the unavailability of animal or cell model for the coeliac disease that could be used to test the CD histopathological, immunological and serological abnormalities (Auricchio, S et al., 1984 and Papista et al., 2012). As the TG2 is active in the lamina propria of human small intestines and believed to be exported from epithelial cells. It would be an advantage to use a small intestinal epithelial cell line to test the therapeutic efficacy and cellular safety of flavonoids that have TG2 inhibition action (kaempferol, morin, and quercetin), (Papista et al., 2012). Although HT29 cells are not ideal for studying CD, however, this cell line may provide a marker about the effects of different reagents on disease-relevant to a human cell type. The HT29 cell line is derived from large intestine carcinomas was used in this study to examine the effect of flavonoids on the cell viability, cell proliferation, *in situ* TG2 activity and identification of proposed TG2 substrates. The data (See Figure 5-2) displayed that the kaempferol and quercetin (4 to 125 µM) did not show a statistically significant inhibition effect on the HT29 cell viability; morin displayed inhibition effect on the viability of the cells (as measured by MTT reduction assay). On the other hand, the three flavonoids did show a statistically significant inhibitory effect on the proliferation of HT29 cell lines (Figure 5-3).

These findings are in agreement with the study that stated the cellular protective effects of flavonoids in colon cancer development should be accompanied with the inhibition of cell proliferation to delete cells carrying mutations and to maintain a normal cell population (Kuntz et al., 1999). It is worth mentioning, that by connecting the inhibitions action of the studied flavonoids for the transamidation of hrTG2 (Figure 3-8 and figure 3-13) with the anti-proliferative data of the same flavonoids (Figure 5-4), could speculate that there is a link in the mechanisms of both actions of these flavonoids. To discover the *in situ* action of the active flavonoids against the TG2 activity (kaempferol, morin, and quercetin) and to identify the TG2 target substrate. Biotin-X cadaverine (a cell-penetrating substrate of TG2) was used to label intracellular proteins in HT29 cells via intracellular TG2 activity (Almami, et al,. 2014). The data (Figure 5-5) showed that the

intracellularTG2 activity was reduced by using kaempferol, which is in agreement with *in vitro* TG2 inhibitory effect (Figure 3-8). While the morin and quercetin inhibition effect on intracellular TG2 activity was statistically insignificant. These findings are inconsistent with *in Vitro* TG2 assays.

The reasons for this dissimilarity might be the fact that the environment of *in vitro* assay was different. As the flavonoids, TG2 and substrates were pure, in contrast, in the cell model were the flavonoids are exposed to metabolism and presence of other TG2 isoforms. In addition, to the presence of various proteins which could act as an alternative substrate to the TG2. Hence, it is necessary to have a knowledge of intracellular TG2 substrates targets, and interacting proteins that may act as novel drug targets or new diagnostic markers. Various proteins with biotin-x-cadaverine were identified by using SDS-PAGE/ western blotting and ExtrAvidin HRP detection. the appropriate proteins were extracted from gel and because of the band was extracted from 1-D gel, many proteins have been detected., The mass spec identified proteins that show high sequence coverage and corresponding to the band size were selected from the data. The peptide sequences obtained from MS/MS were searched for homologies in the databases. Some of the identified proteins have not been previously identified as TG substrates either in HT29 or in other cell lines. These include the BAG family of molecular chaperone regulator 3 proteins, and RAF proto-oncogene serine/threonine-protein kinase (Table5-2).

As mentioned previously, the deamidation activity of TG2 is crucial in the initiation of coeliac disease. However, to date, no effective assay has been produced to quantify the deamidation activity of hrTG2 or the release of ammonia from the target proteins. Therefore, was attempt to develop and optimise an assay to measure the deamidation activity catalysed by TG2. Previous work at NTU (Aldubayan 2014) presents a preliminary attempt to optimise *Vicia faba* storage proteins for a TG2 deamidation assay, by using non-denaturing PAGE. It was reported that was increased electronegativity as result of incubation of *Vicia faba* storage proteins with gplTG2 in presence of 2mM $CaCl_2$. This increase in electronegativity was calcium-dependent compared to samples incubated with EDTA and the blank. These findings were tentatively validated using microplate OPA assay to measure ammonia. It is important to remember that there can be little or no deamidation in the presence of an amine (Fleckenstein, B. et al 2002). Therefore, it is important to have an assay to measure and quantify the deamidating activity of transglutaminases. To mimic the environment of coeliac disease, human recombinant

TG2 was used to visualise TG2 deamidation activity using *Vicia faba* storage proteins (see Figure 6-4). The results confirm deamidation of amide nitrogen from the *Vicia faba* storage proteins by hrTG2 is a calcium-dependent deamidation (See Figure 6-5). These findings support a previous NTU study (Aldubayan 2014) which used gplTG2 while the current study hrTG2 was used. As the aim was to quantify the deamidation activity of TG2. it was necessity to translate the qualitative deamidation assay of *Vicia faba* proteins (non-denaturing PAGE assay) into a quantitative assay. By using an OPA reagent, the flouremetric microplate assay was optimised. The assay is based on the reaction of OPA with NH_{4+} released in the *Vicia faba* storage protein by the enzyme action of hrTG2, resulting in a fluorescence signal. The intensity of the signal would be proportional to the amount of ammonia in solution and consequently level of deamidation induced by hrTG2 in the presence of calcium ions (see introduction section). The results (See Figure 6-7) indicate that there is a high quantity of ammonia released in samples with $Cacl_2$, comparing to other samples, which suggest that the ammonia released from the storage proteins is Ca^{2+} dependent. The conclusion from previous data is the deamidation of seed storage proteins of *Vicia faba* was dependent on the presence of hrTG2 and Ca^{2+} ions.That is in agreement with the published role of transglutaminase, as their activity dependant on the presence of calcium (Chrobok, et al. 2018).

Because of the ingestion of gluten is the critical factor in the initiation of coeliac disease (Siegel and Khosla 2007). The microplate deamidation assay was transferred to the measurement of the deamidation of wheat gliadin peptides by hrTG2. Different time of incubation (Figure 6-11) and series of TG2 concentration (See Figure 6-13) were examined, the finding demonstrates that the deamidation of the gliadin was fast compared with the using storage protein as substrate.As the optimum time give a high quantity of ammonia was varied from 2 to 10 mins this. It must, however, be recommended that care should be taken to measure the ammonia release as quickly as possible while performing the assay. Moreover, these findings confirm the dependence of releasing ammonia on increasing the concentration of TG2 (See Figure 6-13). The finding in Figure 6-10 refers to that the deamidation of gliadin protein was enhanced but not dependent on the presence of calcium ions. As it was unexpected to note that the detectable amount of ammonia in samples with EDTA. The application of commercially available TGase inhibitor (cystamine and Z-DON). Cystamine only at high concentration (10mM) inhibit the ammonia release (deamidation). However, Z-DON were unable to prevent the release of

ammonia from gliadin mixture peptides in the presence of hrTG2 (See Figure 6-16), even though, Z-DON is a good inhibitor of amine incorporation. One possibility expanding this finding could be that deamidation does not require the active site to be in the different conformation as far polyamine incorporation or protein cross-linking. Based on this data the active site Cys277 might be not involved in all the deamidation of the gliadin mixture peptides. This driven the experiments to use inactive human recombinant TG2 (Cys277, cysteine replaced by serine) instead of active TG2. An unexpected observation was that the release of ammonia was similar when using active hrTG2 compared to the control sample with no hrTG2 (see Figure 6-17) suggesting that either the cysteine 277 at the active site of hrTG2 is not exclusively involved in the deamidation reaction of TG2 or that the assay is in some way compromised.Overall, the data of this study suggested the following: the ammonia releasing (deamidation) was fast, increasing by increasing the concentration of TG2, enhanced but not dependent on presence of calcium ions, and suggesting that the cysteine 277 at the active site of hrTG2 is not exclusively involved.

There could be some discussion about these findings, particularly when ammonia also released after incubation of gliadin peptide mixtures with TG2 may not be from deamidation only, but could also be produced by transamidation (see chapter 1). The general notion in the literature is that deamidation has been shown to occur parallel to transamidation proceeding via the same catalytic pathway. The ratio of transamidation versus deamidation is high in the presence of amine (Fleckenstein, B. et al 2002). In order, to confirm that the ammonia released was due to the deamidation activity of hrTG2. The fragment of 33-mer gliadin peptide (100µM) was used as a substrate with active and inactive hrTG2 (this fragment which crucially does not contain any lysine and as such, there can be no transamidation). The data in Figure 6-19 and Figure6-20 did not show significant difference between the quantity of ammonia released between gliadin peptide mixtures and 33-mer peptides after incubation with active hrTG2. These finding, suggest that the ammonia released in the assay was due to the deamidation catalytic action of hrTG2. Furthermore, based on the finding of 33-mer peptides, this assay suggests the specificity of these peptides to deamidated by active TG2. The comparison of active TG2 with inactive TG2 data (See Figure 6-21), the data show that 33mer peptides have not been deamidated by the inactive form whereas gliadin mixture deamidated by inactive TG2.

A set of experiments were performed and analysed by a mass spectrometer to validate this assay, the data from MS displayed insignificant deamidation of peptides and not able to distinguish the deamidated peptide from the undeamidated peptide (data not shown). This was because both peptides eluted at the same position on a C18 mass spec column and with the same % of ACN, the difference between deamidated and un deamidated peptides just one Dalton which made the analysis difficult. An analysis which could be resolved in the time allowed. Further work is currently being undertaken to resolve the problem using HILIC and ion-exchange chromatography. Numerous methods have been used for quantification of protein/peptide deamidation. However, this current research concentrates on the deamidation mediated by TG2 as mentioned previously; to date, the most of published assays are for quantifying the level of deamidation catalysed by TG2 are used either Capillary electrophoresis or mass spectrometry techniques. The mass spectrometry methods have been successfully utilised to characterize peptides in complex mixtures, these results so far have largely been qualitative (Ong and Mann 2005) with the associated high costs. Therefore, this study suggests the OPA assay, which is a low-cost and simple assay that could measure the TG2 deamidation activity and would be essential for testing the effect of the biologically active compound on the TG2 activity as potential treatment of coeliac disease.

Based on the findings of this study, a recommendation can be made for future work. This study focused on the effect of flavonoids on the TG2 transamidation and deamidation activity. Other transglutaminase isoforms might be included in the study of the specificity of kaempferol, morin, and quercetin, which display inhibition effect toward the TG2. In addition, using other dietary flavonoids will give more knowledge about the effect of those flavonoids on the TG2 activity. Moreover, including other food extracts, which contains flavonoids, will be highly recommended as the consumption of food differ from person to another. Future studies are strongly recommended to test those flavonoids on coeliac disease human biopsies to elucidate their effect on the activity of TG2. The successful achievement of the clinical trial for these flavonoids could prove innovative towards the treatment of coeliac disease. This would mean that flavonoids based treatment in the form of an oral tablet could regularly be prescribed for the coeliac disease sufferers. Future studies are necessary to test the efficacy of the flavonoids TG2 inhibitors on other cell lines. For example, the inhibitors could be tested on neuronal cell lines such as N2a or on the white blood leukemia cell line K562; both of these cell lines have active TG2.

In the deamidation section, a further experiment could clarify whether the active site cysteine is required for deamidation. The exact nature of the deamidation conformation of TG2 would have to be determined using X-ray crystallography. Additionally, more experiments using high sensitive mass spectrum with gliadin peptides could confirm and validate the data of this assay.

7.2 Conclusion

This study was aimed at the identification of TG2 inhibitors from natural sources, as the products derived from natural sources are believed less or non-toxic. In this study, the flavonoid was studied as active biological natural products. Three of 13 pure flavonoids were displayed inhibition behaviour on the hr TG2 and in less extent gplTG2 amine incorporation activity, kaempferol, morin, and quercetin. However, the majority of flavonoids have inhibition effects on the cross-linking activity of TG2. Flavonoid did not show significant inhibitory effect on the amount of ammonia released (deamidating activity of hrTG2) comparing to the control. A new method for purification of flavonoids from food extracts was suggested in this study, which is Immobilized Metal Ion Affinity Chromatography (IMAC). In addition, BCA assay was optimised in a new quantification method using quercetin as standard. For amine incorporation activity, most of food extracts display a significant inhibition effect towards the hrTG2 and gplTG2. Whereas in cross-linking activity, the majority of food extracts display inhibition effect on the gpl TG2 cross-linking activity, only Kale have effect on hrTG2 activity. In the deamidation activity, no food extracts were showing inhibition for the ammonia released (deamidation activity) effect after using the optimised fluorescence assay (OPA) assay. The kaempferol and quercetin display an inhibition effect on growth of the HT29 cell line. The fluorescence assay confirmed the TG2 inhibitory characteristic of the kaempferol on HT29 cell lines. Numerous proteins were identified within a single protein band, which could act as TG2 substrates. Moreover, in this study, modified ammonia assay developed to quantify the ammonia released in deamidation activity for hrTG2. In conclusion, this was first study examined the effect of the flavonoids on the activity of TG2, the finding of this study displayed significant inhibition effect of three flavonols (Kaempferol, morin, and quercetin) on the transamidation activity of TG2. In addition, the novel method for the purification (IMAC) of flavonoids extracts from food samples was used in this study. According to the search in publication, this is first study used BCA method as a colorimetric method for quantification of flavonoids in the food extracts. Moreover, new

fluorescence microplate assay for deamidation activity of TG2 was optimised. Further experiments needed to validate this assay in future. Based on the finding of this study, the new biological effect of flavonoids was detected, which could offer new direction in therapeutic of CD, although, not all food containing flavonoid could be recommended to that CD sufferers.

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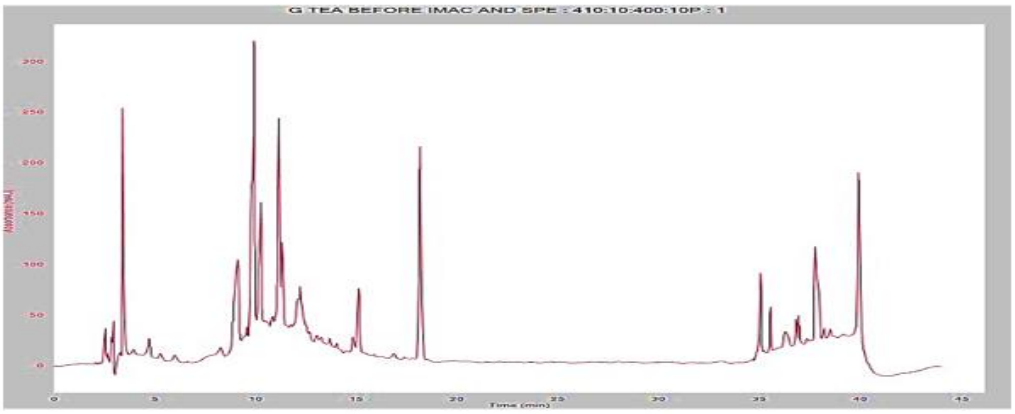
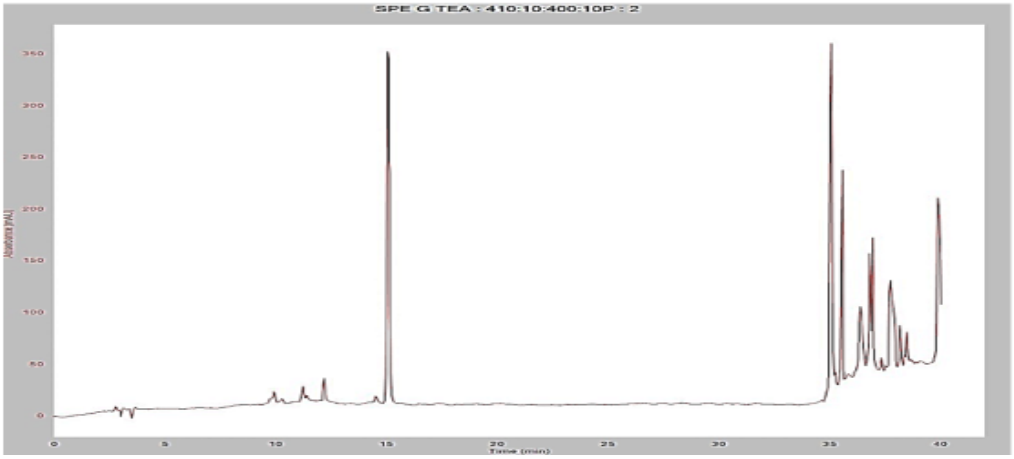
APPENDICES

Comparison between crude, SPE and IMAC fraction of food samples using RP-HPLC

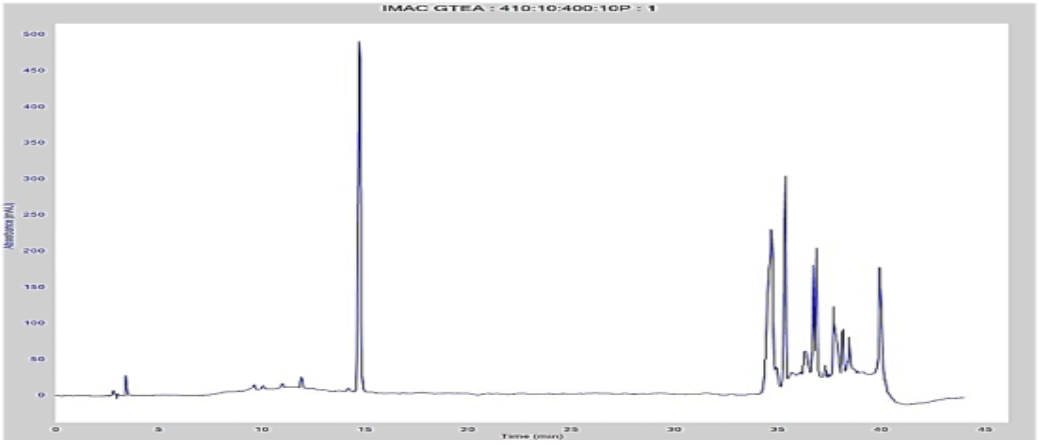
GREEN TEA BEFORE

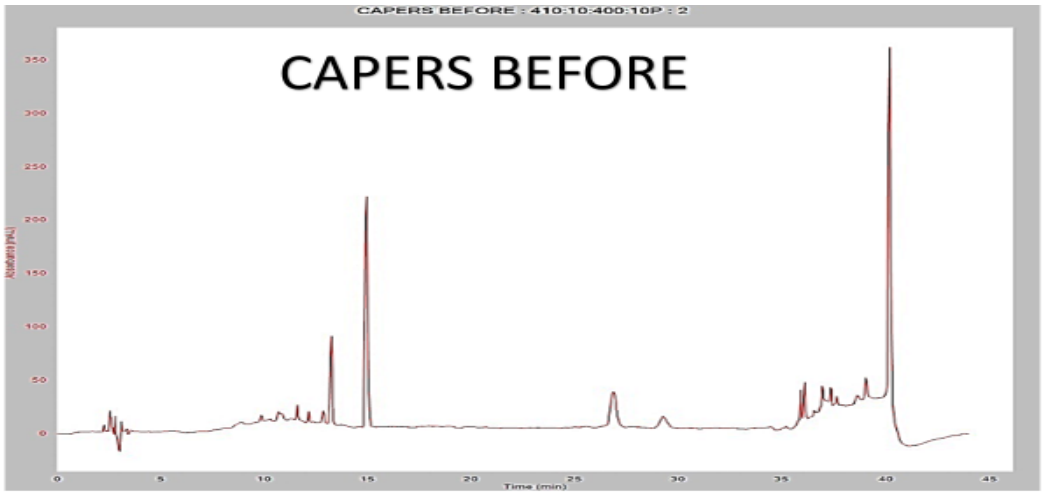


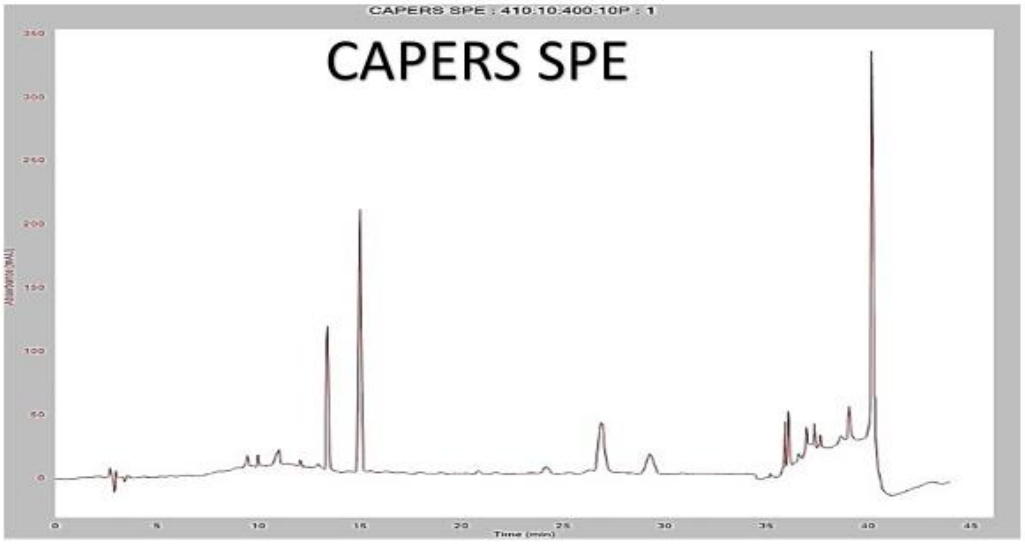
GREEN TEA (SPE)

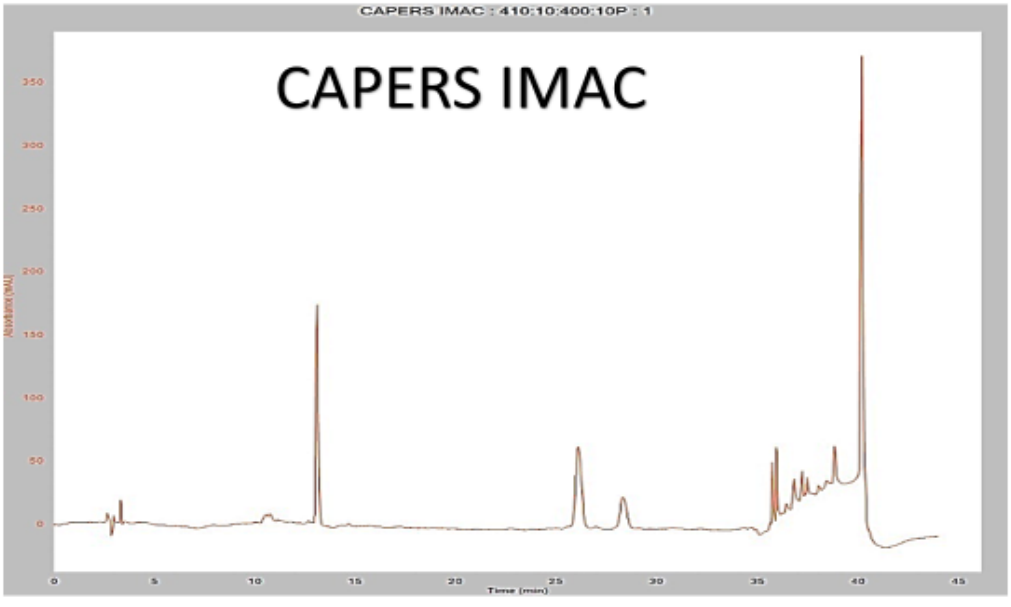


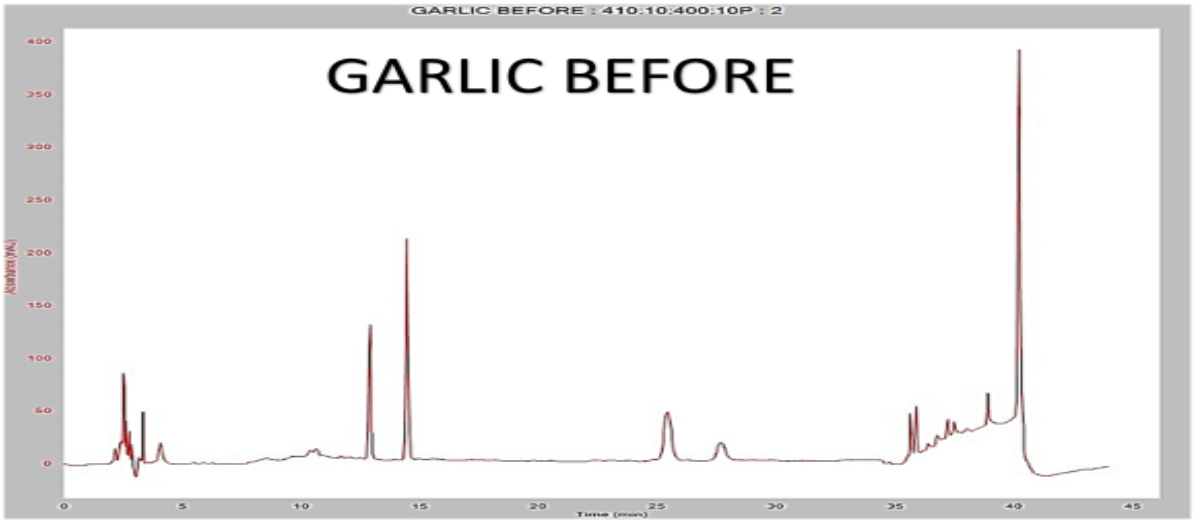
GREEN TEA (IMAC)

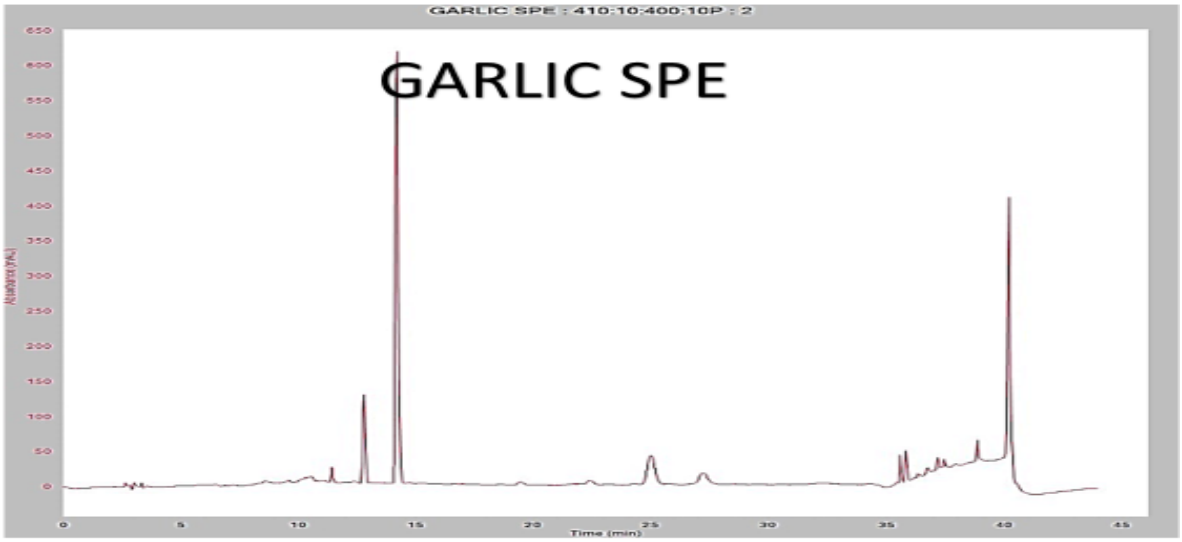


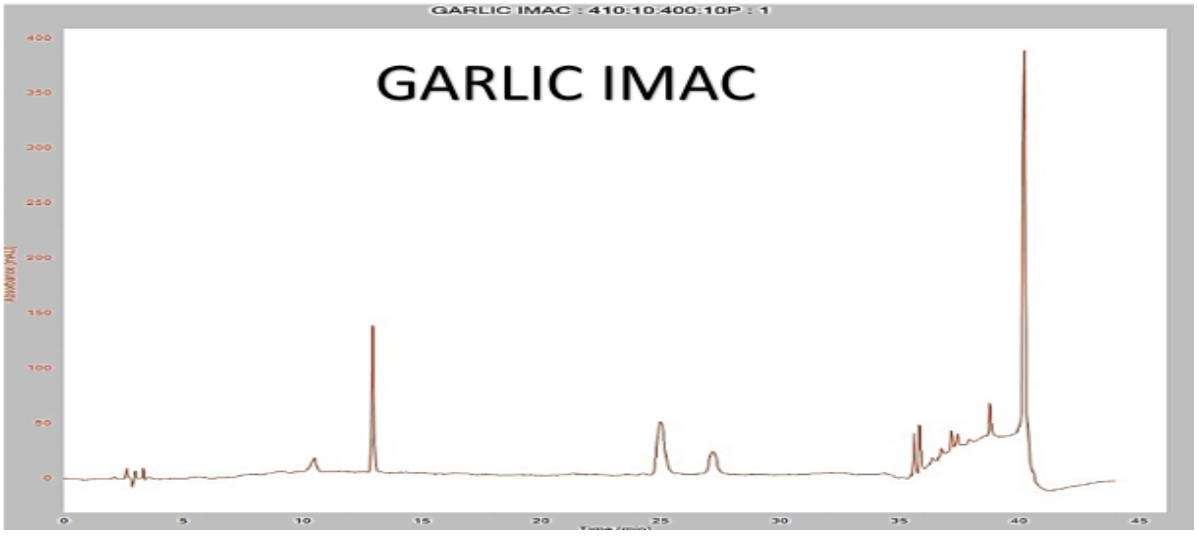

CAPERS BEFORE : 410:10:400:10P : 2
CAPERS BEFORE
Time (min)

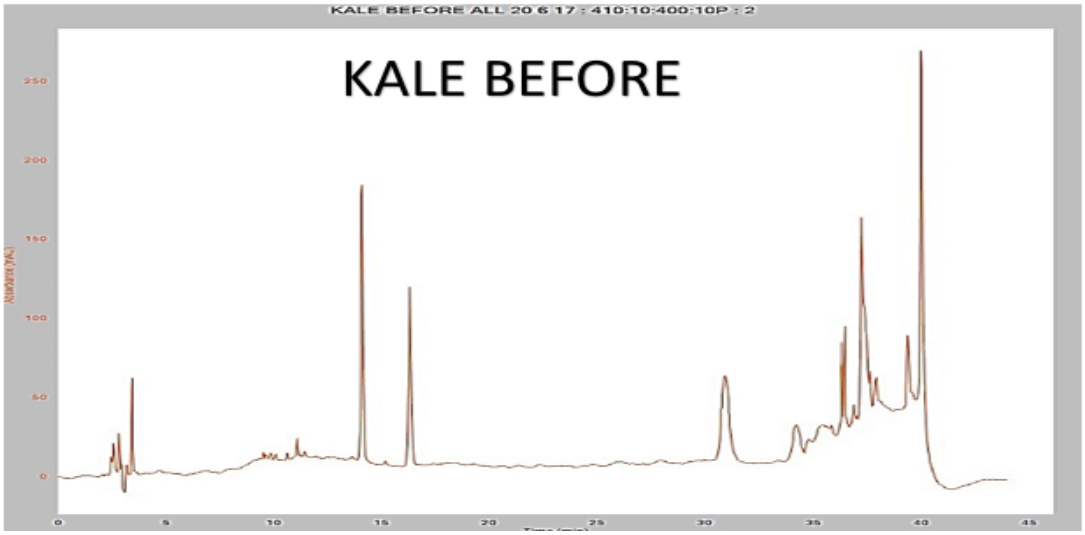

CAPERS SPE : 410:10:400:10P : 1
CAPERS SPE
Time (min)

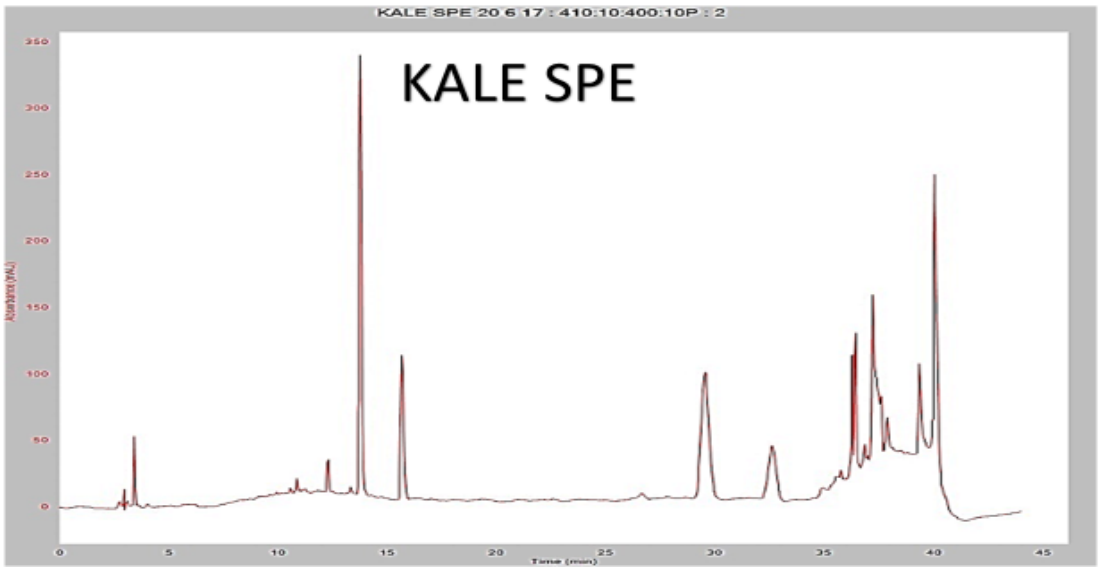

CAPERS IMAC : 410:10:400:10P : 1
CAPERS IMAC
Time (min)

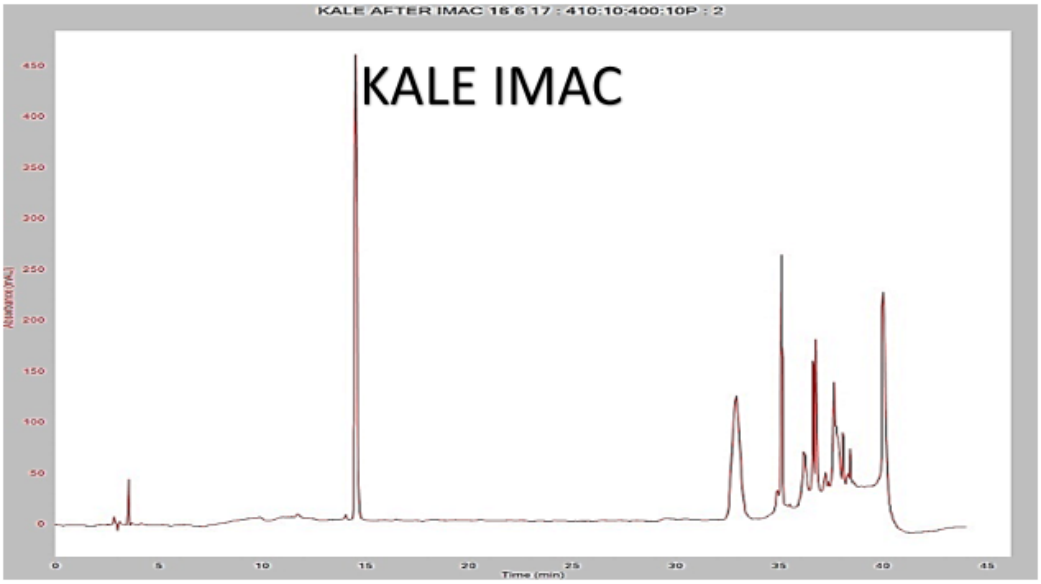

GARLIC BEFORE : 410:10:400:10P : 2
GARLIC BEFORE
Time (min)

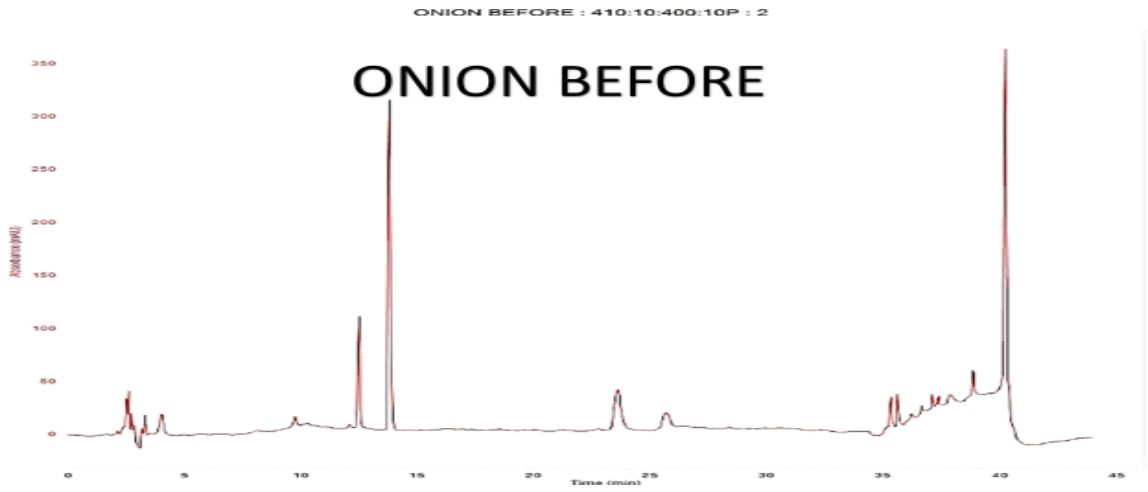

GARLIC SPE : 410:10:400:10P : 2
GARLIC SPE
Time (min)

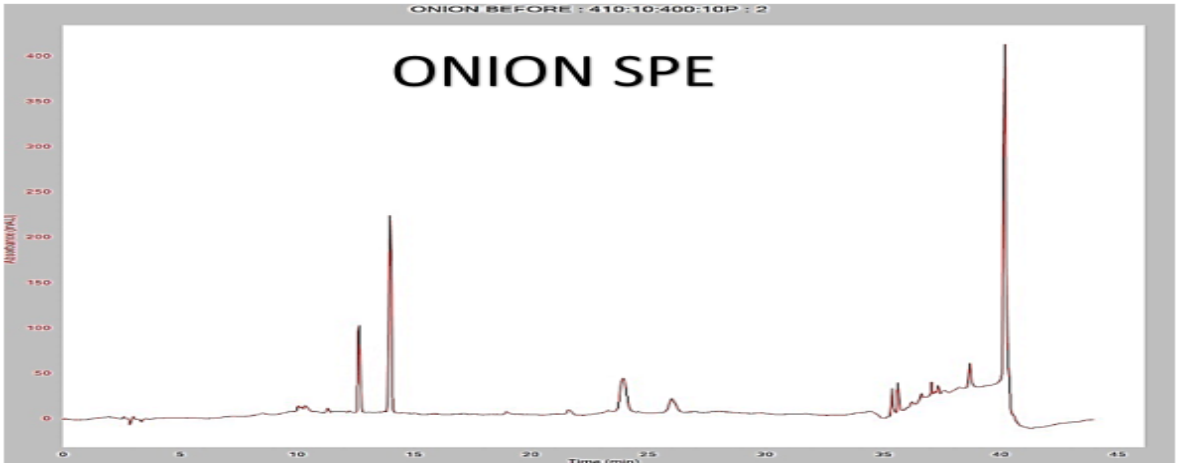

GARLIC IMAC : 410:10:400:10P : 1
GARLIC IMAC

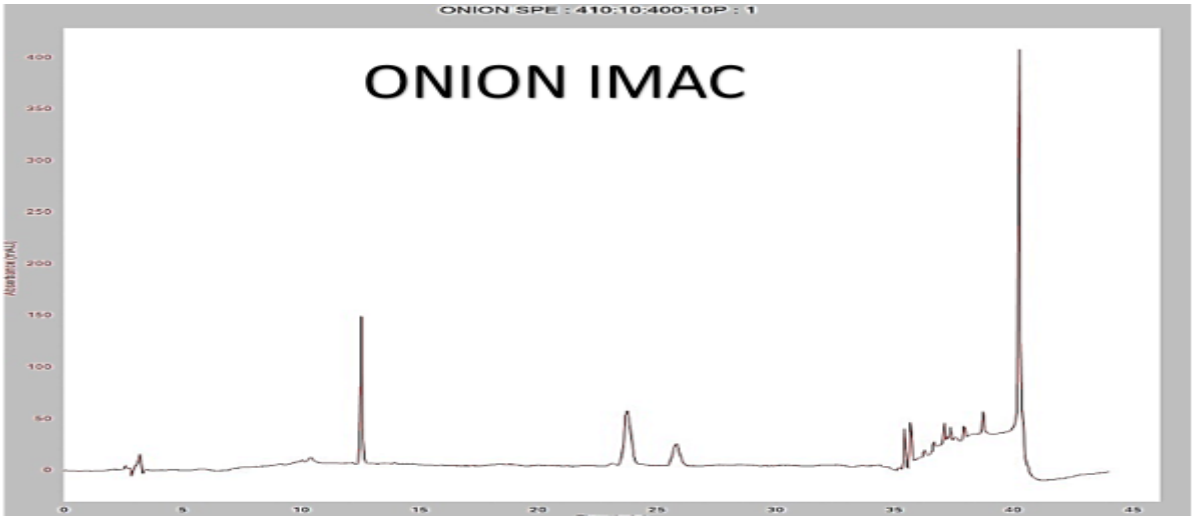

KALE BEFORE ALL 20 6 17 : 410:10:400:10P : 2
KALE BEFORE

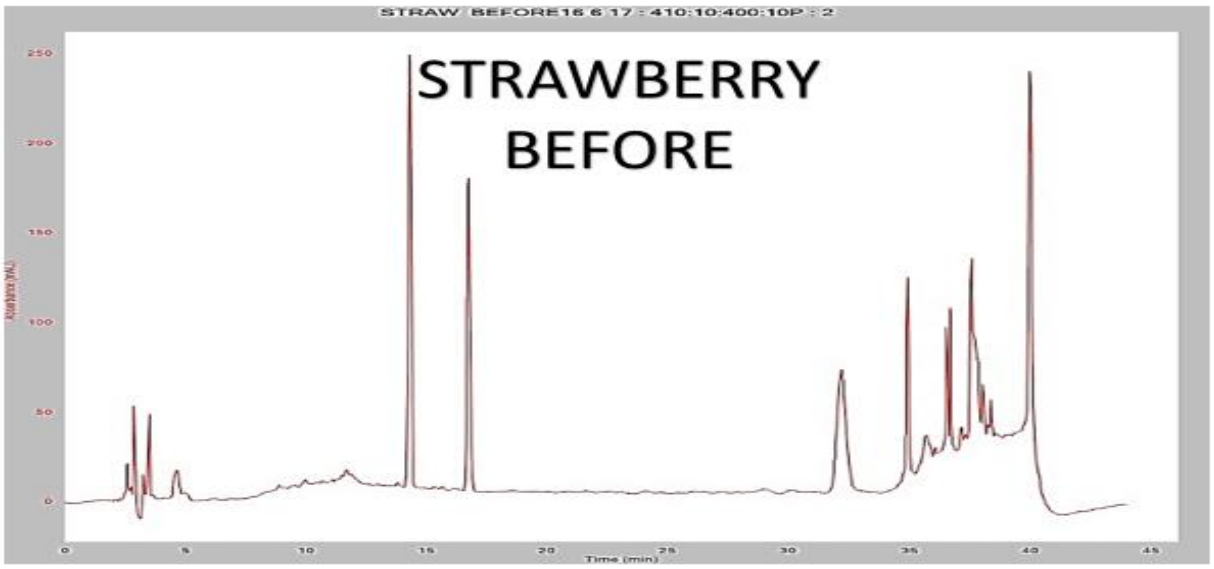

KALE SPE 20 6 17 : 410:10:400:10P : 2
KALE SPE

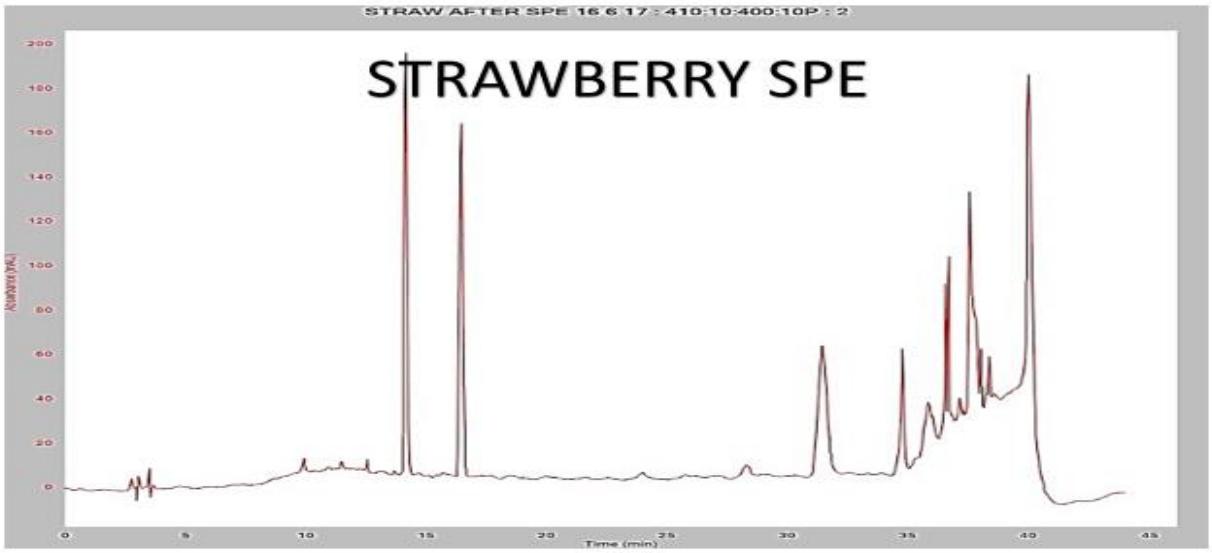

KALE AFTER IMAC 16 6 17 : 410:10:400:10P : 2
KALE IMAC

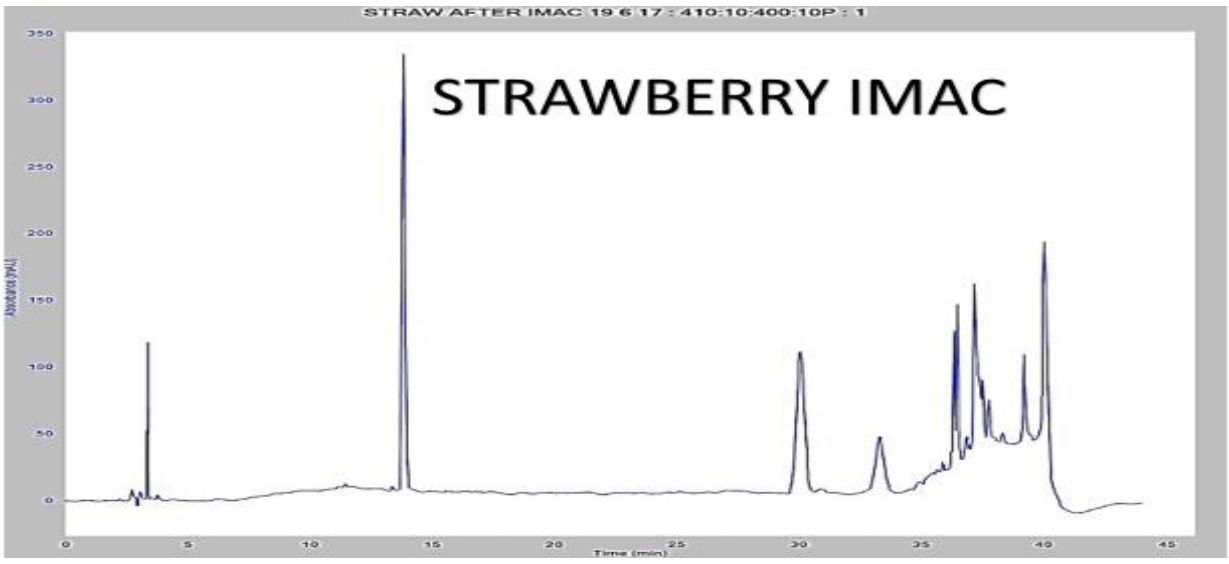

ONION BEFORE : 410:10:400:10P : 2
ONION BEFORE
Absorbance (mAU)
Time (min)


ONION BEFORE : 410:10:400:10P : 2
ONION SPE
Absorbance (mAU)
Time (min)

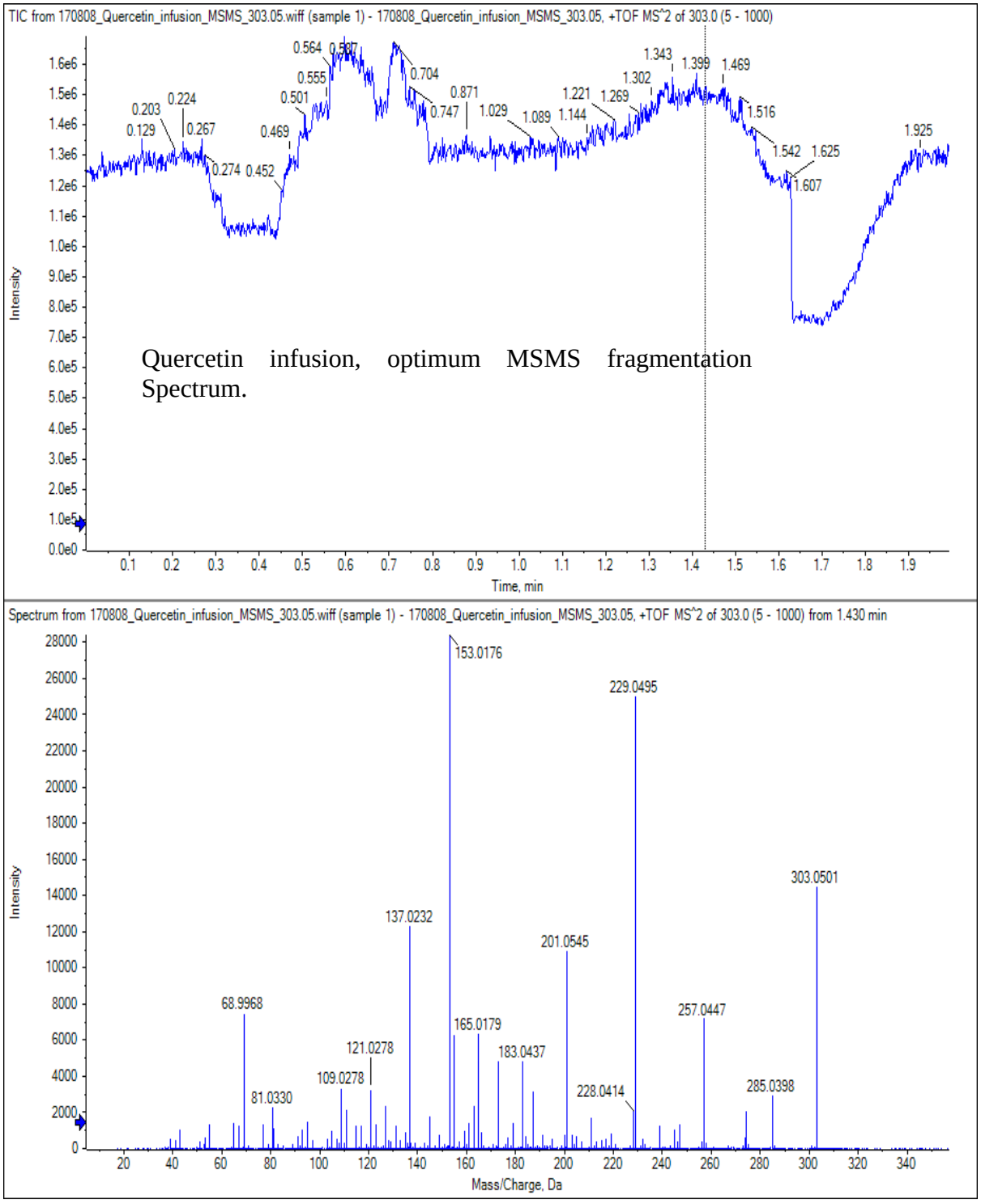

ONION SPE : 410:10:400:10P : 1
ONION IMAC
Absorbance (mAU)


STRAW BEFORE16 6 17 : 410:10:400:10P : 2
STRAWBERRY
BEFORE
Time (min)

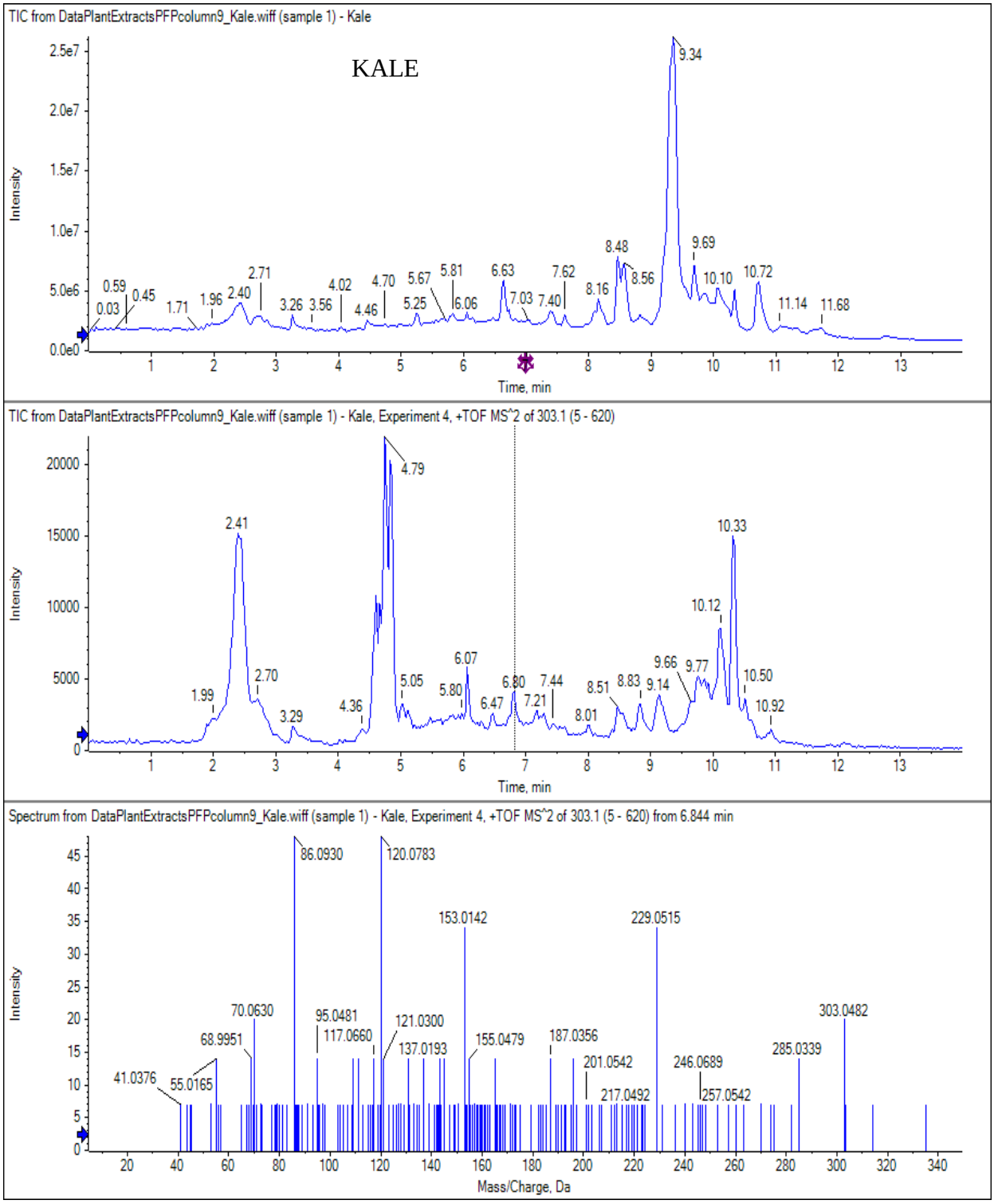

STRAW AFTER SPE 16 6 17 : 410:10:400:10P : 2
STRAWBERRY SPE
Time (min)

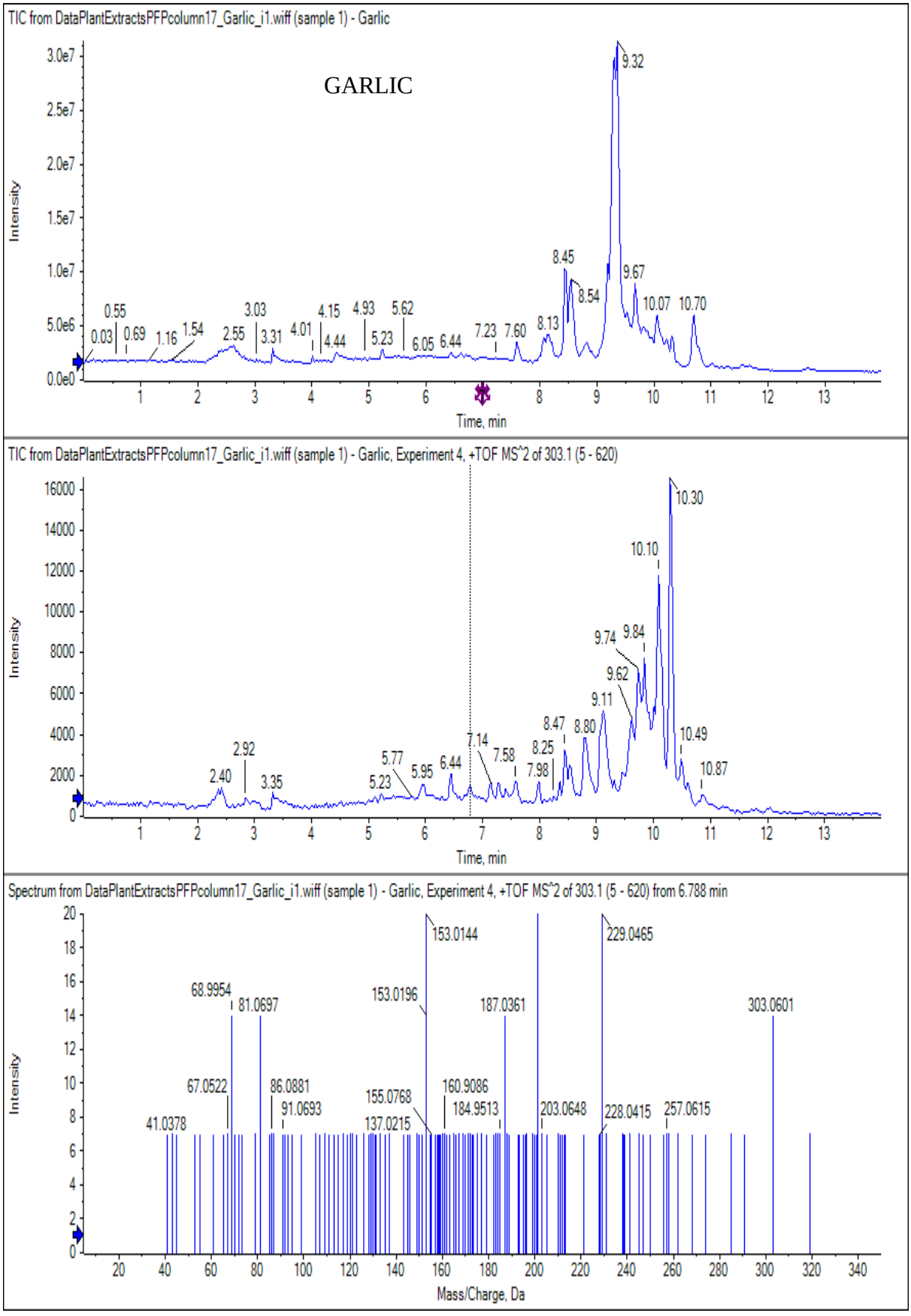

STRAW AFTER IMAC 19 6 17 : 410:10:400:10P : 1
STRAWBERRY IMAC
Time (min)

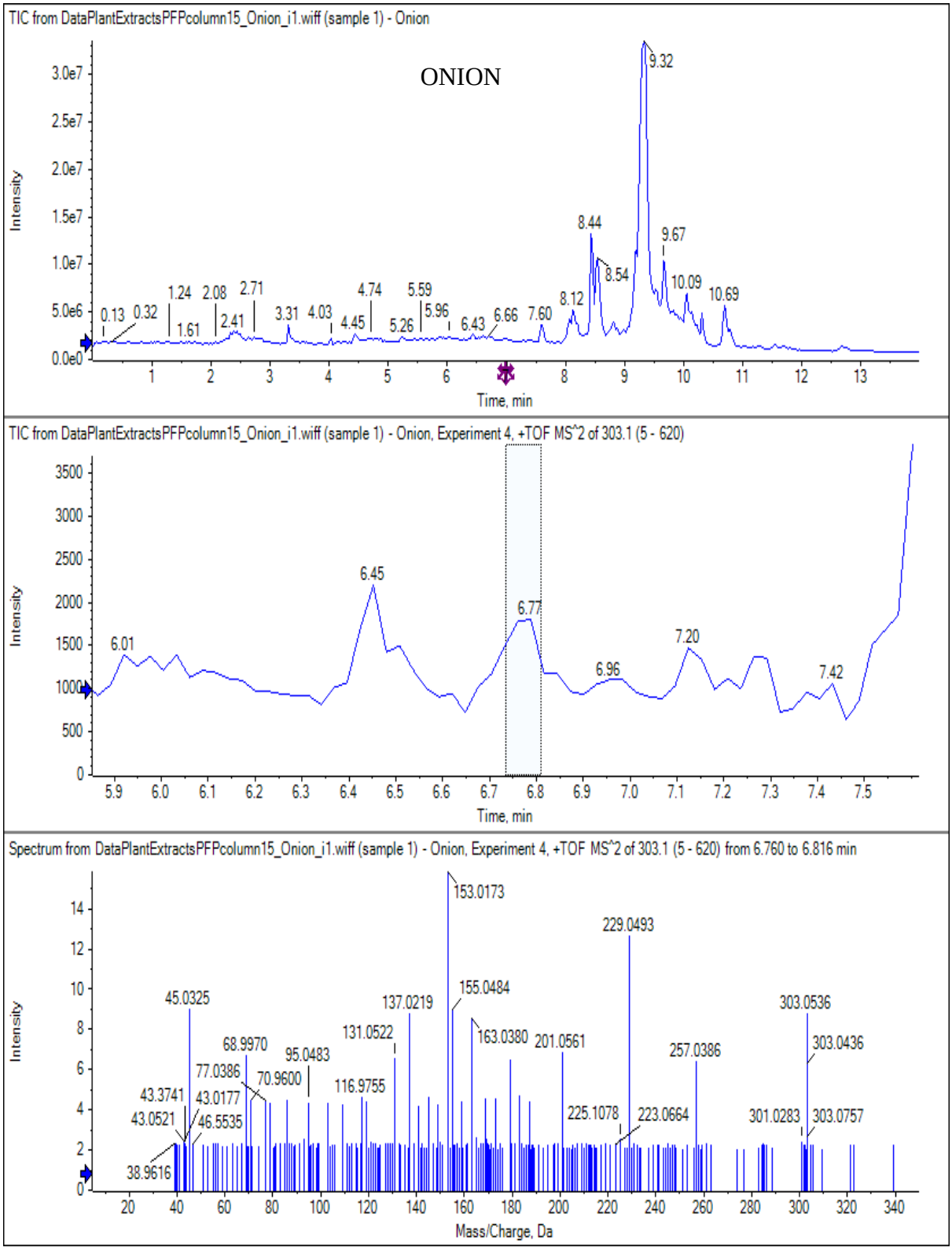
Qualitative analysis using LC/MS/MS

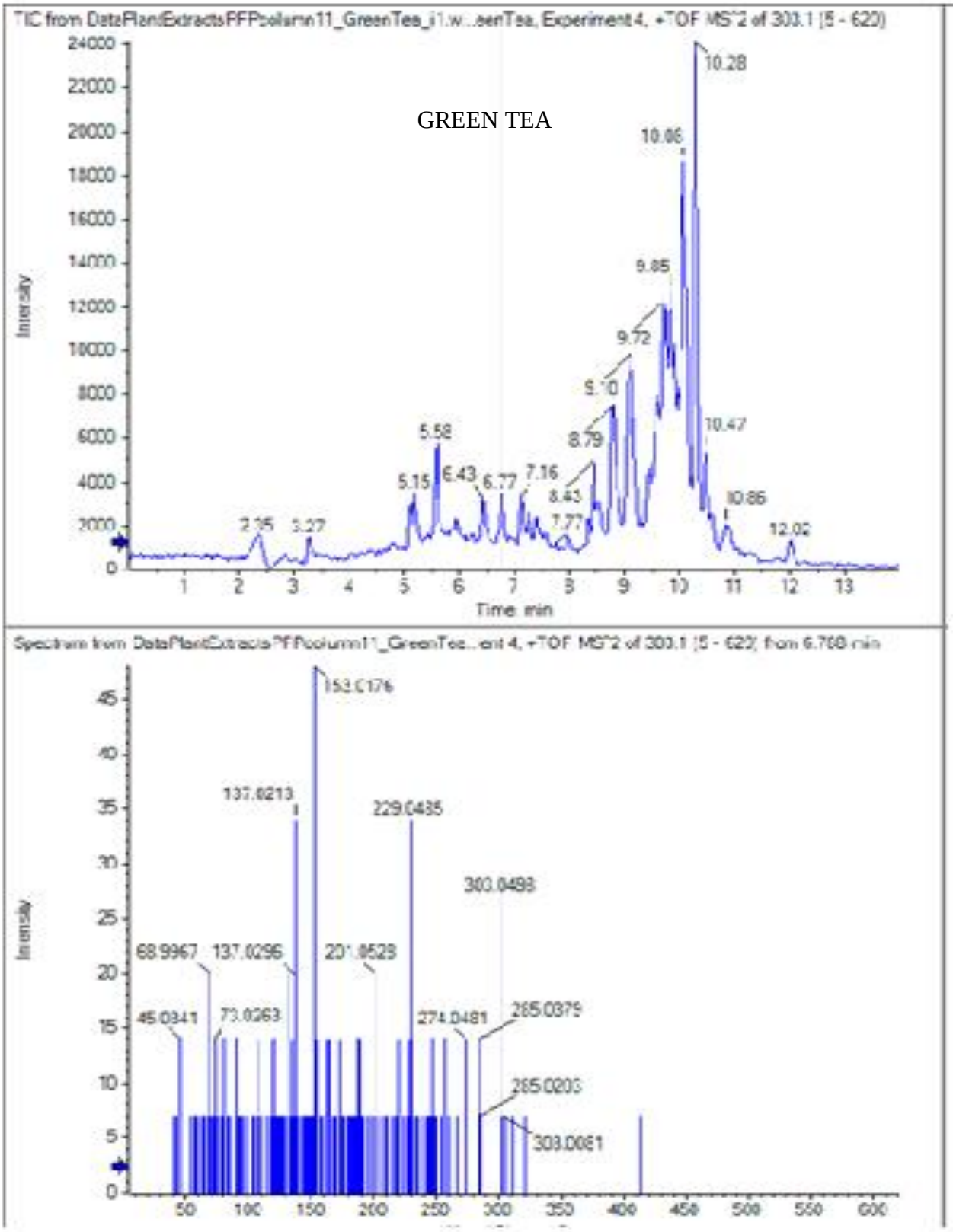


Quercetin infusion, optimum MSMS fragmentation Spectrum.


TIC from DataPlantExtractsPFPcolumn9_Kale.wiff (sample 1) - Kale
KALE
Intensity
Time, min
TIC from DataPlantExtractsPFPcolumn9_Kale.wiff (sample 1) - Kale, Experiment 4, +TOF MS^2 of 303.1 (5 - 620)
Intensity
Time, min
Spectrum from DataPlantExtractsPFPcolumn9_Kale.wiff (sample 1) - Kale, Experiment 4, +TOF MS^2 of 303.1 (5 - 620) from 6.844 min
Intensity
Mass/Charge, Da


TIC from DataPlantExtractsPFPcolumn17_Garlic_i1.wiff (sample 1) - Garlic
GARLIC
Intensity
Time, min
TIC from DataPlantExtractsPFPcolumn17_Garlic_i1.wiff (sample 1) - Garlic, Experiment 4, +TOF MS^2 of 303.1 (5 - 620)
Intensity
Time, min
Spectrum from DataPlantExtractsPFPcolumn17_Garlic_i1.wiff (sample 1) - Garlic, Experiment 4, +TOF MS^2 of 303.1 (5 - 620) from 6.788 min
Intensity
Mass/Charge, Da


TIC from DataPlantExtractsPFPcolumn15_Onion_i1.wiff (sample 1) - Onion
ONION
Intensity
Time, min
TIC from DataPlantExtractsPFPcolumn15_Onion_i1.wiff (sample 1) - Onion, Experiment 4, +TOF MS^2 of 303.1 (5 - 620)
Intensity
Time, min
Spectrum from DataPlantExtractsPFPcolumn15_Onion_i1.wiff (sample 1) - Onion, Experiment 4, +TOF MS^2 of 303.1 (5 - 620) from 6.760 to 6.816 min
Intensity
Mass/Charge, Da


GREEN TEA
Intensity
Time min
10.28
10.06
9.85
9.72
9.10
8.79
10.47
5.58
5.15
6.43
6.77
7.16
8.43
7.77
10.86
12.02
2.35
3.27
153.0176
137.0213
229.0435
300.0498
68.9967
137.0296
45.0341
73.0263
274.0481
265.0379
265.0203
303.0061

Deamidated Peptide Sequencing by Mass Spectrometry: