

**INVESTIGATION OF THE REACTIVITIES OF
MONOMERIC AND POLYMERIC COMPONENTS IN
SOME FOODS IN DIGESTION CONDITIONS *IN VITRO***

***IN VITRO* SİNDİRİM KOŞULLARINDA MONOMERİK VE
POLİMERİK GIDA BİLEŞENLERİNİN
REAKTİVİTELERİNİN İNCELENMESİ**

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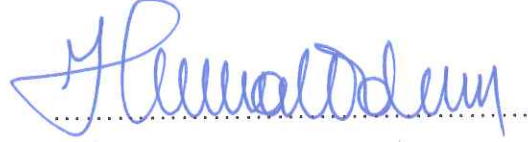
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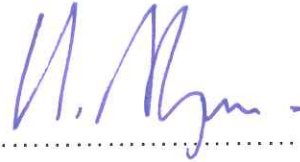
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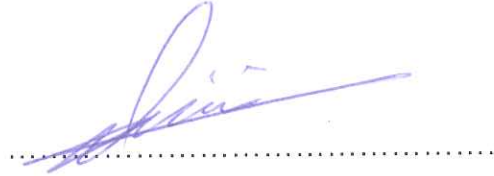
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BERAT AYTÜL HAMZALIOĞLU

ABSTRACT

INVESTIGATION OF THE REACTIVITIES OF MONOMERIC AND POLYMERIC COMPONENTS IN SOME FOODS IN DIGESTION CONDITIONS *IN VITRO*

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Doctor of Philosophy, Department of Food Engineering

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After foods have entered the digestive tract, they are exposed to various changes as a result of varying pH and enzymatic conditions. The changes in food components during digestion are of importance. Thermal process contaminants are the monomeric compounds that exert various harmful effects on health and are formed during thermal treatment in foods. These toxic compounds are found in most of thermally processed foods and enters the body within consumption of these foods. However, after being ingested, it is expected that these compounds will undergo chemical changes under digestion conditions as in the case of other food components. Due to their health concern, to determine the changes they are exposed to is an important issue. Besides, carbohydrates are the main energy sources in human nutrition. One of the complex carbohydrates having polymeric structure is starch and constitutes to several grain-based foods. Since consumption of these foods is related with many chronic diseases, to investigate the fate of this polymeric food component during digestion and factors affecting their digestion behaviour is another important point. The aim of this PhD thesis is the investigation of the fate of these food components and their possible reactions during digestion. To achieve the aim, simulated *in vitro* digestion procedure was used; both model and actual food systems were submitted to digestive process.

In the first part, bakery and fried potato products were tested to understand the fate of acrylamide during *in vitro* multi-step enzymatic digestion system. At the end of digestion, acrylamide decreased in biscuits and it was confirmed in model systems that this was due to Michael addition of amino acids to acrylamide during digestion. In contrast to bakery products, acrylamide levels increased during gastric digestion of fried potatoes. The results exhibited that intermediates like Schiff base accumulated in potatoes during frying are potential precursors of acrylamide under gastric conditions. Similarly, interactions of reactive α -dicarbonyl compounds, particularly methylglyoxal (MGO) and 3-deoxyglucosone (3-DG), and HMF during *in vitro* digestion process were investigated in commercial biscuits. MGO and 3-DG concentrations decreased under intestinal digestion conditions. Results of the model systems composed of MGO and lysine, cysteine or ovalbumin and model biscuits containing lysine, cysteine or ovalbumin revealed that disappearance in dicarbonyl contents was due to interactions of reactive dicarbonyl compounds with the accumulating amino acids during digestive process. On the other hand, HMF contents of biscuits increased during gastric phase. It was confirmed that sugar degradation products such as 3-DG and 3,4-dideoxyglucosone (3,4-DG) accumulated in biscuits during baking were converted to HMF under gastric conditions. However, reactions of HMF with amino acids proceeded with the progress of digestion leading to a significant decrease in the concentrations of HMF during intestinal phases. High-resolution mass spectrometry (HRMS) analysis in both HMF-amino acid model systems and in biscuits confirmed that HMF reacted with amino and sulfhydryl groups through Michael type addition and Schiff base formation. Following the reactions of HMF with amino and sulfhydryl compounds during digestion, reactions of HMF with selected amino acids (arginine, cysteine and lysine) were investigated in HMF-amino acid (high moisture) and Coffee-amino acid (low moisture) model systems at 5, 25 and 50°C. The results presented that HMF reacted efficiently and effectively with amino acids in both high and low moisture model systems. From the kinetic evaluation of these reactions, cysteine was determined as the most reactive amino acid towards HMF owing to its thiol group. As the tendency of cysteine for the reactions with acrylamide and HMF was considered, cysteine was found as effective in reacting with carbonyl compounds. Thus, different sulphur sources were additionally examined for their scavenging abilities of an α -

dicarbonyl compound, glyoxal (GO) in various model systems under simulated physiological conditions (37°C & 7.4 pH, 2 hours). The results presented that some of these sulphur sources could scavenge GO under these conditions. Potassium metabisulphite and cysteine was found to be effective in GO scavenging, however, methionine could not scavenge GO. The results of the extracts of digested raw, steamed and boiled broccoli sprouts and wine indicated that GO was scavenged by these foods.

In the second part, effect of cooking treatment, different shapes and sizes on digestibility of polymeric component, starch was determined. Baking and frying was compared as cooking treatments and potatoes were cut into thick strip, thin strip, cube, and disk shapes prior to cooking. Cooked potatoes were subjected to *in vitro* digestion to determine their nutritionally important starch contents (rapidly, slowly digestible and available starch). Frying and baking differed on available starch formation in potatoes for all shapes and sizes. The amounts of different digestible starch fractions were correlated with surface-to-volume ratio. The results suggested that the surface-to-volume ratio could be a potential factor on controlling different digestible starch contents of cooked potatoes. Additionally, the effect of consumption of other foods together with potato on digestibility of potato starch was examined in different binary and ternary systems of baked potato with olive oil, wine, wheat bran or meat. The results of binary systems showed that consumption of potato together with olive oil, wine, wheat bran or meat caused to significant decrease in amylolytic hydrolysis. Wine could amount to the highest inhibition in digestibility of potato and it was followed by olive oil, wheat bran and meat. Lastly, more inhibition was observed in ternary combinations of these foods.

Keywords: *in vitro* digestion, acrylamide, dicarbonyl compounds, 5-hydroxymethylfurfural, sulphur compounds, potato, starch

ÖZET

***IN VITRO* SİNDİRİM KOŞULLARINDA MONOMERİK VE POLİMERİK GIDA BİLEŞENLERİNİN REAKTİVİTELERİNİN İNCELENMESİ**

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Gıdalar sindirim borusundan geçtikten sonra, değişen pH ve enzimatik koşullar nedeniyle çeşitli değişimlere maruz kalırlar. Gıda bileşenlerinin sindirim esnasında geçirdiği bu değişimler önem arz etmektedir. Termal proses kontaminantları, sağlık üzerine çeşitli zararlı etkileri olan ve gıdalarda ısıtma işlemi esnasında oluşan monomerik bileşiklerdir. Bu toksik bileşikler genel olarak termal proses edilmiş gıdalarda bulunmakta ve bu gıdaların tüketimiyle vücuda alınmaktadır. Ancak, tüketildikten sonra, diğer gıda bileşenlerinde olduğu gibi bu bileşiklerin de sindirim süresince çeşitli kimyasal değişimler geçirmesi beklenmektedir. Sağlık üzerine yarattıkları zararlı etkilere bağlı olarak, bu bileşiklerin sindirim süresince geçirdikleri değişimlerin belirlenmesi önemli bir konudur. Bunun yanında karbonhidratlar, insan beslenmesinde önemli enerji kaynaklarından biridir. Bu kompleks karbonhidratlardan biri polimerik yapıya sahip olan nişastadır ve birçok tahıl bazlı gıdanın temelini oluşturmaktadır. Bu tür gıdaların tüketimi birçok kronik hastalıkla ilişkilendirildiğinden, bu polimerik gıda bileşeninin sindirim sürecindeki gidişatı ve sindirim davranışını etkileyen faktörlerin belirlenmesi de diğer önemli bir

noktadır. Bu doktora tezinin amacı, bu gıda bileşenlerinin sindirim sürecindeki gidişatlarının ve muhtemel reaksiyonlarının belirlenmesidir. Bu bağlamda, simüle edilmiş *in vitro* sindirim prosedürü kullanılmış, hem model hem de gerçek gıda sistemleri sindirim sürecine tabi tutulmuştur.

İlk kısımda, akrilamidin *in vitro* çok aşamalı enzimatik sindirim sistemindeki gidişatının belirlenmesi amacıyla fırınlanmış ürünler ve kızarmış patates ürünleri test edilmiştir. Sindirim sonunda, bisküvilerdeki akrilamid miktarı azalmış ve bunun amino asitlerin akrilamide Michael tipi eklenmesi yoluyla gerçekleştiği model sistemlerle doğrulanmıştır. Fırıncılık ürünlerinin aksine, kızarmış patateslerdeki akrilamid seviyeleri gastrik sindirim süresince artmıştır. Sonuçlar, Schiff bazı gibi patateslerin kızartılması esnasında biriken ara ürünlerinin gastrik koşullarda akrilamidin potansiyel öncülleri olduğunu ortaya koymuştur. Benzer şekilde, bisküvilerdeki reaktif dikarbonil bileşiklerin, özellikle metilglioksal (MGO) ve 3-deoksiglukozon (3-DG), ve 5-hidroksimetilfurfural (HMF) 'ın sindirim süresinceki interaksiyonları incelenmiştir. Sindirim bağırsak koşullarında MGO ve 3-DG konsantrasyonları azalmıştır. MGO ve lizin, sistein ya da ovalbumin içeren model sistemler ve lizin, sistein ya da ovalbumin içeren model bisküvi sonuçları dikarbonil içeriğindeki bu azalmanın dikarbonil bileşiklerin sindirim sonucunda biriken amino asitlerle interaksiyonlarına bağlı olduğunu göstermiştir. Diğer yandan, bisküvilerdeki HMF miktarı gastrik koşullarda artış göstermiştir. Fırında pişirme esnasında bisküvilerde biriken şeker degradasyon ürünlerinden 3-DG ve 3,4-dideoksiglukozonun (3,4-DG) gastrik koşullar altında HMF 'ye dönüştürüldüğü doğrulanmıştır. Bununla birlikte, HMF ile amino asitlerin reaksiyonları sindirim süresince devam etmiş ve bağırsak fazında HMF konsantrasyonlarında azalmaya sebep olmuştur. HMF-amino asit model sistemlerinde ve bisküvilerde gerçekleştirilen yüksek rezolüsyonlu kütle spektrometresi analizleriyle HMF ile amino ve sülfidril gruplarının Michael tipi eklenme ve Schiff bazı oluşumu yoluyla reaksiyona girmiş olduğu doğrulanmıştır. HMF ile amino ve sülfidril bileşiklerin sindirim süresinceki reaksiyonlarından hareketle, HMF 'nin bazı amino asitler (arjinin, sistein ve lizin) ile reaksiyonları 5, 25 ve 50°C 'de gerçekleştirilen HMF-amino asit (yüksek nem) ve Kahve-amino asit (düşük nem) model sistemlerinde incelenmiştir. Sonuçlar, HMF 'nin hem yüksek hem düşük nemli model sistemlerde amino asitlerle etkili bir biçimde reaksiyona girdiğini göstermiştir. Bu reaksiyonun kinetik çözümlenmesi sonucunda, sisteinin yapısındaki tiyol grubuna bağlı olarak

HMF 'ye karşı çok reaktif olduđu bulunmuştur. Sisteinin akrilamid ve HMF ile reaksiyona girmedeki eğilimi düşünöldüğünde, karbonil bileşiklerle reaksiyon vermede etkili olduđu görölmüştür. Dolayısıyla, buna ek olarak farklı kükürt kaynakları dikarbonil bir bileşik olan glioksal (GO) sönümleme yeteneğinin belirlenmesi için farklı model sistemlerde simüle fizyolojik koşullar (37°C & 7.4 pH, 2 saat) altında test edilmiştir. Sonuçlar, bu kükürtlü bileşiklerin bazılarının glioksalı bu koşullar altında sönümleyebileceğini gösterdi. Potasyum metabisüfit ve sistein GO sönümlemede etkin bulunmuşken metiyonin herhangi bir etkiye sebep olmamıştır. Sindirilmiş çiğ, buharda pişirilmiş ve kaynatılmış brokolilerin ekstraktları ve şarapla yapılan çalışma sonuçları, glioksalın bu gıdalar tarafından sönümlendiğı sonucunu göstermiştir.

İkinci kısımda, pişirme yöntemi ve farklı şekil ve boyutların polimerik bileşen olan nişastanın sindirilebilirliği üzerine etkisi belirlenmiştir. Pişirme yöntemi olarak fırında pişirme ve kızartma işlemleri karşılaştırılmış ve patatesler pişirme öncesinde kalın çubuk, ince çubuk, küp ve disk şeklinde kesilmiştir. Pişirilmiş patatesler besinsel olarak önemli nişasta içeriklerinin (hızlı, yavaş sindirilebilir ve toplam sindirilebilir nişasta) belirlenmesi için *in vitro* sindirime tabi tutulmuştur. Kızartma ve fırında pişirme bütün şekil ve boyutlarda patateslerdeki sindirebilir nişasta açısından fark oluşturmuştur. Sonuçlar, pişirilmiş patateslerdeki farklı sindirilebilir nişasta miktarlarının kontrolü açısından yüzey-hacim oranının potansiyel bir faktör olabileceğini ortaya koymuştur. Ek olarak, patatesle birlikte diğçer gıdaların tüketiminin nişastanın sindirim davranışı üzerine etkisi farklı ikili ve üçlü patates sistemlerinde belirlenmiştir. İkili sistem sonuçları, patatesle birlikte zeytinyağı, şarap, buğday kepeğı veya et tüketiminin amilolitik hidrolizde önemli bir düşüşe neden olduğunu göstermiştir. Patatesin sindirim davranışı açısından en yüksek inhibisyon şarap tarafından sağlanmış ve bu, zeytin yağı, buğday kepeğı ve et tarafından izlenmiştir. Son olarak, bu gıdaların üçlü kombinasyonlarında daha fazla oranda inhibisyon gözlenmiştir.

Anahtar Kelimeler: *in vitro* sindirim, akrilamid, dikarbonil bileşikleri, 5-hidroksimetilfurfural, kükürt bileşikleri, patates, nişasta

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SYMBOLS AND ABBREVIATIONS

Symbols

E_a	Activation energy
k	Reaction rate constant
K	Chemical equilibrium constant
T	Temperature t
t	time

Abbreviations

Acr	Acrylamide
AGE	Advanced Glycation End products
Arg	Arginine
AS	Available Starch
Asn	Asparagine
BB	Boiled broccoli
BSA	Bovine Serum Albumin
Cys	Cysteine
CIE	International Commission on Illumination
Cys	Cysteine
DAD	Diode array detector
3-DG	3-deoxyglucosone
3,4-DG	3,4-dideoxyglucosone
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
ESI	Electrospray Ionization
GI	Glycemic Index
Glc	Glucose
GO	Glyoxal
GR	Glucoraphanin
HFCS	High fructose corn syrup
HILIC	Hydrophilic Interaction Chromatography
HMF	5-Hydroxymethylfurfural

HMFA	5-Hydroxymethyl-2-furoic acid
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
IARC	International Agency for Research on Cancer
LC/MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
Lys	Lysine
MCX	Mixed-mode cation exchange
MGO	Methylglyoxal
MRM	Multiple reaction monitoring
ND	Not detected
NOAEL	No Observed Adverse Effect Level
MCX	Mixed-mode cation exchange
PDA	Photodiode array
PMS	Potassium metabisulphite
RAG	Rapidly Available GLucose
RB	Raw Broccoli
RCS	Reactive carbonyl species
RDS	Rapidly Digestible Starch
RID	Refractive Index Dedector
RS	Resistant Starch
RSLC	Rapid Seperation Liquid Chromatography
SAG	Slowly Available Glucose
SB	Steamed Broccoli
SDF	Simulating Dudodenal Fluid
SDS	Slowly Digestible Starch
SGF	Simulating Gastric Fluid
SIM	Selected ion-monitoring mode
SMF	5-sulfoxymethylfurfural
SPSS	Statistical Package for the Social Sciences
SSF	Simulating Saliva Fluid
TDI	Tolerable daily intake

TQ	Triple quadrupole
UFLC	Ultra fast liquid chromatography
UHPLC	Ultra high-performance liquid chromatography
UPLC	Ultra performance liquid chromatography



INTRODUCTION

Ingested food enters the digestive tract, which significantly alters its structure and chemical composition by varying the pH and the action of several enzymes in the mouth, stomach and intestine. Each step of digestion might be considered as a reaction media with different specific conditions. In addition to changing pH in each step, enzymes acting on food components lead to the hydrolysis and transformations of these components. Under these conditions, some of these may interact with certain components released from the food matrix in the gastrointestinal tract. As a result of these interactions, the amount of food components may be reduced, as well as new compounds may be formed, which might also behave as reactants. Therefore, the total amount of a food component in the ingested food is not always the net amount that is available for the body. A lot of studies about the investigation of available fraction of food components have been done to date. However, it has not been considered that they may also be reactive during digestion.

The main objective of this PhD thesis is to understand the reactions of food components with each other during digestive process. To achieve this, targeted food components were monitored after each step of digestion.

Background information about *in vitro* digestion and the reactions of thermal process contaminants as “monomeric food components” are given in **Chapter 1**. Additionally, digestion behaviour and the interactions of potato starch as “polymeric food components” were summarized.

Chapter 2 discusses the fate of process contaminants namely acrylamide, 5-hydroxymethylfurfural (HMF) and alpha-dicarbonyl compounds during *in vitro* digestion. The reactions of these contaminants with food components, especially amino acids and protein side chains were investigated in simulated *in vitro* mouth, stomach, small intestine and colon conditions. Within the acquired data about the high reactivity of thiol compounds, especially cysteine, the scavenging effect of different sulphur compounds was investigated in the presence of glyoxal (GO) under simulated physiological conditions. To understand the effect of sulphur compounds on the scavenging of GO, potassium metabisulphite and sulphur rich foods such as wine and broccoli was tested. The effect of thiol compounds together with amino compounds was also investigated for their reactions with

HMF. The kinetics of the reaction between HMF and either amino or thiol compounds were evaluated to understand its reactivity under moist and dry conditions.

The results reported in **Chapter 2** have been published in the following articles:

Hamzalıoğlu, A.; Gökmen, V., Formation and elimination reactions of 5-hydroxymethylfurfural during in vitro digestion of biscuits, *Food Research International*, doi: 10.1016/j.foodres.2017.05.034, **2017**.

Hamzalıoğlu, A.; Gökmen, V., Investigations on the reactions of α -dicarbonyl compounds with amino acids and proteins during in vitro digestion of biscuits, *Food & Function*, 7, 2544-2550, **2016**.

Hamzalıoğlu, A.; Gökmen, V., Investigation of the reactions of acrylamide during *in vitro* multistep enzymatic digestion of thermally processed foods, *Food & Function*, 6, 109-114, **2015**.

Chapter 3 evaluated the effect of shape and type of cooking on digestibility of polymeric compounds namely potato starch. The potatoes, which were prepared in thick, thin strip, disk and cube shape, were baked and fried at different temperatures for various times. Then, the digestible fractions including slowly digestible, rapidly digestible and available starch were determined under *in vitro* digestion conditions. Addition of wine, oil, meat and wheat bran with potato to resemble a mixed meal was performed to understand their combined effect on digestibility of potato starch.

1. CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

This chapter gives the fundamental knowledge on the topics covered in the thesis. Throughout the thesis, *in vitro* digestion is the common subject of every chapter. Therefore, this chapter starts with digestive process, then, gives the basics about some of thermal process contaminants including acrylamide, α -dicarbonyl compounds and HMF as “monomeric food components” and their possible reactions. Lastly, literature about the digestion behaviour of potato starch as “polymeric food component” is covered.

1.2 *IN VITRO* DIGESTION

Food mostly enters the body as large particles that are unable to cross through the intestinal walls. To be absorbed, it must be firstly dissolved and broken down to small particles [1] This process, digestion, is both mechanical and chemical breakdown of foods to small molecules thus making their passage to bloodstream easier. Human digestive system consists several stages and during these stages, food components are subjected to structural changes. Digestive system mainly includes mouth, stomach, small intestine and colon.

In vivo methods, including human or animal subjects, are better for obtaining more precise results. Bu these methods are both expensive and time-consuming. *In vitro* models have the advantage of being rapid, cheap and lack of ethical restrictions [2] For this reason, to develop an *in vitro* procedure simulating the body conditions well is a useful alternative to *in vivo* studies and allows rapid screening of target compounds.

Simulated digestion models typically include the oral, gastric, duodenal (small intestine) and colon phases. These models mimic the physiological conditions with the digestion time and the presence of digestive enzymes, pH, salts [3, 4] Using a multi-step digestion model and collection of samples at the end of each step enables to follow the changes in targeted food components during phases of digestion individually. Simulated digestion conditions are summarized in Figure 1.1.

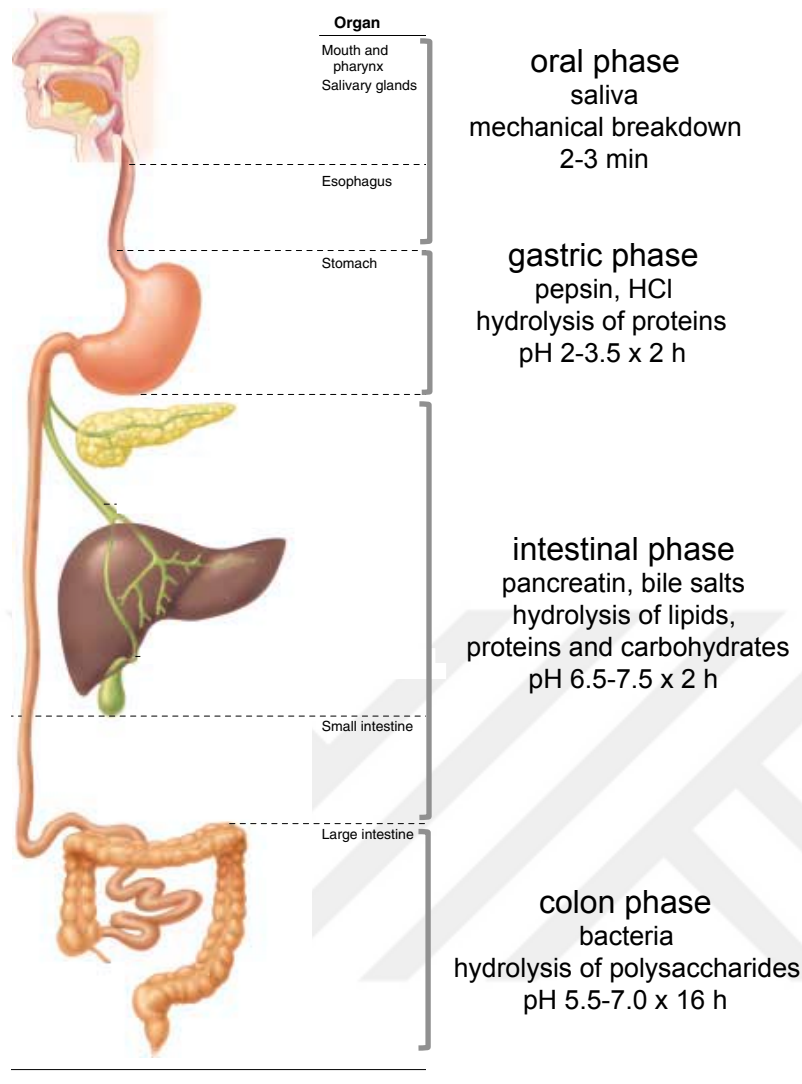


Figure 1.1. *in vitro* digestion adapted from [1].

Food enters the body through the mouth and mechanic digestion starts here within the chewing and swallowing effect of saliva for the solid foods. Saliva is composed of mostly water, enzyme (amylase) and electrolytes such as sodium, potassium, calcium, chloride, bicarbonate and phosphate. Ground solid foods are mixed with simulating saliva fluids and then homogenized to mimic the chewing conditions in oral phase for 2-3 minutes. For the liquid foods, oral phase is omitted thus they reach directly to stomach. The homogenised and swallowed food having higher surface area reaches to stomach.

Stomach is the part of gastrointestinal tract where the preparation of digesta takes place prior to its deliver to the small intestine for the further digestion. During the gastric phase, movements in the stomach provide the continuous mixing of food components. Food is diluted and solubilized by gastric juice, which contains both

enzymes and hydrochloric acid. pH generally varies between 2.0 and 3.5 depending on the emptiness of the stomach. It is about 2.0 in the fasting state whereas it increases depending on ingested food. After that, it decreases again within the secretion of hydrochloric acid. Several proteases, collectively known as pepsin, are present in gastric juice and the hydrolysis of proteins occurs in stomach with the action of pepsin enzyme. Even polysaccharides are dissolved and partially digested, fat is not dissolved in stomach. Since the molecular size of food components is still large to cross the epithelium of gastric wall, absorption occurring in the stomach is negligible [3]. Digestion time in gastric phase differs depending on gastric emptying rate of consumed food. Generally the digestion in the stomach takes 2 hours.

The next stage is the duodenal phase including pancreatic juice for the further digestion of partially digested food molecules. Pancreatic juice is mainly composed of bile salts that function as emulsifier to make the lipophilic food components and fat molecules soluble. Duodenal pH is generally between 6.5- 7.5 and duodenal phase takes 2 hours. Since pancreatin, mixture of enzymes in small intestine, consists the amylolytic enzymes, lipase and trypsin; hydrolysis of lipids, proteins and carbohydrates occur during duodenal phase. In consequence of digestion of food components, the absorption mostly takes place in small intestine, as well.

Considering this, *in vitro* digestion models do not consist the colon phase [5]. However, colon contains around 300-500 species of bacteria whose concentration is up to 10^{11} or 10^{12} cells/g luminal contents. The digestion of undigested polysaccharides such as fibers by these bacteria also occurs in colon thus the absorption of these products in colon phase is also possible [6, 7]. Instead of presence of bacteria, bacterial enzymes might be added to simulate the colon phase. pH in the colon varies between 5.5-7.0 and the total time for colon phase is up to 16 hours.

In terms of mimicking the physiological conditions, temperature for the simulated digestion models is adjusted to 37.4 °C.

1.3 THERMAL PROCESS CONTAMINANTS AS MONOMERIC FOOD COMPONENTS

Thermal process is applied to foods for processing or preservation purposes. Several chemical reactions including Maillard reaction proceed during thermal process and results in formation of chemical compounds. While these compounds provide many desired characteristics in foods, they may also cause to the formation of undesired compounds having adverse effects on human health. Such compounds are thus referred as “thermal process contaminants” or “thermal process toxicants” [8].

Although Maillard reaction is a series of complex reactions, basically it begins with carbonyl-amine reactions proceeding between amino and carbonyl groups. Thermal process contaminants are formed in the further stages of this reaction. As thermal process contaminants, acrylamide, 5-hydroxymethylfurfural (HMF) and α -dicarbonyl compounds are covered in this chapter.

1.3.1 Formation mechanism and reactivity

Acrylamide, 2-propenamid, is a reactive amide and has a chemical formula C_3H_5NO . (Figure 1.2)

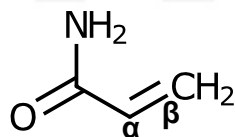


Figure 1.2. Chemical structure of acrylamide

Acrylamide has been classified as a ‘probable human carcinogen’ by the International Agency for Research on Cancer (IARC) in 1994 and so much effort to evaluate its formation mechanism has been done so far [9-11]. Maillard reaction, the reaction between amino acids and reducing sugars as a result of heating food, was found to be the key responsible mechanism for the formation of acrylamide in heated foods. In addition, asparagine was found to be the main precursor amino acid leading to the formation of acrylamide. Even asparagine itself is converted to acrylamide; studies carried out with different carbonyl compounds showed that hydroxycarbonyl, dicarbonyl or bioactive carbonyl compounds react rapidly with asparagine yielding acrylamide [12-15]. In this manner, reaction yield depends on

carbonyl source and its ability to decarboxylate Schiff base. Formation mechanism of acrylamide through Maillard reaction is given in Figure 1.3 [16].

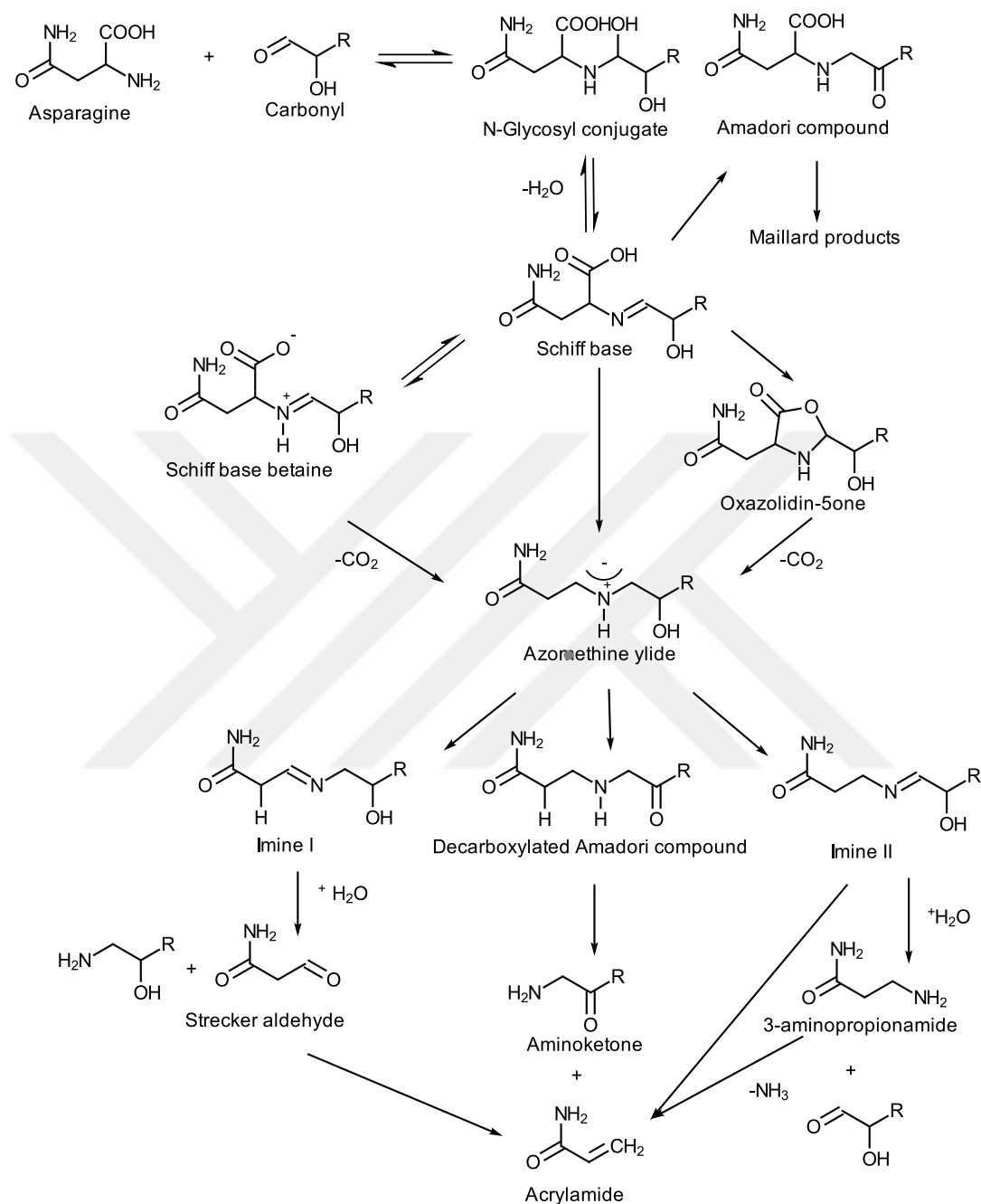


Figure 1.3. Formation pathway of acrylamide [16].

Firstly, α -amino group of asparagine reacts with the carbonyl group of carbonyl source yielding N-glycosyl-asparagine (carbinolamine) which then forms Schiff base through the removal of one molecule of water as a result of heating under dry conditions at temperatures above 120°C. When the reaction condition is high-moisture, Schiff base may rearrange to form Amadori compounds. Since these

stable compounds could not decarboxylate, rearrangement of Schiff base is not prerequisite for the formation of acrylamide in this mechanism. Lately they decompose to form colour and flavour compounds [17]. Decarboxylation of Schiff base, yielding an azomethine ylide that may form imine I and II, is the second step of acrylamide formation. It may also proceed through Schiff base betaine or oxazolidin-5-one [18, 19]. Imine II could form acrylamide through 1,2-elimination, β -elimination. Additionally, formation of 3-aminopropionamide, which then deaminates to form acrylamide, as a result of hydrolysis of imine II is also possible [19].

On the other hand, it is believed that Strecker aldehyde, which is formed through hydrolysis of imine I, is one of the direct precursors of acrylamide [20]. There are also minor pathways for the formation of acrylamide other than Maillard reaction. Acrylamide is formed in oils through the formation of acrolein due to decomposition of oils when they are heated at high temperatures. Acrolein is then converted to acrylic acid that reacts with ammonia to form acrylamide [21].

Acrylamide is highly electrophilic due to its α , β -unsaturated carbonyl group Figure 1.2). For this reason, it is possible for acrylamide to be involved in 1,2 nucleophilic addition or Michael addition (1,4 nucleophilic addition) [22]. Detailed information about nucleophilic addition and Michael type addition will be given in following sections in this chapter.

It easily reacts with nucleophilic groups such as amino, thiol and imidazolic NH groups found mostly in biologic molecules [22, 23]. Michael addition of different amino and thiol sources to acrylamide was reported to be a useful mitigation strategy for acrylamide in foods [24-26].

As it is known, one of the compounds formed as a result of Maillard reaction and caramelization reaction of sugars is the α -dicarbonyl compounds. From the hexose sugars, the 6-carbon backbone is protected to form dicarbonyl compounds such as glucosone and 3-deoxyglucosone (3-DG). Breaking the C-C bond of hexoses, short chain dicarbonyl compounds such as glyoxal (GO) and methylglyoxal (MGO) are formed [27]. Chemical structures of α -dicarbonyl compounds are exemplified in Figure 1.4.

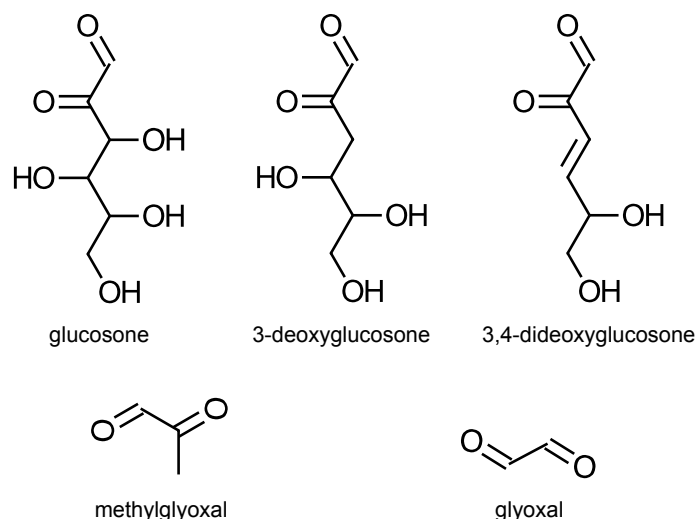


Figure 1.4. Chemical structures of α -dicarbonyl compounds

As in acrylamide formation, formation of dicarbonyl compounds through Maillard reaction starts with carbonylation of amino compound by a reducing sugar resulting in N-glucosylamine formation. Following this, glycolaldehyde-N-alkylimine is formed [28] and glycolaldehyde is produced as a result of its hydrolysis. GO is formed within the oxidation of glycolaldehyde and subsequent elimination of amino compound from it [29].

On the other hand, formation of 3-DG from Amadori product is also possible. After Amadori product (ketosamine) is enolized through 1,2 enolization, hydrolysis of this 1,2-eneaminol proceeds. 3-DG is formed in consequence of the addition of water and elimination of amino acid [30].

Under acidic conditions, enolization and elimination of water molecules from reducing sugars result in formation of different sugar dehydration products [30]. Dehydration of hexose sugars progress with the removal of a water molecule from the monosaccharide structure and ends with the elimination of three molecules of water. These dehydration reactions proceed with heating under low moisture conditions and in the absence of amino compounds [30, 31]. 3-deoxyosone is formed as a result of elimination of a water molecule from the aldo or keto-hexose. 3-DG is the formed α -dicarbonyl compound when the hexose is glucose or fructose [32]. Dehydration of 3-DG results in formation of a new α -dicarbonyl compound, 3,4-dideoxyglucosone (3,4-DG). And lastly, HMF is produced through the removal of a water molecule from 3,4-DG [33, 34]. Formation pathway of 3-DG

and 3,4-DG is illustrated in Figure 1.5. These α -dicarbonyl compounds are also the precursors of short chain α -dicarbonyl compounds such as GO and MGO.

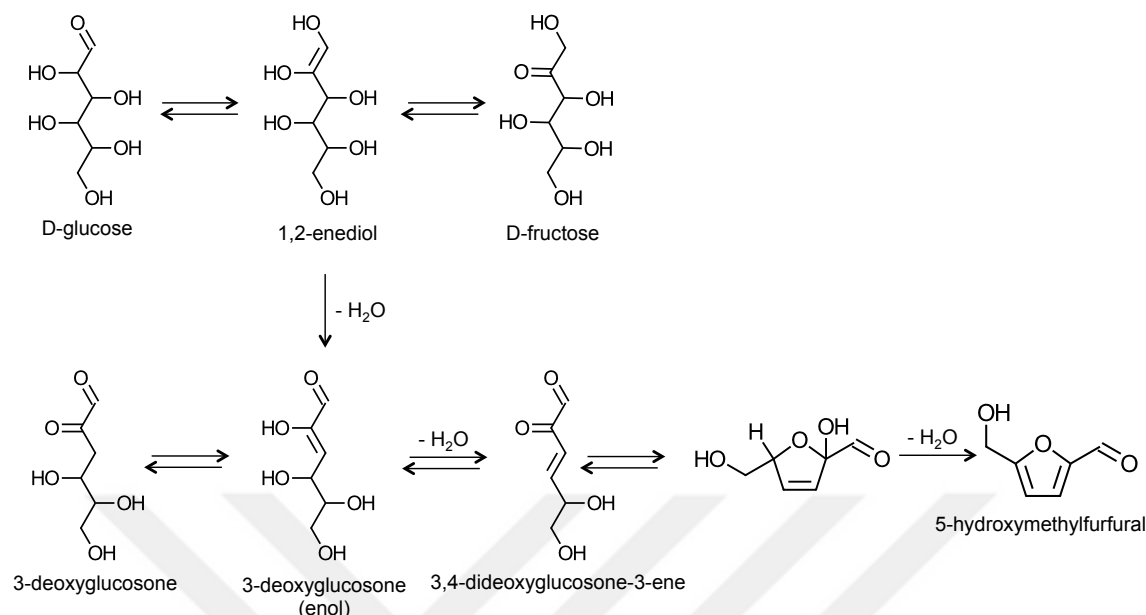


Figure 1.5. Formation pathway of 3-DG and 3,4-DG through sugar degradation adapted from [30].

GO is formed as a result of cleavage of C2-C3 bond from glucosone [35]. Formation of GO with the removal of 2 water molecules from glucose and further cleavage of C2-C3 is also possible (Figure 1.6) [36].

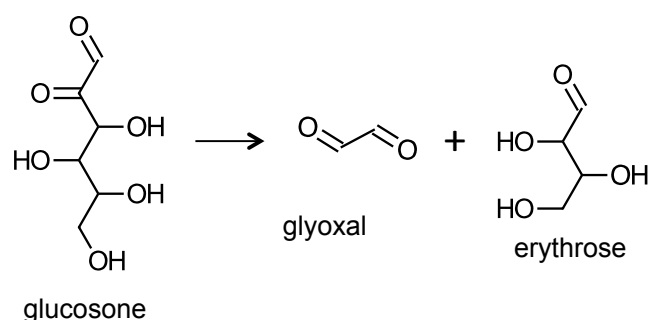


Figure 1.6. Formation of GO from glucosone adapted from [35].

Similarly, formation of MGO proceeds with the cleavage of C3-C4 bond from deoxyglucosone [37] and 3-DG [38]. Formation pathway of MGO from 3-DG is given in Figure 1.7.

In addition to sugar degradation, GO and MGO are also formed as a result of lipid oxidation [39] and degradation of ascorbic acid [40].

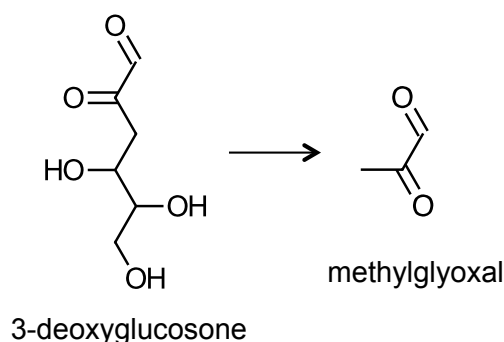


Figure 1.7. Formation of MGO from 3-DG adapted from [36]

α -dicarbonyl compounds readily react with amino and sulfhydryl groups owing to electrophilic nature provided by their carbonyl groups. They react with N terminals as well as side groups of proteins yielding advanced glycation end products (AGEs) [41]. This means that α -dicarbonyl compounds react with food proteins during thermal treatment [42, 43].

In the case of lysine as an amino acid, formation of N-carboxymethyllysine (CML) provided by the reactions of GO and lysine residues is possible [44]. CML is one of the AGEs formed both in foods and in the body. In addition to reactions of GO with lysine residues, CML formation might proceed from oxidation of an Amadori product, N- ϵ -fructosyllysine [45]. N- ϵ -fructosyllysine is produced through the reactions of lysine residues (ϵ -amino group) with glucose [46]. It is the early Maillard reaction product and furosine is formed as a result of its hydrolysis under acidic conditions. Furosine is used as a marker of early glycation in foods [47]. Decrease in furosine content provides information about the direction of the glycation, in terms of advanced stages.

Glycation reactions of α -dicarbonyl compounds with tissue proteins also occur under physiological conditions. AGEs are known to be involved in some chronic-degenerative diseases in humans such as diabetes mellitus [48], Alzheimer's disease [49] and atherosclerosis [50].

In addition to their nucleophilic addition reactions with protein-side chains, dicarbonyl compounds could polymerize with other dicarbonyl compounds through Aldol condensation [27].

Furthermore, α -dicarbonyl compounds are scavenged by several polyphenols. Epicatechins and theaflavins were reported to be able to reduce MGO concentration under physiological conditions through trapping by their catechin-like structure [51, 52]. In addition, genistein, derived isoflavone from soy products, was found to be effective in trapping MGO by forming mono and di-MGO adducts under physiological conditions [53]. Similarly, resveratrol could inhibit the glycation of bovine serum albumin (BSA) in BSA-MGO and arginine-MGO model systems due to its reactions with MGO [54].

As it was mentioned before, HMF is an intermediate formed in dehydration of hexoses under acidic conditions. Additionally, it is also formed through Maillard reaction. Basically, it is formed through dehydration of 3 moles of water from glucose and fructose [31]. It has molecular formula $C_6H_6O_3$. (Figure 1.7)

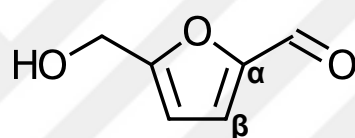


Figure 1.8. Chemical structure of HMF

Since fresh and raw foods are lack of HMF, formation of HMF is related with the thermal load applied to foods. HMF easily accumulates in carbohydrate-rich foods during thermal process or storage. For this reason, it is mostly used as an indicator of quality loss in carbohydrate-rich foods as a consequence of thermal treatment or improper storage. It is also used as a marker of Maillard reaction or hexose degradation [22, 31, 55].

Heating at higher temperatures is prerequisite for the formation of HMF through caramelization whereas it is formed at lower temperatures and neutral pH through condensation with amino compounds.

As given in Figure 1.9, HMF is formed through 3-DG as a result of caramelization of glucose and fructose. On the other hand, carbonyl group of HMF prefers condensing with amino groups in the presence of amino acids yielding Schiff base. Schiff base turns into Amadori product and then 1, 2-eneaminol intermediate which then forms imine cation through elimination of water. 3-DG is formed by the

hydrolysis and the elimination of amine from this imine cation. Formation of HMF following these steps is the same as in caramelization [22].

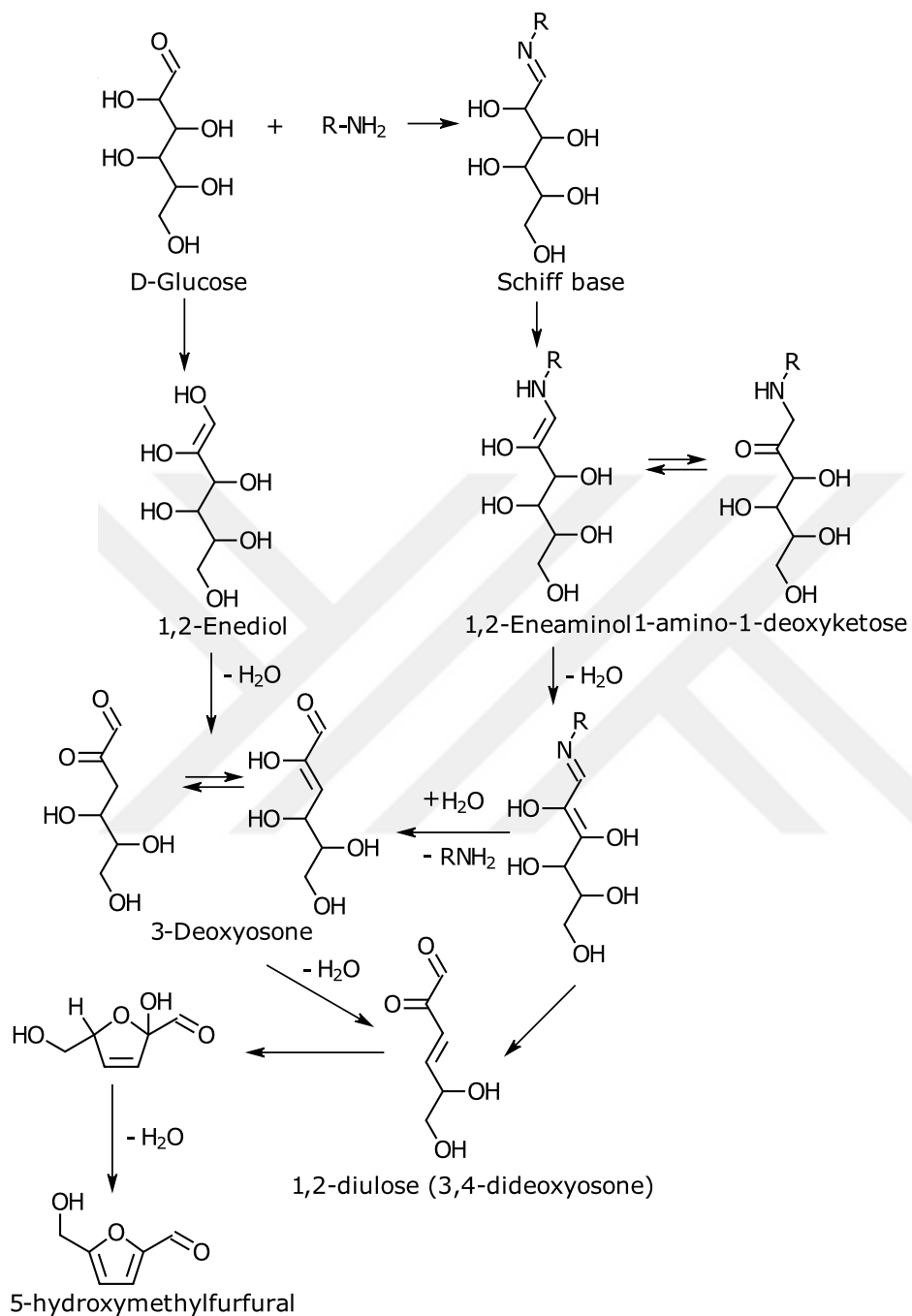


Figure 1.9. Formation of HMF [34, 56]

As it is illustrated in Figure 1.7, it contains a furan ring, an allylic hydroxyl group and an α , β -unsaturated carbonyl group. Owing to highly electrophilic carbonyl group, it is prone to react with amines through Schiff base formation and Michael addition [57]. Thereby, it was shown that HMF reacted with primary amines such as glycine or lysine and generated Schiff base adducts. Accordingly, HMF also

reacted with secondary amino acids or secondary amines including proline or piperidine leading to the formation of N-substituted 5-(aminomethyl)furan-2-carbaldehyde derivatives [33]. In addition, it was proven that, carbonyl group of HMF reacted with amino acid asparagine yielding acrylamide [58]. Moreover, Zou et al [59] indicated that reactions of HMF with amino acids were found to be an effective mitigation strategy both in model systems and biscuits.

1.3.2 Occurrence in foods and dietary exposure

After the acrylamide has been firstly detected in heated foods, European Commission started to have data about acrylamide concentrations in foods. Recently, in June 2015, the European Food Safety Agency (EFSA) reported the acrylamide levels in monitored foods. As indicated in this report, acrylamide is formed in baked or fried carbohydrate-rich foods mostly in French fries, potato chips, breads, biscuits and coffee. Table 1.1 gives the summary of the recent results reported by EFSA [60].

Table 1.1. Acrylamide levels ($\mu\text{g kg}^{-1}$) of foods in 2015, adapted from EFSA report [22]

	n	Median ($\mu\text{g kg}^{-1}$)	Mean ($\mu\text{g kg}^{-1}$)	Maximum ($\mu\text{g kg}^{-1}$)
French fries	1378	196	332	1115
Potato crisp	800	389	580	1841
Bread	535	17	40	137
Breakfast cereals	561	67	113	348
Biscuits, crackers	1974	120	264	1077
Coffee	682	221	317	878
Baby foods	348	15	24	70
Cereal based baby foods	394	15	103	200
Other products	120	36	330	1510

French fries, potato crisps and coffee amounted to the highest amounts of acrylamide. Besides, other products including roasted nuts, black olives and vegetable chips were found out to contain acrylamide almost the same amounts as in the others. Acrylamide amounts greatly varied depending on both process conditions and precursor concentrations.

According to the report published by EFSA, infants, toddlers and children were found to be the most exposed group of people to acrylamide. It was reported that, estimated dietary exposure of children varied between 0.5 and 1.9 $\mu\text{g kg body weight day}^{-1}$ and fried potato products was found as the main food group contributing to acrylamide exposure in this group. Bread, breakfast cereals, biscuits and crackers were the other contributors in children as well as in adults and adolescents. For the adults, adolescents and elder people, acrylamide exposure ranged between 0.4 and 0.9 $\mu\text{g kg body weight day}^{-1}$ and coffee was confirmed to be the main contributor for this group of people [60].

Concentration of α -dicarbonyl compounds in foods varies depending on food composition. From the α -dicarbonyl compounds, 3-DG is determined as the most concentrated one. Accumulation of MGO in foods differs because it is highly reactive. Therefore, concentration of 3-deoxygalactosone is mostly higher than MGO in foods. Sugar rich foods including juices, honey, beer, cookies and high fructose corn syrup (HFCS) are the main dietary sources of α -dicarbonyl compounds [61, 62]. In addition, foods having additives such as honey, caramel or HFCS could be considered as source of α -dicarbonyl compounds [62]. α -dicarbonyl levels of some foods are given in Table 1.2

Table 1.2. alpha dicarbonyl levels of foods, adapted from [28, 29].

	3-DG		3-DGal		MGO	
	Range	Mean (mg kg ⁻¹)	Range	Mean (mg kg ⁻¹)	Range	Mean (mg kg ⁻¹)
soft drinks	0-28	1.6	0-60	1.3	0-2.2	-
beer	18-54	34	0-16	11	0-1	0.5
winegar	4.6-2622	341	1.1-162	14	1.7-53	8.9
jellies, jams	1.7-1061	165	0-22	0	0-13	3.6
honey	271-1641	626	14-46	34	0-463	1
bread	13-619	45	0-47	4.8	0-28	3
biscuits	8.5-385	129	0-88	14	1.8-68	8.3
potato fries	0-18	6.9	0	0	0	0
HFCS	184-730		10-60		1.4-11	

There are limited data about the dietary exposure to dicarbonyl compounds. However, Arribas-Lorenzo and Morales [63], studied the dietary exposure of Spanish population to MGO and GO from biscuits. The results of the study indicated that daily exposure to GO and MGO was calculated quietly similar, as 213 µg/person and 216 µg/person, respectively.

In the study of Degen et al [61], they calculated the dietary 3-DG and MGO intake provided by two different diets including 3-DG poor and 3-DG rich diets. For this, they used fruit juices and beer consumption data for 3-DG rich diets whereas they took consumption of fruits, vegetables and milk products into account to exemplify 3-DG poor diets. As a consequence, they calculated the total daily intake of 3-DG as 50 mg. Within this data, they also stated that daily intake of MGO varied between 5 and 20 mg.

Concentration of HMF in foods is mainly related with thermal process applied, thus it could be used as a marker for monitoring the thermal process [64-66]. Besides, HMF is produced in honey at room temperature within the normal acidity acting on its sugars [22]. Use of caramel or honey as ingredients in food formulations might also be a potential source of HMF in foods [34]. Coffee, dried fruits, caramel,

bread and bakery products are the most important sources of dietary HMF [67, 68]. HMF levels of some foods are given in Table.

Table 1.3. HMF content of selected food products [34].

Food product	HMF content, mg kg ⁻¹
Bread	3.4-68.8
Breakfast cereals	6.9-240.5
Biscuits, crackers	0.5-74.5
Coffee	100-1900
Honey	10.4-58.8
Beer	3.0-9.2
Dried fruits	25-2900
Milk based baby foods	0.18-0.25
Cereal based baby foods	0-57.18

According to daily consumption, most important contributors of HMF exposure were found as bread and coffee [67]. According to Jansowski et al [69] HMF intake was found to be varying between 30-150 mg/person. Rufian Henares and de la Cueva [70] reported that dietary intake of HMF in Spanish population ranged between 2.1-23 mg day⁻¹ meanwhile they calculated average intake of HMF as 9.7 mg day⁻¹. In a similar study carried out with Spanish adolescents, daily HMF intake was calculated as 5.1 mg [71]. In a recent study that is developed in pre-adolescent population, mean dietary HMF intake was reported to be 13.72 mg/day. From the meals, lunch was determined as the meal contributing most part of daily HMF intake (%27) and it was followed by dinner, afternoon snack, midmorning and breakfast [72]. In the study of Husoy et al [73], it was reported that mean daily HMF intake was found to be 5.6 in the studied Norwegian volunteers (n=53). Besides, urinary 5-hydroxymethyl-2-furoic acid (HMFA), which is the major HMF metabolite in humans, was found out 12.4 mg/person corresponding to dietary HMF exposure, as well.

1.3.3 Bioavailability and toxicity

Acrylamide is highly hydrophilic and absorption rate from the intestines is quite high. Ingestion of acrylamide through foods is the fastest way for its absorption. It was reported that acrylamide is rapidly absorbed in mice and rats. Bioavailability of acrylamide from both food and aqueous gavage was monitored in rats and mice. It was found that around 38% and 23% of acrylamide from the food and around 80% and 42% of acrylamide from aqueous gavage was bioavailable for the rats and

mice, respectively [74, 75]. Additionally, in a study in which bioavailability of acrylamide in crisp bread was monitored, it was exhibited that acrylamide was completely absorbed [76]. Recently, it was shown that acrylamide was rapidly absorbed after the consumption of water containing acrylamide and reached to the maximum concentration in plasma just after 0.5 hours [77]. Besides animal studies, Sorgel et al [78] indicated that acrylamide was absorbed in humans as a result of crisp bread and potato products consumption. Similarly, Boettcher et al [79] studied the acrylamide bioavailability in male subjects and 57% of metabolites of acrylamide was recovered in urine 2 days later.

After both *in vivo* and *vitro* considerations, acrylamide was found to be toxic [80, 81], genotoxic [82] and neurotoxic [83]. Epidemiology studies demonstrated that there was a link between acrylamide exposure and cancer in humans [84-90]. However, World Health Organization (WHO) reported [91] that acrylamide is not lethal if the levels are not higher than 250 mg kg bw day⁻¹ whereas it is moderate toxic with LD₅₀ value of 150–180 mg kg⁻¹ for rodents [92]. In addition, by the results of the toxicity studies in rat, no observed adverse effect level (NOAEL) was observed as 2 mg kg bw day⁻¹ [93].

Bioavailable part of acrylamide interacts with tissue proteins and DNA. As mentioned above, since it is highly electrophilic, it easily reacts with nucleophilic groups in biologic molecules [23]. It is also converted to metabolites mostly to glycidamide in the body through oxidation. Due to its highly electrophilic nature as in acrylamide, it reacts with nucleophilic groups modifying DNA or body proteins, as well [94].

Published data so far report bioavailable part of dicarbonyl compounds are varying. GO and MGO concentrations of urine of healthy subjects were reported to be between 0.29-14.5 mg L⁻¹ and 1.44-7.2 mg L⁻¹, respectively [95]. Zhang et al [96], reported lower concentrations for these dicarbonyl compounds, 11.9 µg L⁻¹ and 20.6 µg L⁻¹, respectively. In addition, they reported the higher levels in diabetic patients urine samples as 67.9 µg L⁻¹ for GO and 76.0 µg L⁻¹ for MGO.

Similarly, higher serum levels of dicarbonyl compounds were reported for diabetic patients. Mean serum concentrations of GO and MGO were respectively 45 µg L⁻¹ and 50 µg L⁻¹ for healthy subjects whereas those concentrations were 205 µg L⁻¹

and $215 \mu\text{g L}^{-1}$ for diabetic subjects [97]. On the other hand, MGO concentration in blood (not serum) was found to be $18.4 \mu\text{g L}^{-1}$ and $34.5 \mu\text{g L}^{-1}$ for healthy subjects and diabetic patients, respectively [98]. In addition, plasma concentrations of 3-DG for healthy and diabetic individuals were reported as 7.8 and $12.8 \mu\text{g L}^{-1}$. However, in urine, concentrations were found as 0.44 and 0.94 mg/ g creatine for healthy and diabetic individuals [99].

Reactive carbonyl species (RCS) including α -dicarbonyl compounds are important reactive precursors initiating carbonyl stress in human cells [100-102]. As it was mentioned above, α -dicarbonyl compounds are prone to react with tissue proteins in the body forming AGEs. It was reported that, α -dicarbonyl compounds and AGEs have cytotoxic effects provided by reactive oxygen species, which cause to the damage of cells [103, 104]. In addition, there is a link between accumulation of AGEs in the tissues and organs by age due to dietary intake of these compounds and the development of so many chronic diseases such as retinopathy, nephropathy, arteriosclerosis and cataract formation [105]. Above all, since plasma dicarbonyl levels of diabetic patients are significantly higher, this group of people is also highly affected by carbonyl stress [106, 107].

According to the data reported in literature, it is still not clear that if human exposure to HMF poses potential health risks. It was reported that, HMF was rapidly absorbed in gastrointestinal tract when rats were orally fed by different concentrations of HMF (0.08-500 mg/kg body mass) [34]. Delgado-Andrade et al [108] reported that both absorption and transport of HMF was promoted while the cells were subjected to higher concentrations of HMF. In addition, microbial transformation of some part of HMF, which is not exposed to gastrointestinal digestion, to furfuryl alcohol by enteric bacteria was also possible [109].

With respect to the results of these studies, HMF exhibits low or no mutagenicity. But the major concern about dietary HMF is based on its conversion to 5-sulfoxymethylfurfural (SMF) [34]. Since SMF bears a reactive sulphate group, it readily reacts with DNA and macromolecules resulting toxicity and mutagenicity [110, 111]. Mutagenicity or genotoxicity of 5-sulfoxymethylfurfural (SMF) was confirmed by both in vitro and in vivo studies carried out with animals [111, 112]. In a very recent study, SMF was detected in plasma samples of humans. In addition, presence of DNA adducts with SMF was confirmed in blood cells [72]. SMF has

also been reported to initiate tumour formation in mice skin [111, 112]. Furthermore, conversion of HMF into acrylamide and furan through Maillard reaction, which are classified as “possibly carcinogenic to humans” is possible [58, 113, 114].

1.4 POSSIBLE REACTIONS OF THERMAL PROCESS CONTAMINANTS DURING DIGESTION

As it was mentioned above, acrylamide and HMF has α , β -unsaturated carbonyl group that might be involved in Michael type addition reactions to amine or thiol compounds. Additionally, α -dicarbonyl compounds are also prone to react with amino and thiol compounds. Mechanism of these reactions is discussed under this section.

1.4.1 Nucleophilic addition

If a molecule or ion has a free pair of electrons, it is nucleophile. So, nucleophiles are electron donators to electrophiles thus forming a chemical bond as a consequence. On the other hand, electrophiles are reactive species attracting electrons since they have free orbitals attached to electron rich center. Therefore, they are electron acceptors in order to form a bond with nucleophiles. In other words, nucleophiles are Lewis bases whereas electrophiles are Lewis acids. Carbonyl compounds are one of the well-known electrophiles. Amines, ammonia or amides are important examples of nitrogen containing nucleophiles. Besides, because sulfur is very nucleophilic, hydrogen sulfide or thiol compounds are the most reactive sulfur containing nucleophiles [115].

Nucleophilic addition is the addition reaction of a nucleophile with an electrophile. This process has 2 main steps; nucleophilic attack on the electrophile's carbon atom, formation of an intermediate and the proton transfer. Finally, when the electrophilic molecule has a double bond, double bond is substituted with a single bond. Nucleophilic addition of amine molecule to a carbonyl compound is illustrated in Figure 1.10.

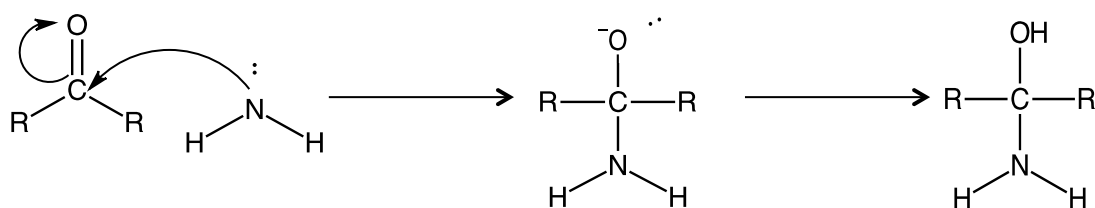


Figure 1.10. Nucleophilic addition of an amine to carbonyl compound

In the 1st step, amine attacks to the carbon of carbonyl molecule thus forming a new bond between the amine and carbonyl molecule. Following step is the cleavage of C=O bond and formation of an anion intermediate. Last step consists the proton transfer to anions O atom resulting in the formation of OH.

Reactivity of nucleophile plays an important role here. For instance, anionic nucleophiles attack to the C=O and formed intermediate becomes protonated with dilute acid. On the other hand, C=O bond needs to be activated before nucleophilic attack by neutral nucleophiles. Presence of an acid catalyst working as a protonater could provide activation [115].

Moreover, if the carbonyl compound is an α , β unsaturated carbonyl (Figure 1.11), which has conjugated double bonds, electrophilic character of carbonyl carbon is substituted with double bond of β -carbon

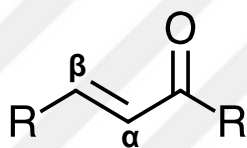


Figure 1.11. α , β unsaturated carbonyl

In this case, nucleophilic molecule may directly attack at carbonyl carbon as well as at β -carbon. Therefore, nucleophilic addition is called as 1,2 addition when the reaction proceeds from carbonyl carbon otherwise it is called as 1,4 addition. 1,4 addition is generally known as “Michael addition”.

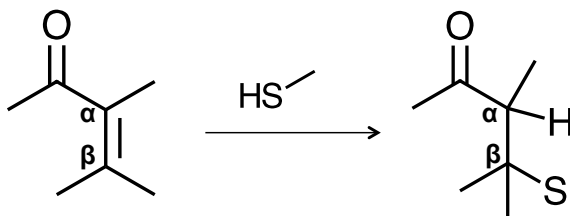


Figure 1.12. 1,4 addition (Michael addition) between α , β unsaturated carbonyl and a thiol compound

Nucleophile competes in attacking at carbonyl or β -carbon and the reactivity of nucleophile gives direction to this reaction. In the case of amines, Michael addition

is more favorable since it keeps carbonyl group. Main difference from 1,2 addition is the addition of hydrogen to oxygen in 4 position in protonation step. (Figure 1.13)

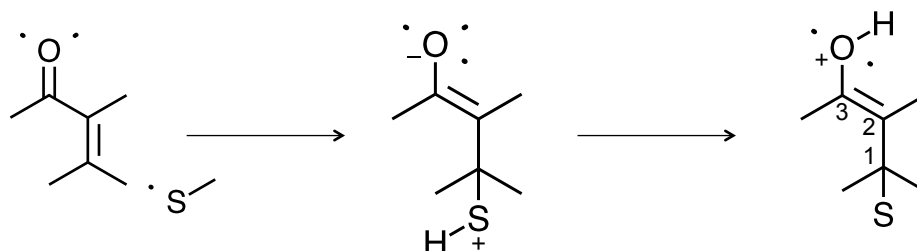


Figure 1.13. Protonation step of 1,4 addition (Michael addition) between α , β unsaturated carbonyl and a thiol compound

Following step is the tautomerization, which results in formation of the molecule having the same carbon skeleton, but positions of protons and electrons differ. (Figure 1.14)



Figure 1.14. Tautomerization step of 1,4 addition (Michael addition) between α , β unsaturated carbonyl and a thiol compound

1.4.2 Imine (Schiff base) formation

In the case of reaction of carbonyl compound with a primary amine or ammonia, imine is formed. Imines are similar to aldehydes or ketones whose $C=O$ is substituted with $C=N$. They are also known as Schiff bases. Since a small molecule leaves from the adduct, imine formation also refers to condensation reactions. Leaving small molecule is generally a water molecule. (Figure 1.15)

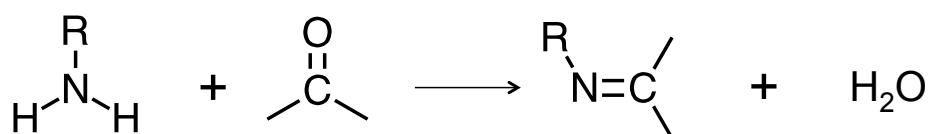


Figure 1.15. Formation of a Schiff base between carbonyl compound and an

amine

Imine formation proceeds with the nucleophilic attack of amine to carbonyl carbon.

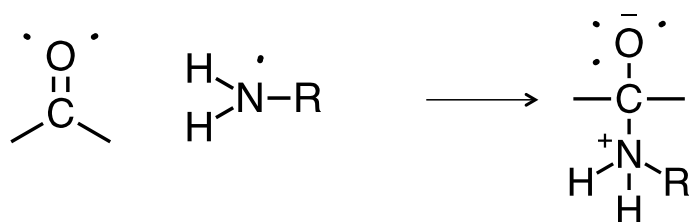


Figure 1.16. First step, nucleophilic attack, of Schiff base formation

Transfer of proton to oxygen atom and deprotonation of nitrogen atom follows it resulting in formation of an intermediate, carbinolamine. (Figure 1.17)

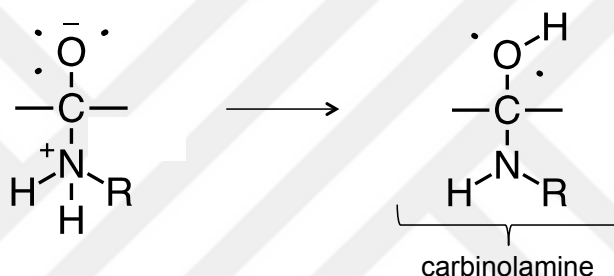


Figure 1.17. Carbinolamine formation

Following step is the protonation of OH in carbinolamine for subsequent elimination of water molecule from it. Acidic pH is prerequisite for the elimination of water because there should be enough acid for the protonation of OH in carbinolamine. Optimum pH is about 4-5 and at lower pH, nucleophilic amine molecules become too protonated R-NH_3^+ thus will lose its nucleophilicity.

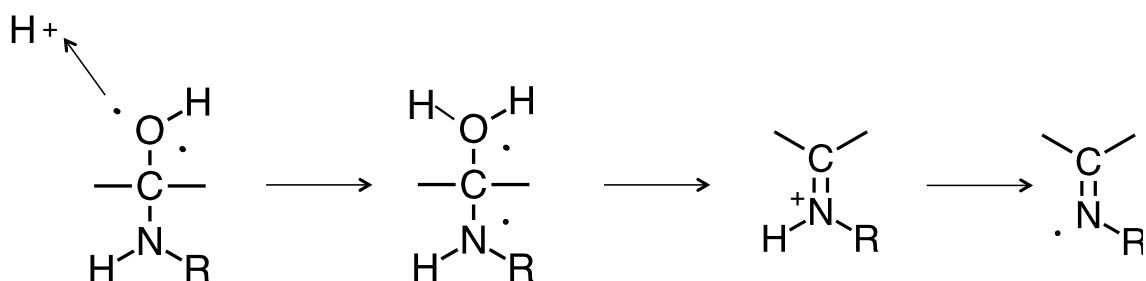


Figure 1.18. Elimination of water molecule from carbinolamine yielding Schiff base

1.5 POTATO STARCH AS A POLYMERIC FOOD COMPONENT

Potato is one of the most preferred crops owing to its nutritional values. In addition

to being an important energy source provided by its high carbohydrate content [116], it also contains various nutrients other than carbohydrates. It is composed of 70-80% water, 1-2% protein, 0.1-0.5% lipids, 10-18% starch, 0.5% fiber and other minor compounds including vitamins, minerals and phenolic compounds [117]. Phenolic compounds come into prominence [118, 119] due to consumption by different age groups in higher amounts, and for this reason, might possess beneficial effects on health [120, 121]. Besides beneficial sides of potato, its consumption is associated with several diseases over time depending on its digestion behaviour.

1.5.1 Digestion and GI of potatoes

Starch digestion refers to its breakdown to monomers in consequence of the action provided by amylolytic enzymes. These amylolytic enzymes are composed of salivary α -amylase and pancreatic amylase. The digestion of starch starts in the mouth by the salivary α -amylase but after it arrives to stomach, it becomes inactive in acidic media. For this reason, digestion of starch in the oral phase is considered as minor. However, decomposition of starch mostly occurs in small intestine. Firstly, amylolytic enzymes should diffuse into food matrix and bind to substrate [122]. As a result, breakdown of starch by α -amylase refers to random hydrolysis of α -1,4 glycosidic bonds in amylose and amylopectin of starch [123] forming maltose, maltotriose and maltotetraose [124]. Since α -amylase does not have ability for the hydrolysis of α -1,6 glycosidic bonds of amylopectin, dextrans and some other oligosaccharides are formed as a result. By subsequent hydrolysis of these big saccharides by α -glucosidase, they are completely hydrolysed to glucose to be absorbed through the walls of small intestine.

Due to their digestibility behaviours, starches are classified as rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) [125]. RDS is the proportion of starch that is digested within 30 minutes of enzymatic digestion [126]. It refers to gelatinized starch becoming accessible to digestion as a consequence [127]. SDS is the proportion of starch that is digested during 100 minutes of digestion [126]. It refers to physically inaccessible or retrograded forms of starch. RS [128] is the remaining non-hydrolysable part of starch at the end of digestion (120 minutes) [129]. Since this portion of starch is not absorbed in small intestine, it reaches colon intact for the further fermentation by microorganisms in

colon [130]. It is also utilized as a dietary fiber providing so many beneficial health effects especially on colon health [131].

It is believed that polysaccharides provide controlled increase in blood glucose levels in contrast to rapid increase induced by mono or disaccharide consumption.

In vitro assessment of these fractions refer to rapidly available glucose (RAG) and slowly available glucose (SAG) in means of the rate of available glucose for the absorption from the small intestine cells [132].

Foods having higher RDS fractions cause to the rapid increase whereas higher SDS and RS containing foods provide controlled increase in blood glucose levels [123]. Effect of carbohydrates on blood glucose level is expressed as glycaemic index (GI) [133]. Therefore, RDS contributes to food to be classified as high GI product [132]. On the other hand, SDS provides food to be classified as low GI product and the advantage of a slow increase in blood glucose level [132, 134, 135].

GI, is the incremental area of blood glucose during the 2 hours digestion of 50 g available carbohydrate. For the GI measurement, glucose is referred as the reference food with having a value of 100, and the GI value of other foods is compared to this reference food. When the foods are classified according to their GI values, high GI foods refer to foods having GI value higher than 70. Medium GI foods have GI values between 55 and 70, and low GI foods have GI values lower than 55 [136].

Low GI diets are believed to be in relation with so many beneficial effects on health. For instance, controlled blood glucose and lipid levels prevent the risk of cardiovascular disease and type 2 diabetes [137]. It was reported that, there is a negative relation between GI and high-density cholesterol levels [138, 139]. In addition, a positive correlation was found between consumption of low GI foods and decrease in the risk of diabetes [140, 141]. and cardiovascular disease [142]. On the other hand, no significant interaction was observed between GI and cardiovascular disease in men with body weight lower than 23 kgm⁻². Similarly, in a Zutphen study, no significant relationship was found between GI and cardiovascular disease in old men [143]. Low GI diet consumption provided the improvement in cardiovascular disease risk factors in type 2 diabetic patients

[144]. From the cancer point of view, GI may have importance for the prevention of some types of cancers [133]. Augustin et al [137] reported that there was a significant increase in breast cancer incidence with the consumption of high GI diets. In addition, results of the study for colon cancer showed significant relation with GI [145].

Table 1.4. GI values of some foods [146].

Food	Glycemic index (glucose=100)	Food	Glycemic index (glucose=100)
Baguette, white	95	Chickpeas	10
White bread	71	Soybeans	15
Wheat tortilla	30	Green peas	51
Coca cola	63	Spaghetti (boiled)	46
Cornflakes	93	Pizza (plain baked dough)	80
White rice	89	Boiled potato	82
Banana (ripe)	62	Instant mashed potato	87

According to the GI values given in Table 1.4 vegetables, milk and spaghetti corresponds to low GI foods. Contrarily, bakery products and cereals comprise to high GI foods. Among the foods, potatoes have high GI values.

1.5.2 Effects on digestibility of potato starch

From the digestibility point of view, undigested part of starch accounts RS whereas digestible part corresponds to available starch (AS) [147]. Potato starch is resistant to digestion when it is raw, however it becomes digestible with the modification. There are so many factors affecting the digestibility of starch including potato varieties, structural characteristics of starch, processing and storage conditions. Published data revealed that GI of cooked potatoes from different varieties varied too much. For example, GI for boiled Pontiac variety was reported to be around 56 whereas it was found to be around 101 for Desiree variety [148]. Amylose: amylopectin ratio has also important role on resistance to digestion. High amylose containing starches are reported to be the starches containing digestible starch fractions lower amounts [149].

Potato is mostly composed of water and starch. Water comprises 80% of potato whereas starch constitutes to 70-90% of dry matter [117]. Even potato starch

molecule is resistant to digestion in its raw form, it loses its intact structure when it is heated in the presence of water, which is also called gelatinization, becoming accessible for enzymatic action. For this reason, water availability is important from the starch digestibility point of view. For gelatinization, water content should be high enough in terms of water: starch ratio higher than 0.75 [150]. In addition to water content, heating time and thermal load has also importance [151, 152].

In Table 1.5, available starch (DS) and RS contents of potatoes subjected to different heating applications were given.

Table 1.5. AS and RS contents of potatoes as a result of different cooking applications [153].

Food	RS (%)	AS (%)	% moisture
Raw	69.0	81.2	81.2
Boiled	1.2	81.2	81.2
Mashed	2.1	69.9	86.2
Wholly oven-cooked	3.7	62.2	79.6
Raw flakes	2.8	68.6	8.6
French-fry	6.6	52.7	17.7
Crisp	3.3	65.1	2.57

*RS and AS contents were given in dry basis.

As it is shown, uncooked potatoes were having the highest RS content, in contrast, boiled or mashed potatoes were having the lowest. These differences are attributed to differences in moisture contents and thermal processing conditions. Since boiling is the cooking process in excess water, digestibility of potatoes increases too much [129, 154]. On the other hand, water availability is limited in cooking processes such as baking and frying, therefore fried or baked potatoes were having comparably lower levels of AS. With the rapid evaporation of water in outer parts, potato surface becomes compact which might also be a physical barrier for the action of amylolytic enzymes. In addition, in fried potatoes, lower AS is because of the formation of amylose-lipid complexes, which are also RS, on the surface of potatoes [147, 155].

One of the ways to decrease GI through increasing RS contents is reported as cooling potatoes after cooking. In that case, formation of RS is provided by the

retrogradation of gelatinized starch [156]. Tahvonen et al [154] reported that mashed potatoes were having GI of 106 ± 42 whereas cooling and cold storage caused to significant decrease in GI (75 ± 17). Similarly, Monro et al [157] reported that RS content of different boiled potatoes ranged between 3.3-6.5%, however RS content of cooled potatoes varied between 7.9% and 10.9%.

The factors affecting the amount of RS could also be used for increasing SDS content of potatoes. Controlled starch gelatinization is a good way of increasing SDS. As in biscuits, limited gelatinization of starch under low moisture conditions provides the starch granules to be retained intact form [132]. Similarly, to enhance the SDS content of potatoes might be possible. In addition, formation of Maillard reaction products, in terms of starch-protein complexes, was reported to be related with lower digestible starch [158].

1.5.3 Interactions of potato starch with other food components

In addition to cooking and storing conditions, presence and/or addition of some food components has also a role on RS content of potatoes. Sucrose is one of these food components. It was reported by Escarpa et al [159] that, sucrose addition caused to the RS formation in potatoes.

Free-fatty acid addition to starch induced the formation of amylose-lipid complexes. However, interactions of starch with lipids lead to the reduction in RS content of potato starch. Addition of an emulsifier, glycerol monopalmitin, to potato starch inhibited the RS formation [160]. In the aforementioned study [159], potato starch was autoclaved with different additives. The results indicated that addition of olive oil led to 4.6-6.9% decrease in RS content. On the other hand, the results of the study of Crowe et al [161] indicated that the addition of different free fatty acids to potato mixture composed of starch, amylose and amylopectin caused to 35% inhibition in hydrolysis of starch, in mostly amylose fraction. This inhibition was related with formation of RS in consequence of complex formation between amylose and fatty acids [162].

It is believed that presence of protein fractions leading to protein-starch interactions retarding digestion of starch. This is attributed to encompassing of starch thus becoming inaccessible for amylolytic enzymes. For instance, action of amylolytic enzymes on starch in pasta is mostly restricted by the formation of

protein network [126, 163]. Additionally, it was also reported that it might be due to prevention of hydrolysis provided by protein network in the small intestine [164].

Moreover, there is an increased interest on the effect of dietary fiber on starch hydrolysis. Jenkins et al [165] reported that dietary fibers provide higher viscosity to bolus enabling the movement of bolus slower, therefore slower increase in glucose levels.

In addition to all these additional food components, there are so many molecules that could act as amylose inhibitors. Polyphenols are the molecules mostly associated with this. Antocyanins and tea phenolics were reported to be capable of inhibiting α -amylase activity [166, 167]. Besides, potato phenolics (in some varieties) are also reported to prevent glucose hydrolysis through inhibition of amylolytic enzymes in the intestine [168].

2. CHAPTER 2

REACTIVITIES OF PROCESS CONTAMINANTS AS MONOMERIC FOOD COMPONENTS DURING *IN VITRO* DIGESTION

2.1 INTRODUCTION

Ingestion of food is considered as the major route of exposure to many contaminants in human health risk assessment. Since possible reactions may occur under the varying conditions of digestive process, total amount of a contaminant found in the ingested food does not always reflect the exact amount available to the body. And therefore, determination of the fate of ingested contaminant during digestion is an important issue for human health.

As it is mentioned before, acrylamide is one the process contaminants which is classified as probable carcinogen in humans. α -dicarbonyl compounds are the intermediates formed in thermally processed foods that react with the amino and sulfhydryl residues of proteins and peptides leading to formation of AGEs. AGEs are known as being involved in some chronic-degenerative diseases in humans such as diabetes mellitus [48], Alzheimer's disease [49] and atherosclerosis [50]. In addition, HMF is rich in mostly consumed foods and its toxicity arises since it is consumed too much. Considering the possible impact of these compounds on human health, amount of ingested process contaminants with foods is of importance.

Despite acrylamide is one of the most widely encountered thermal process contaminants in foods, information about its fate during the digestion of processed foods is lacking. There are few reports about the role of exogenous α -dicarbonyl compounds in gastrointestinal tract. Daglia and others [169] reported that MGO concentration decreased after digestion because of its reaction with digestive enzymes by carbonylating their free amino groups. Similarly, Henle and his group reported that [170] only 5-10% of the initial MGO of manuka honey was recovered after *in vitro* digestion and they concluded that dietary MGO is rapidly degraded during digestion process thus becoming safer for human health. In a recent study of same group, MGO was rapidly scavenged by creatine forming N-(4-methyl-5-oxo-1-imidazolin-2-yl) sarcosin (MG-HCr) under physiological conditions [171]. On the contrary, Papetti and others [172] reported that *in vitro* simulated digestion conditions reduced free α -dicarbonyl compounds in coffee, whereas it strongly

increased free α -dicarbonyl content in barley and soy sauce. However, still little is known about the interactions of food-derived α -dicarbonyls during digestion. About the fate of HMF during digestion, limited and controversial data was reported so far. Delgado-Andrade et al [108] studied the bioavailability of HMF in breakfast cereals and reported that some part of HMF retained in the non-soluble fraction after digestion affecting its availability depending on food matrix. They also reported that HMF degraded to some extent during gastrointestinal digestion. On the other hand, Rufian-Henares and Delgado-Andrade [173] reported that most part of the Maillard reaction products and HMF showed stability and resistance to *in vitro* digestion.

Owing to their highly reactive natures provided by carbonyl groups, process contaminants such as acrylamide, HMF and dicarbonyl compounds present in foods may interact with certain components, especially with amino and thiol compounds, released from the food matrix under the varying conditions of gastrointestinal tract. Moreover, these conditions may also favour the conversion of precursors into them. For this purposes, it is aimed to investigate the reactions of acrylamide, dicarbonyl compounds and HMF with other food components during digestion in this chapter. Different model systems and foods were subjected to *in vitro* digestion process and then scanned by high-resolution mass spectrometry (HRMS) to explain the interactions between these contaminants and amino or thiol compounds. Within the obtained data from the reactions of these contaminants during digestion, kinetics of the reactions of HMF with amino and thiol compounds was evaluated. Moreover, reactions of dicarbonyl compound, namely GO, with different sulphur compounds under simulated physiological conditions were investigated.

2.2 EXPERIMENTAL

2.2.1 Chemicals and consumables

Acrylamide (99%), 3-deoxyglucosone (75%), glyoxal (40%), quinoxaline (99%), 2-methylquinoxaline (97%), methylglyoxal (40%), 2,3-dimethylquinoxaline (97%), o-phenylenediamine (98%), diethylenetriaminepentaacetic acid (98%), hydroxymethyl furfural (HMF), albumin from chicken egg white, gluten from wheat, L-cysteine (>99%), L-Methionine, L-Lysine, L-Asparagine (98%) and ammonium formate were purchased from Sigma (Deisenhofen, Germany). Potassium

chloride, sodium chloride, magnesium chloride, ammonium bicarbonate, potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). Potassium hexacyanoferrate (II) trihydrate and zinc sulphate heptahydrate were purchased from Merck (Darmstadt, Germany). Potassium metabisulphite (PMS) was purchased from a local shop where winemaking equipments are sold.

Carrez I and Carrez II solutions were prepared by dissolving 15 g of potassium hexacyanoferrate in 100 mL of water, and 30 g of zinc sulfate in 100 mL of water, respectively. The enzymes: pepsin (≥ 250 U/mg solid) from porcine gastric mucosa, pancreatin (4 x USP) from porcine pancreas, protease from *Streptomyces griseus* (≥ 3.5 U/mg solid) and viscozyme L were purchased from Sigma–Aldrich (Deisenhofen, Germany). Bile extract, porcine was also purchased from Sigma Aldrich (Deisenhofen, Germany). Formic acid (98%), acetonitrile and methanol (HPLC grade) were purchased from J. T. Baker (Deventer, Holland). Oasis MCX solid phase extraction cartridges (1 mL, 30 mg), UPLC HSS T3 column (150 mm x 4.6 mm i.d.; 3 μ m), Atlantis T3 column (250 mm x 4.6 mm id; 5 μ m), Oasis HLB (1 mL, 30 mg) solid-phase extraction cartridges, Atlantis HILIC column (250 x 4.6 mm, 5 μ m), Atlantis dC18 (250 x 4.6 mm, 5 μ m) column, polyvinylidene fluoride (PVDF) filters and 0.45 μ m nylon syringe filters were supplied by Waters (Millford, MA). Purospher Star RP-18e column (150 x 4.6 mm, 5 μ m) was supplied by Merck (Darmstadt, Germany). Zorbax SB C18 column (2.1 100 mm; 1.8 μ m) was supplied from Agilent (Santa Clara, CA, USA).

2.2.2 Preparation of foods

To determine the reactivity of thermal process contaminants and their interactions with other food components during *in vitro* digestion, various food samples were used. All food samples were ground and freeze dried prior to the digestion process.

2.2.2.1. Preparation of foods for the investigation of the reactions of acrylamide during digestion

For the determination of reactivity of acrylamide during digestion, different non-sweet and sweet biscuits as well as potato products were subjected to digestion. Besides bakery products, potato products were also digested in order to better understand the fate of acrylamide during digestion. Both commercial biscuits and

potato chips were obtained from a local market, and fried potato samples were obtained from a fast food restaurant in Ankara. Laboratory made biscuits were prepared using a recipe adapted from the American Association of Cereal Chemists (AACC) method 10-54. Ingredients were as follows; wheat flour (80 g), shortening (32 g), sucrose (35 g), glucose (0.6 g), fructose (0.6 g), nonfat milk powder (0.8 g), NaHCO_3 (0.8 g), NH_4HCO_3 (0.4 g), NaCl (1 g), and water (17.6 g). All ingredients were thoroughly mixed in accordance with the procedure described in AACC method 10–54 using a dough mixer Artisan Kitchen Aid 5KSM150 (MI, USA). Dough was rolled in 4 mm thickness, cut into discs having 6 cm diameter. The discs were baked in a conventional oven (Memmert, UNE 400, Germany) set at 200 °C for 12 min.

2.2.2.2. Preparation of foods for the investigation of the reactions of dicarbonyl compounds during digestion

High fructose corn syrup is usually added to formulation with the technological purposes and sugar degradation products are formed during baking of commercial biscuits. Since regular biscuits, baby biscuits and twice baked biscuits are the important examples of mostly consumed biscuits; they were subjected to digestion in order to understand the fate of sugar degradation products during digestion. To prepare the model biscuits, 2.5 g of cysteine, lysine or ovalbumin was added to 2.5 g of biscuits prior to digestion.

2.2.2.3. Preparation of foods for the investigation of GO-scavenging

To determine the carbonyl scavenging ability of bioaccessible broccoli under physiological conditions; raw, steamed and boiled broccoli were subjected to digestion. For this reason, 250 grams of raw broccoli sprouts were boiled for 30 min in water bath adjusted to 90°C. To prepare the steamed broccoli samples, 250 grams of raw broccoli sprouts were steamed by the steam of water bath adjusted to 90°C for 5 min. Raw, steamed and boiled broccoli sprouts were quickly frozen at -80°C and were freeze-dried. Dried samples were ground prior to *in vitro* digestion.

2.2.2.4. Preparation of foods for the investigation of the reactions of HMF during digestion

Commercial biscuits including regular, twice baked and baby biscuits were used to determine the fate of HMF during *in vitro* digestion. These biscuits were chosen since they contain sugar degradation products that are formed during baking

process. Even the consumers from all ages consume baby biscuits, they are really important due to higher consumption by babies and kids (ages ranging between 6 months and 7 years). In addition, twice-baked biscuits are highly heat-treated forms of regular biscuits. For these reasons, they were obtained from a local market and subjected to *in vitro* digestion. Main nutritional ingredients were given as 75.7 g carbohydrate (24.5 g sugar), 5.7 g protein and 11.4 g fat for 100 g regular biscuits. These values were given as 79.9 carbohydrate (22.9 g sugar), 7.4 g protein and 9.6 g fat for 100 g twice-baked and 74.1 carbohydrate (24.1 g sugar), 4.6 g protein and 14.9 g fat for 100 g baby biscuits. Biscuit samples were ground and freeze dried prior to the digestion process.

2.2.3 Preparation of model systems

Different model systems composed of thermal process contaminants and amino acids or proteins were prepared to determine both their fate and interactions with amino acids or proteins during *in vitro* digestion.

2.2.3.1. Preparation of acrylamide-amino acid model systems

For the determination of the reactivity of acrylamide, model systems composed of acrylamide alone for “ACR”, acrylamide and cysteine for “ACR-Cys”, and acrylamide and lysine for “ACR-Lys” were prepared. 10 μ moles of each reactant were dissolved in 10 mL of deionized water, and directly subjected to digestion process. Another model system; “Asn-Glc”; composed of asparagine (10 μ mol) and glucose was also prepared. It was heated at 180 °C for 10 min to form the Maillard reaction products, including acrylamide and intermediate compounds like the Schiff base formed between asparagine and glucose. Then, the heated model system was dissolved in deionized water (10 mL) and subjected to *in vitro* digestion to determine changes in the levels of acrylamide and the Schiff base formed between asparagine and glucose.

2.2.3.2. Preparation of dicarbonyl-amino acid model systems

For the determination of the reactivity of α -dicarbonyl compounds during digestion, model systems composed of MGO; “MGO, MGO and lysine; “MGO-Lys” and MGO and cysteine; “MGO-Cys” were prepared. 100 μ moles of each reactant were dissolved in 10 mL of deionized water, and directly subjected to digestion process. For the subtraction of the interaction with the enzymatic proteins; 100 μ moles of MGO were dissolved in 10 mL of deionized water to obtain “MGO w/o Enzymes”

model system. They were not subjected to enzyme addition but digestion procedure was also applied with the pH adjustment and the addition of simulating juices. On the other hand, model systems containing both sugar and protein during digestion was also tested in order to understand if glycation occurs or α -dicarbonyl compounds are formed under gastrointestinal conditions. For this purpose, lysine rich ovalbumin and cysteine rich gluten was chosen as target proteins. 2.5 g of both glucose and ovalbumin or gluten was weighed into flasks for “Glc-Ovalbumin” and “Glc-Gluten” model systems and they were subjected to digestion as in food samples.

2.2.3.3. Preparation of HMF-amino acid model systems

For the determination of the reactivity of HMF during digestion, model systems composed of HMF; “HMF”, HMF and lysine; “HMF-Lys” and HMF and cysteine; “HMF-Cys” were prepared. For the model systems, 10 μ moles of each reactant were dissolved in 10 mL of deionized water, and directly subjected to digestion process. To test the effect of higher amounts of cysteine on interactions between cysteine and HMF, model system containing 70 μ moles of cysteine with 10 μ moles of HMF; “HMF-7Cys” was subjected to *in vitro* digestion. For the subtraction of the interaction with the enzymatic proteins; 10 μ moles of HMF were dissolved in 10 mL of deionized water for “HMF w/o Enzymes” model system. They were not subjected to enzyme addition but the digestion procedure was also applied with the pH adjustment and the addition of simulating juices.

In addition, for the kinetic evaluation of the reactions of HMF with amino acids, model systems simulating both low and high moisture conditions were prepared. For the preparation of high moisture model systems, 8 μ moles of HMF and 30 μ moles of amino acids were dissolved in 1.5 mL of 0.05% benzoic acid solution. Since HMF content of foods is minor compared to amino acids and not to limit the possible reactions of HMF with amino acids, excess amount of amino acids was added to reaction medium. Benzoic acid was used in order to prevent microbial growth during the reaction period. The pH of these model systems was measured as 3.5. “HMF” model system without the addition of amino acid was prepared to monitor the self-degradation of HMF during storage. Arginine, cysteine and lysine were added to HMF solution to prepare “HMF-Arg”, “HMF-Cys” and “HMF-Lys” model systems, respectively.

Roasted coffee was selected to simulate the low moisture conditions to investigate the reactions of HMF with amino acids. Green coffee beans were roasted in an oven at 220°C for 10 min. This condition was selected since it cause to the formation of HMF at highest amounts in roasted coffee [174]. One gram of ground roasted coffee was put into a glass flask. A total of 8 μ moles arginine, cysteine or lysine was added to prepare “Coffee-Arg”, “Coffee-Cys” or “Coffee-Lys” model systems, respectively. Ground roasted coffee alone, as “Coffee” model system was also used to monitor the reactions of HMF with amino acids.

The flasks containing the mixtures of model systems were placed in water bath set at 5, 25 and 50°C to monitor the reactions. These conditions were selected to reveal the reactions of HMF with amino acids at cold and high storage conditions besides the body conditions. Changes in the concentrations of HMF were determined at the end of 1, 2, 3, 5 and 7 days.

2.2.3.4. Preparation of dicarbonyl-sulfur model systems

To determine the carbonyl scavenging ability of different sulphur containing sources under physiological conditions, model systems composed of GO and GO and wine were prepared. For the model systems of “GO” and “GO-Wine”, 10 μ moles of GO was dissolved in 30 mL of phosphate buffer saline (PBS) solution (pH=7.4) whereas 10 μ moles of GO was dissolved 25 mL of PBS with 5 mL of wine. To understand the GO scavenging ability of potassium metabisulfite, 20, 40 and 100 μ moles of potassium metabisulfite was added to 10 μ moles of GO solution to obtain “GO-PMS”, “GO-2PMS” and “GO-5PMS” model systems. Besides, 50 μ moles of potassium metabisulfite added to GO-wine solution to have “GO-Wine-PMS” model system. “GO-Cys” and “GO-Met” model systems composed of 10 μ moles of GO with cysteine or methionine was also prepared to test the effect of sulphur containing amino acids on GO under body conditions. All of the model systems were reacted in water bath at 37°C up to 2 hours. Aliquots from the model systems were collected initially, at the end of 1st and 2nd hour.

2.2.4 In vitro digestion

Digestion fluids simulating the saliva, gastric juice and duodenal juice were used to mimic the conditions of gastrointestinal tract. Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated duodenal fluid (SDF) were prepared according to the recipe given in Table 2.2.

Table 2.1. Preparation of simulated gastrointestinal fluids

	SSF		SGF		SDF	
	pH=7.0		pH=3.0		pH=7.0	
	Volume (mL)	stock (g/L)	Volume (mL)	Stock (g/L)	Volume (mL)	Stock (g/L)
KCl	10	46.7	28	46.7	5.4	46.7
KH ₂ PO ₄	20	68	0.9	68	0.8	68
NaHCO ₃	4	84	6.5	168	42.5	42.5
NaCl	1	120	10	120	8	120
MgCl ₂ (H ₂ O) ₆	1	30	2	30	1.1	30

The final volume of the solution was made up to 500 mL with distilled water and frozen at -20°C.

In vitro digestion procedure was adapted from procedure reported by Papillo et al [175]. 5 grams of dry ground food or 10 mL of the model system were transferred to a glass flask with screw cap. For food samples, 5 mL of SSF was added and the flask was shaken for 2 min to simulate the oral passage. Liquid model system samples were not exposed to the oral phase, thus they were put directly into the gastric phase. After 5 mL of pepsin solution (12.5 mg/ml in 0.1 M HCl) and 10 mL of SGF were added, the mixture was adjusted to pH 2.0. Then, the acidified mixture was incubated at 37°C by shaking for 2 h at an agitation speed of 60 strokes per min to simulate the gastric phase. Bile salts were dissolved in the SDF solution to a concentration of 10 mg/mL. The pH was adjusted to 7.5 after the gastric phase. After that, 20 mL of the mixture of SDF with bile salts and 5 mL of pancreatin solution (10 mg/ml in water) were added to the flask. The mixture was incubated at 37°C by shaking for 2 h at an agitation speed of 60 strokes per min to simulate the duodenal phase. The colon phase was simulated by the addition of bacterial enzymes of the flora found in colon. It is supposed to be the consecutive hydrolysis of proteins and polysaccharides occur in the sample during colon phase. For this, 5 mL of protease solution (1mg/ml, pH 8.0) was added, and the mixture was incubated at 37°C by shaking for 1 h. Then, 150 µL of Viscozyme L was added, and the mixture was incubated at 37°C by shaking for 16 h at an agitation speed of 30 strokes per min. Aliquots of samples were withdrawn from the flask at the end of simulated gastric, duodenal and colon phases for the analyses of acrylamide, its precursors and reaction products. All samples were

digested with three parallels as described above.

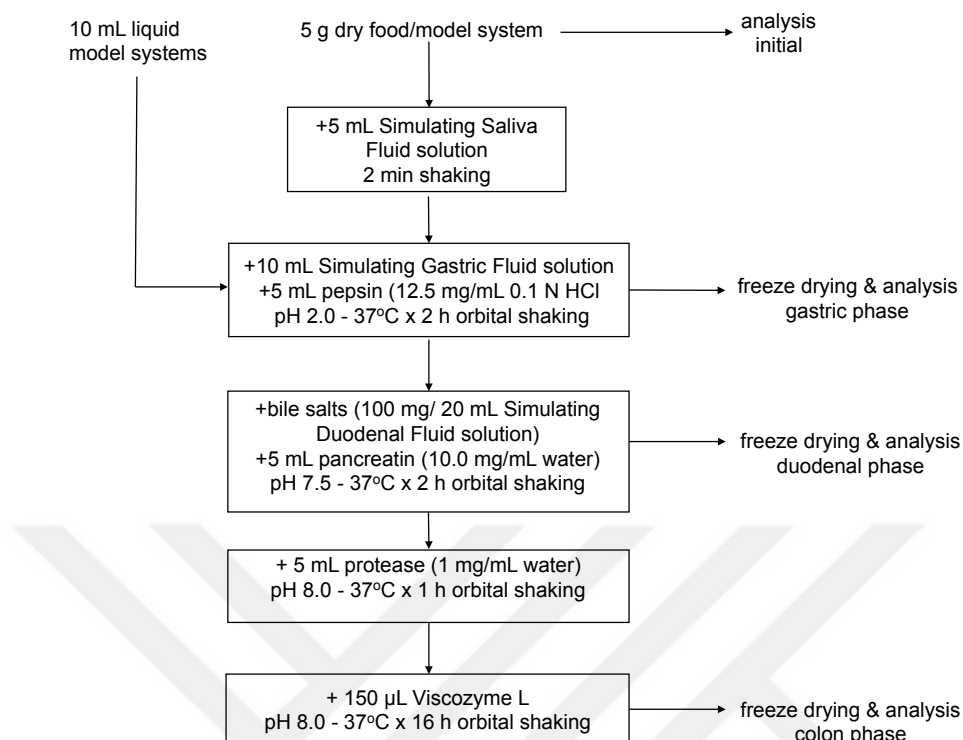


Figure 2.1. Schematic representation of the steps of simulated *in vitro* gastrointestinal digestion process

2.2.5 Analysis of acrylamide by LC-MS/MS

Aliquots of the digests withdrawn from the samples of model systems were centrifuged at 11180 g for 5 min. The supernatant was filtered through 0.45 µm filter into an autosampler vial, and analysed for acrylamide using LC-MS/MS. Aliquots from food samples were transferred to petri dishes and dried in an oven at 37°C. Extraction of dried powders and analysis of acrylamide was carried out by using LC-MS/MS according to a published procedure by Hamzalioglu and Gökmen [15].

2.2.6 Analysis of α -dicarbonyl compounds in the digests by HPLC-DAD

Aliquots of the digests withdrawn from the samples of model systems were centrifuged at 11180 g for 5 min. Aliquots of the digests withdrawn from biscuits were lyophilized. Extraction of dried powders, derivatization prior to analysis and analysis of extracts by using HPLC-DAD were done according to procedure published by Kocadagli and Gökmen [176].

2.2.7 Analysis of HMF in the digests by HPLC-PDA

Aliquots of the digests withdrawn from the samples of model systems were centrifuged at 11180 g for 5 min. Aliquots of the digests withdrawn from biscuits were lyophilized. Extraction of dried powders and analysis of these extracts by using HPLC-PDA were carried out according to procedure published by Kocadagli et al [174].

2.2.8 Analysis of furosine in the digests by HPLC

Analysis of furosine in the digests of “GLC-Ovalbumin” and “GLC-Gluten” model systems composed of glucose and ovalbumin or gluten was performed by using HPLC according to Gökmen et al [177]. Prior to analysis, hydrolysis procedure was carried out.

2.2.9 Analysis of free amino acids in wine and broccoli digests by UPLC-ESI-MS/MS

For the analysis of all free amino acids wine was diluted with mixture of water and acetonitrile (50:50 v/v) and centrifuged at $7000 \times g$ for 3 min. 1 g of broccoli digests were triple extracted with 10, 5 and 5 mL and centrifuged at $7000 \times g$ for 3 min each time. After supernatants were collected at the end of each extraction, 1 mL aliquot was mixed with 1 mL mixture of water and acetonitrile (50:50 v/v) and centrifuged at $7000 \times g$ for 3 min. All the extracts were passed through a syringe filter and collected into an autosampler vial. By using UPLC-ESI-MS/MS, analysis of amino acids was performed according to procedure published by Kocadagli et al [178].

2.2.10 Analysis of glucoraphanin in the digests by HPLC-MS

40 mg of lyophilized broccoli powder or digests was put into flasks was diluted with deionized water (1:9) in an autosampler vial. Extraction and HPLC-MS analysis of glucosinolates were done according to the procedure recently published [179]. Compounds were identified using their nominal mass and by comparing data with the published in that paper. The SIM ion of $[M + H]^-$ was 436 for glucoraphanin (GR) and it was also used for quantitation.

2.2.11 Analysis of reaction products, precursors, intermediates and adducts in the digests by HRMS

Aliquots of the digests withdrawn from model systems and food samples were freeze-dried and dried powder (200 mg) was extracted with 2 mL of water by

vortexing for 2 min. Then, the extract was centrifuged at 11180 $\times g$ for 5 min. After that, supernatants of extracts were passed through a 0.45 μm filter into an auto sampler vial prior to analysis. HRMS was used to confirm the interactions of amino acids or proteins with HMF in the digests of biscuits. The scan analysis were performed according to procedure published by Hamzalioglu and Gökmen [15]. The corresponding ions were extracted from the total ion chromatograms to confirm the presence of the reaction precursors, intermediates and adducts of amino acids or proteins with acrylamide, dicarbonyl compounds or HMF in the digests. In order to confirm the molecular structures of adducts, their observed masses were compared with corresponding theoretical masses. The difference between observed and theoretical masses were calculated in ppm and given as Δ .

2.2.12 Measurement of color in biscuits

Color measurements (CIE $L^*a^*b^*$) were acquired by using a computer vision-based image analysis technique as described previously [180]. The surface color of biscuits was given as average L^* (lightness), a^* (redness), and b^* (yellowness) values. The colors of all three biscuit replicates were individually determined, and data were reported as the average $\pm$ standard deviation.

2.2.13 Statistical analysis

The data were subjected to analysis of variance (one-way ANOVA) by using SPSS 17.0 statistical package. Duncan test was applied to the data in order to evaluate the statistical significant differences between mean values. Difference between results were found to be significant when $p < 0.05$.

2.3 RESULTS AND DISCUSSION

2.3.1 Investigation of the reactions of acrylamide during *in vitro* digestion of thermally processed foods

Bakery and fried potato products as main sources of dietary acrylamide were selected as typical examples of thermally processed foods. A total of 4 commercial biscuits (2 sweet and 2 non-sweet), one laboratory made biscuit, 2 potato chips and one potato fry were used for *in vitro* multistep enzymatic digestion test. Initially, acrylamide concentrations were found as 232 ± 4 and 348 ± 18 ng/g for non-sweet biscuits, 270 ± 5 and 318 ± 2 ng/g for sweet biscuits, and 173 ± 3 ng/g for laboratory made sweet biscuit. Acrylamide concentrations of potato chips and potato fry samples were found as 178 ± 8 , 239 ± 18 and 263 ± 18 ng/g, respectively.

Table 2.2 gives the amounts of acrylamide remaining in the digests of biscuit samples after gastric, duodenal and colon phases of the digestion process. There were significant reductions in the amounts of acrylamide in the digests of all sweet and non-sweet biscuits at the end of gastric and duodenal phases. Taking the entire enzymatic digestion process into account, acrylamide reduction ratio was found to range between 49.2% and 73.4% for biscuit samples. The reduction was significantly higher in the gastric and duodenal phases than in the colon phase. For biscuits, the ratio of acrylamide reduction ranged between 17.4% and 49.9% in the gastric phase, and between 23.9% and 58.1% in the duodenal phase.

Gastric phase was the simulation of stomach where pepsin hydrolyzed proteins into smaller peptides or amino acids at low pH conditions. Duodenal phase included the addition of bile salts capable of promoting digestion and absorption of lipids together with pancreatin having the activities of amylase, lipase and trypsin. In the colon phase, proteolytic enzymes of microbial flora of colon continued to favor the hydrolysis of proteins and peptides. So, the simulated digestion process created a pool of amino acids that might be interacting with acrylamide. It has been previously reported that Michael type addition of amino acids to acrylamide, which is a potential way to decrease acrylamide content of foods, can take place under certain conditions [181, 182]. Due to its highly electrophilic nature, each molecule of amino acid could form adducts with one or two molecules of acrylamide [26].

Table 2.2. Changes in the amount of acrylamide in the digests of biscuits during in vitro digestion

	Acrylamide, μmol			
	<i>initial</i>	<i>gastric phase</i>	<i>duodenal phase</i>	<i>colon phase</i>
<i>Non-sweet</i>				
sample 1	16.32 $\pm$ 0.40 ^a	10.90 $\pm$ 0.10 ^b	6.68 $\pm$ 1.39 ^c	5.73 $\pm$ 1.09 ^c
sample 2	24.48 $\pm$ 1.79 ^a	12.27 $\pm$ 0.45 ^b	7.95 $\pm$ 0.50 ^c	7.60 $\pm$ 0.56 ^c
<i>Sweet</i>				
sample 1	18.96 $\pm$ 0.45 ^a	13.33 $\pm$ 2.44 ^b	5.59 $\pm$ 0.05 ^c	5.24 $\pm$ 0.35 ^c
sample 2	22.33 $\pm$ 0.15 ^a	11.57 $\pm$ 2.44 ^b	7.28 $\pm$ 0.25 ^c	5.94 $\pm$ 0.35 ^c
sample 3*	12.13 $\pm$ 0.25 ^a	10.02 $\pm$ 0.25 ^b	7.63 $\pm$ 0.45 ^c	6.16 $\pm$ 0.25 ^d

* Laboratory made biscuit sample.

a–d Values marked with different letters in each row are significantly different (P < 0.05).

Three model systems, namely acrylamide “ACR”, acrylamide-lysine “ACR-Lys”, and acrylamide-cysteine “ACR-Cys” were used to understand the mechanism of acrylamide reduction. Table 2.3 gives the amounts of acrylamide remaining in the digests of these model systems after gastric, duodenal and colon phases.

Table 2.3. Changes in acrylamide content of different model systems during *in vitro* digestion

	Acrylamide, μmol			
	<i>initial</i>	<i>gastric phase</i>	<i>duodenal phase</i>	<i>colon phase</i>
ACR	10.25 $\pm$ 0.56 ^a	9.29 $\pm$ 0.19 ^{a,b}	8.10 $\pm$ 0.07 ^{b,c}	7.79 $\pm$ 0.05 ^c
<i>Binary Models</i>				
ACR-Lys	10.25 $\pm$ 0.56 ^a	8.22 $\pm$ 0.01 ^{b,c}	8.27 $\pm$ 1.25 ^{b,c}	8.36 $\pm$ 0.43 ^{b,c}
ACR-Cys	10.25 $\pm$ 0.56 ^a	8.31 $\pm$ 0.13 ^{b,c}	5.32 $\pm$ 0.77 ^d	4.02 $\pm$ 0.41 ^e

The values were expressed as $\mu\text{mol}/\text{model system}$.

a–e Values marked with different letters in each row are significantly different (P < 0.05).

At the end of gastric phase, there were slight but statistically significant ($p < 0.05$) reductions in the amounts of acrylamide for both “ACR-Cys” and “ACR-Lys” model systems. The amount of acrylamide remained relatively stable after duodenal and colon phases in the digest of the “ACR-Lys” model system. However, it tended to decrease significantly ($p < 0.05$) after duodenal and colon phases in the digest of the “ACR-Cys” model system. These findings indicated the potential of acrylamide to react with nucleophilic groups ($-\text{SH}$, $-\text{NH}_2$) of amino acids side chains under the stated digestion conditions. Hidalgo et al [24, 26] reported a rapid reduction of acrylamide upon heating in the presence of N-acetyl-cysteine or lysine as a consequence of the Michael type addition the nucleophilic groups to the carbon–carbon double bond of acrylamide. Cysteine owing to its highly nucleophilic $-\text{SH}$ group was thought to be more favourable in reacting towards acrylamide. [25].

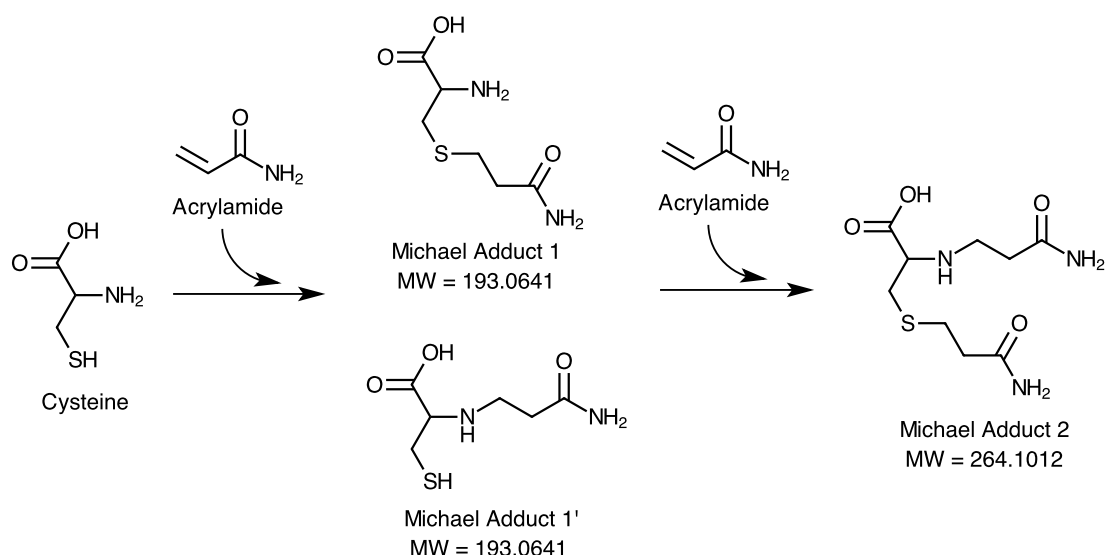


Figure 2.2. Proposed mechanism for the reduction of acrylamide during in vitro digestion through the formation of Michael adducts with cysteine

Figure 2.2 shows the proposed mechanism of acrylamide elimination through Michael type addition of cysteine during digestion process. According to this mechanism, cysteine may react with one or two moles of acrylamide from both nucleophilic groups (-SH or NH₂) forming Michael-adducts 1, 1' or 2, respectively. Scan HRMS analyses of the digests of “ACR-Cys” model system confirmed the formation of these adducts with very high mass accuracy ($\Delta < 2$ ppm) under the simulated digestion conditions (Figure A1.a and Figure A1.b). Only the formation of Michael adduct 1 was observed in the digests of “ACR-Lys” model system under the same conditions. These results suggest that cysteine released through proteolytic activity of enzymes in the gastrointestinal tract might be responsible for the elimination of acrylamide during digestion.

Table 2.4 gives the amounts of acrylamide remaining in the digests of fried potato samples after gastric, duodenal and colon phases of the digestion process. Contrary to biscuits, the amounts of acrylamide increased significantly ($p < 0.05$) during gastric digestion of fried potatoes. At the end of gastric phase, acrylamide levels increased 3.95, 1.20 and 1.45 times in the digests of potato fry and potato chips, respectively. Acrylamide levels tended to decrease significantly ($p < 0.05$) in the digests of fried potato samples after duodenal and colon phases. The ratio of acrylamide reduction ranged between 78.2% and 96.8% in the duodenal phase, and between 48.3% and 90.2% in the colon phase.

Table 2.4. Changes in the amount of acrylamide in the digests of fried potatoes during *in vitro* digestion

	Acrylamide, μmol			
	<i>initial</i>	<i>gastric phase</i>	<i>duodenal phase</i>	<i>colon phase</i>
<i>Potato fry</i>				
sample 1	18.47 $\pm$ 1.74 ^a	73.02 $\pm$ 6.17 ^b	2.32 $\pm$ 0.40 ^c	0.34 $\pm$ 0.18 ^d
<i>Potato chips</i>				
sample 1	12.49 $\pm$ 0.75 ^a	14.98 $\pm$ 1.29 ^b	1.20 $\pm$ 0.70 ^c	0.62 $\pm$ 0.10 ^c
sample 2	16.81 $\pm$ 1.79 ^a	24.41 $\pm$ 0.10 ^b	5.31 $\pm$ 1.04 ^c	0.52 $\pm$ 0.03 ^d

The values were expressed as $\mu\text{mol}/5\text{ g}$ potato sample.

a–d Values marked with different letters in each row are significantly different ($P < 0.05$).

Eriksson et al [183] determined the effects of pH and different enzymes on the extraction of acrylamide from foods. According to their results, extraction yield of acrylamide from foods at pH values ranging from 2.0 to 7.5, or with pepsin enzyme was similar to the yield obtained with water. So, increase of acrylamide levels in the digests of fried potato products during the gastric digestion cannot be attributed to its increased extractability under acidic conditions. It is a fact that asparagine and reducing sugars are the main precursors of acrylamide [184]. According to its formation, Schiff base, decarboxylated Schiff base and 3-aminopropionamide form as critical intermediates upon heating a mixture containing asparagine and glucose [19, 185, 186]. Since raw potato is relatively rich in asparagine and reducing sugars, frying may form high quantities of above-mentioned intermediates together with acrylamide in fried potatoes. Therefore, these intermediates may be considered as potential precursors of acrylamide during the gastric digestion phase. To understand better, model systems containing asparagine and glucose namely “Asn-Glc” were prepared and heated at 180°C for 10 min in an oil bath. They were then subjected to *in vitro* digestion. As in potato products, there was 41.3% increase in acrylamide content of the digests of “Asn-Glc” model systems at the end of gastric phase. Acrylamide content decreased during the following steps with the ratio of 86.4% and %95.7, respectively. Scan HRMS analyses confirmed the presence of Schiff base formed between asparagine and glucose initially in both fried potato samples and model systems with very high mass accuracy ($\Delta < 1\text{ ppm}$) (Figure A1.c and Figure A1.d). In the gastric phase, decrease of the signal response of parent $[M+H]^+$ ion of Schiff

base was compatible with the increase of acrylamide level. More than 90% of the Schiff base present initially in the fried potatoes disappeared in their digests just after the gastric phase. The results suggest that intermediates like Schiff base accumulated in fried potatoes during frying are converted to acrylamide under gastric conditions as proposed in Figure 2.3.

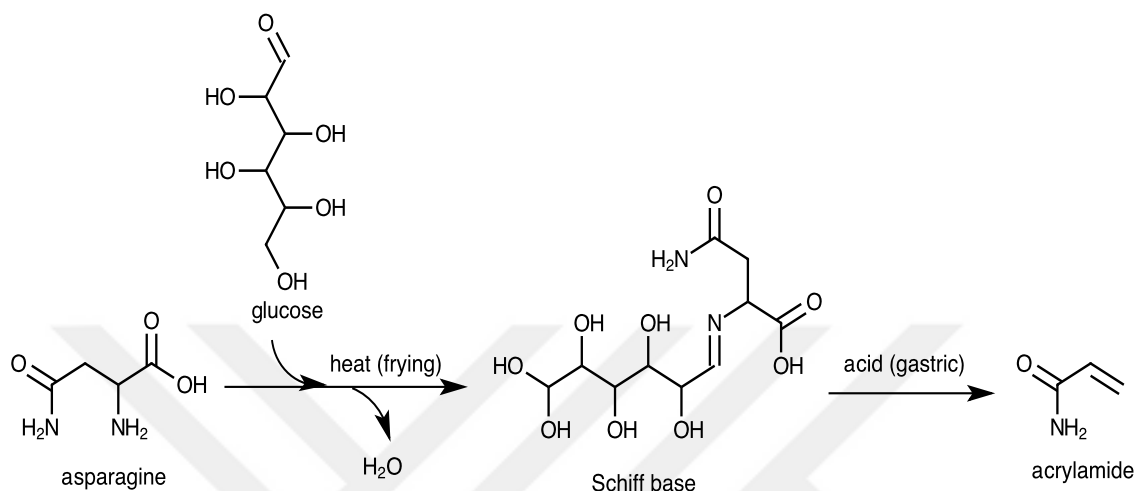


Figure 2.3. Proposed mechanism for the formation of acrylamide during gastric digestion from the precursors existing in fried potato products

2.3.2 Investigation of the reactions of α -dicarbonyl compounds during *in vitro* digestion of biscuits

It is known that biscuits are one of the major dietary sources for α -dicarbonyl compounds since they contain varying amounts of high fructose corn syrup. For this reason, commercial baby, regular and twice-baked biscuits were selected as typical samples to be used for *in vitro* digestion. Initial concentrations of MGO were 19.44 ± 0.93 , 31.35 ± 0.39 and 39.64 ± 4.57 $\mu\text{g/g}$ in baby, twice-baked and regular biscuits, respectively. The biscuit samples were found contain significantly higher amounts of 3-DG. Initial concentrations of 3-DG were 147.57 ± 0.84 , 73.68 ± 7.35 and 97.52 ± 10.93 $\mu\text{g/g}$ in baby, twice baked and regular biscuits, respectively. Trace levels of dimethylglyoxal and GO were also detected in all biscuit samples.

Biscuits alone (control) or added with lysine, cysteine and ovalbumin were digested separately to understand the interactions of dicarbonyl compounds towards free $-\text{NH}_2$ and $-\text{SH}$ groups of amino acids and proteins under gastrointestinal conditions. Table 2.5 and Table 2.6 gives the concentrations of MGO and 3-DG remaining in the digested biscuits after gastric, duodenal and colon phases of the digestion process. A significant decrease was observed in 3-

DG contents of biscuits at the end of gastric phase and this suggested that it might be converted to HMF under gastric conditions. This will be discussed in following section (Section 2.3.4) in detail. However, gastric conditions did not cause any significant change in MGO concentrations in all biscuits. Decrease in MGO content of biscuits started with the duodenal phase and reached to 75%, 50% and 66% in regular, twice baked and baby biscuits after the colon phase, respectively. Similarly, 3-DG contents of all biscuits significantly decreased within the progress of digestion. Overall disappearance in 3-DG of digested biscuits at the end of digestion was found to be comparably lower than MGO contents of biscuits.

As it is well known, dicarbonyl compounds are capable of reacting with arginine, lysine and cysteine residues in proteins [41]. It was also reported that reactive MGO is trapped by the guanidino compound creatine under physiological conditions [171]. Since proteins are hydrolysed with the action of proteolytic enzymes under the conditions of gastric, duodenal and colon phases, it is conceivable that the decrease in dicarbonyl content of biscuits is due to its interactions with free amino acids accumulating in the digestion medium as a result of protein hydrolysis.

There were small, but statistically significant reductions in the concentrations of MGO and 3-DG during the digestion of biscuits with added lysine. Addition of ovalbumin to biscuit caused higher reductions in the concentrations of MGO and 3-DG in comparison to the addition of lysine.

Table 2.5. Changes in the contents of MGO in different biscuits during the stages of *in vitro* digestion

	MGO, µg/g			
	<i>initial</i>	<i>gastric phase</i>	<i>duodenal phase</i>	<i>colon phase</i>
<i>Regular Biscuits</i>				
control	39.64±4.57 ^a	33.39±0.82 ^a	12.87±3.67 ^b	9.77±0.11 ^{c,d}
+ Lysine	42.45±1.82 ^a	32.14±0.85 ^a	11.36±0.16 ^c	7.21±2.66 ^{c,d}
+ Ovalbumin	37.50±2.57 ^a	9.21±0.75 ^{c,d}	7.19±2.59 ^{c,d}	5.34±0.38 ^{c,d}
+ Cysteine	39.84±1.96 ^a	ND ^e	ND ^e	ND ^e
<i>Twice Baked Biscuits</i>				
control	31.35±0.39 ^a	31.58±2.69 ^a	22.14±3.31 ^b	15.65±3.48 ^c
+ Lysine	31.31±1.34 ^a	27.47±2.28 ^a	12.38±3.15 ^d	9.67±1.38 ^{d,e}
+ Ovalbumin	32.01±2.20 ^a	9.77±1.16 ^{d,e}	6.16±1.23 ^e	5.61±2.03 ^e
+Cysteine	31.51±1.55 ^a	3.04±2.39 ^e	ND ^f	ND ^f
<i>Baby Biscuits</i>				
control	19.44±0.93 ^a	22.38±3.13 ^a	13.39±0.31 ^b	6.53±0.93 ^c
+ Lysine	19.90±0.85 ^a	19.19±2.74 ^a	12.75±0.66 ^b	7.28±2.36 ^d
+ Ovalbumin	19.34±1.33 ^a	6.57±1.49 ^d	4.81±0.41 ^{d,e}	4.38±0.36 ^{d,e}
+Cysteine	19.54±0.96 ^a	1.38±1.95 ^{e,f}	ND ^f	ND ^f

^{a–f} Values marked with different letters for each types of biscuits are significantly different (P < 0.05). ND: Not detected.

Table 2.6. Changes in the contents of 3-DG in different model systems during the stages of *in vitro* digestion

	3-DG, µg/g			
	<i>initial</i>	<i>gastric phase</i>	<i>duodenal phase</i>	<i>colon phase</i>
<i>Regular Biscuits</i>				
control	97.52±10.93 ^a	68.48±4.53 ^b	58.24±5.88 ^{b,c}	47.44±5.54 ^{c,d}
+ Lysine	104.20±4.47 ^a	60.32±3.98 ^{b,c}	51.86±5.23 ^{c,d}	37.95±4.43 ^d
+ Ovalbumin	92.48±6.35 ^a	22.33±0.32 ^e	13.48±0.66 ^{e,f}	23.62±0.32 ^e
+Cysteine	98.01±4.83 ^a	ND ^f	ND ^f	ND ^f
<i>Twice Baked Biscuits</i>				
control	73.68±7.35 ^a	62.72±1.81 ^b	59.52±1.36 ^b	56.16±0.23 ^b
+ Lysine	78.09±3.35 ^a	62.88±2.49 ^b	62.44±1.43 ^b	31.64±0.14 ^c
+ Ovalbumin	70.53±4.84 ^a	24.43±0.61 ^c	13.68±1.20 ^d	13.53±0.09 ^d
+Cysteine	74.05±3.65 ^a	ND ^e	ND ^e	ND ^e
<i>Baby Biscuits</i>				
control	147.60±0.84 ^a	130.08±2.13 ^b	124.0±2.49 ^b	60.64±2.94 ^{c,d}
+ Lysine	145.51±6.24 ^a	129.6±6.79 ^b	108.4±2.19 ^e	56.67±2.76 ^c
+ Ovalbumin	150.60±8.48 ^a	66.65±3.85 ^d	15.02±0.79 ^f	16.06±1.27 ^f
+Cysteine	148.31±7.30 ^a	1.38±1.95 ^g	ND ^g	ND ^g

^{a–g} Values marked with different letters for each types of biscuits are significantly different (P < 0.05). ND: Not detected

Interestingly, no MGO and 3-DG was found in the digests of biscuits added with cysteine after duodenal phase. These results confirmed potential interactions of free amino acids and proteins with dicarbonyl compounds leading to significant decreases in their concentrations during *in vitro* digestion process. Cysteine having highly reactive –SH group was very effective towards dicarbonyl compounds.

To understand better the interaction mechanism, model systems “MGO” composed of MGO, “MGO-Lys” composed of MGO and lysine and “MGO-Cys” composed of MGO and cysteine were prepared and subjected to simulated digestion process. To exclude the interaction between MGO and the side chains of

enzymes as a source of proteins during gastrointestinal digestion, model system containing MGO alone was also subjected to digestion in the absence of enzymes “MGO w/o Enzyme”. Table 2.7 gives the amounts of MGO remaining in the digests of these model systems after gastric, duodenal and colon phases. Among all model systems, initial amount of MGO was significantly lower in the “MGO-Cys” model system. This indicated that MGO interacted very effectively with cysteine. Gastric conditions did not cause any significant change in the amounts of MGO in model systems. Significant reduction of MGO started with the increasing pH of intestinal conditions. With the effect of pH and digestive fluids, MGO decomposed in the absence of enzymes during duodenal and colon phases. In a previous study, MGO was found to be stable under gastric pH (2.0) compared to intestinal pH (7.5) due to less reactivity with proteins at pH 2.0 [170].

Moreover, the amount of MGO in “MGO” model system was relatively lower than that of “MGO w/o Enzymes” after duodenal and colon phases. It is known that, dicarbonyl compounds bind and modify some proteins including enzymes [187]. Previous studies reported that dicarbonyl compounds react with digestive enzymes during *in vitro* simulated gastro duodenal digestion, so that decreasing amounts of free dicarbonyl compounds could be explained by their reaction with pepsin and pancreatin to produce carbonylated proteins [188]. Accordingly, this difference might be originating from the interactions of MGO with arginine, lysine and cysteine residues of enzymes under basic intestinal conditions. It was reported that addition of protein lead to more interactions with MGO besides its interaction with enzymatic proteins [170]. In another study, it was reported that MGO prefers reacting with the digestive enzymes to a greater extent if it is digested alone, while its reaction with digestive enzymes is partially inhibited by other components when it is together with food matrix [169].

Table 2.7. Change in the contents of MGO in different model systems during the stages of in vitro digestion

	MGO, μ mole			
	<i>initial</i>	<i>gastric phase</i>	<i>duodenal phase</i>	<i>colon phase</i>
MGO w/o Enzyme	97.40 $\pm$ 3.68 ^a	96.56 $\pm$ 0.96 ^a	69.34 $\pm$ 3.83 ^b	47.25 $\pm$ 5.75 ^c
MGO	100.00 $\pm$ 3.68 ^a	110.24 $\pm$ 4.98 ^{a,d}	49.03 $\pm$ 5.75 ^c	16.34 $\pm$ 1.92 ^e
MGO-Lys	99.80 $\pm$ 0.29 ^a	102.52 $\pm$ 6.00 ^a	42.93 $\pm$ 0.71 ^{c,f}	37.88 $\pm$ 1.43 ^f
MGO-Cys	46.05 $\pm$ 1.69 ^c	38.06 $\pm$ 2.11 ^f	28.71 $\pm$ 2.30 ^g	6.33 $\pm$ 0.37 ^h

a–h Values marked with different letters are significantly different ($P < 0.05$).

To confirm the reaction between sulfhydryl or amino groups of amino acids with dicarbonyl compounds, digests of both model systems and biscuits were analysed by HRMS. Figure 2.4 illustrates the chemical structures of lysine and cysteine adducts of MGO as confirmed with very high mass accuracy ($\Delta < 2$ ppm) by scan HRMS analyses in the digests of model systems (Figure A2.a). Using the digests of biscuit samples, the chemical structures of cysteine, lysine, arginine and histidine adducts of MGO and 3-DG could also be confirmed with very high mass accuracy (Figure A2.b and Figure A2.c).

Signal responses of these adducts remarkably increased towards the end of digestion (Figure A2.d, Figure A2.e and Figure A2.f).

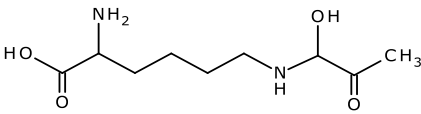
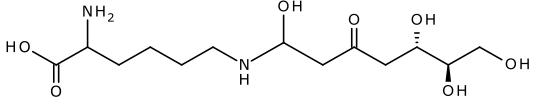
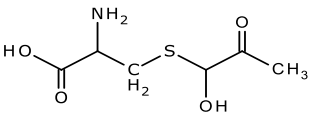
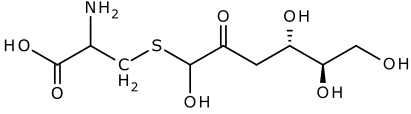
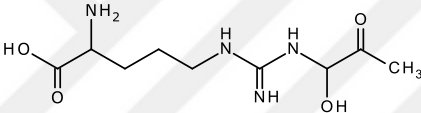
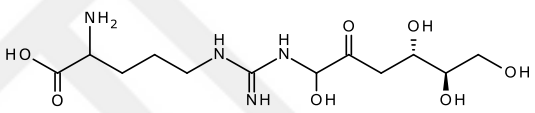
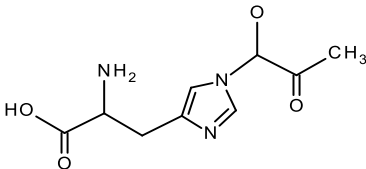
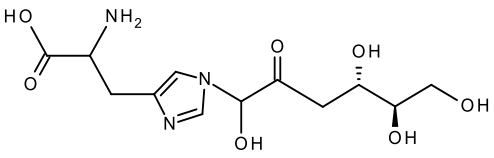
	MGO	3-DG
Lysine	 $C_9H_{18}N_2O_4$ $m/z: 219.1339$ $(\Delta = 0.0368 \text{ ppm})$	 $C_{12}H_{24}N_2O_7$ $m/z: 309.1656$ $(\Delta = 0.2681 \text{ ppm})$
Cysteine	 $C_6H_{11}O_4NS$ $m/z: 194.0481$ $(\Delta = 0.3232 \text{ ppm})$	 $C_9H_{17}O_7NS$ $m/z: 284.0798$ $(\Delta = 0.1619 \text{ ppm})$
Arginine	 $C_9H_{18}N_4O_4$ $m/z: 247.1400$ $(\Delta = -0.5178 \text{ ppm})$	 $C_{12}H_{24}O_7N_4$ $m/z: 337.1717$ $(\Delta = 0.2950 \text{ ppm})$
Histidine	 $C_9H_{13}N_3O_4$ $m/z: 228.0978$ $(\Delta = 0.2126 \text{ ppm})$	 $C_{12}H_{19}N_3O_7$ $m/z: 318.1295$ $(\Delta = -0.2676 \text{ ppm})$

Figure 2.4. Proposed chemical structures of lysine and cysteine adducts of MGO and 3-DG as confirmed by scan HRMS analysis of the digests of model systems and biscuits

Likewise, to test the formation of dicarbonyl compounds and their interactions with the residual groups of proteins during digestion process, model systems composed of glucose; gluten and ovalbumin were also subjected to digestion. As given in Table 2.8, results confirmed that 3-DG was formed predominantly in these model systems. Besides, no significant change was observed in 3-DG content of

the “Glc” model system during digestion. On the contrary, after gastric phase, significant decrease was observed when protein was present. At the end of digestion, 3-DG content decreased 81% in the “Glc-Gluten” model system whereas it decreased nearly completely in the “Glc-Ovalbumin” model system. Since ovalbumin is highly rich in lysine compared to gluten, 3-DG might find more residual free NH₂ groups to interact in the “Glc-Ovalbumin” model system.

Table 2.8. Changes in 3-DG and furosine content of different model systems during the stages of *in vitro* digestion

	<i>initial</i>	<i>gastric phase</i>	<i>duodenal phase</i>	<i>colon phase</i>
3-DG (nmol/mg protein)				
Glc	241.6±1.2 ^a	248.1±11.2 ^a	275.7±11.9 ^a	277.8±0.8 ^a
Glc- Ovalbumin	241.6±1.2 ^a	22.4±1.8 ^b	19.2±0.7 ^b	3.1±0.2 ^c
Glc-Gluten	241.9±0.6 ^a	62.2±1.1 ^b	48.9±3.1 ^c	46.7±0.6 ^d
Furosine (nmol/mg protein)				
Glc	ND	ND	ND	ND
Glc- Ovalbumin	ND ^a	0.8±0.1 ^b	30.4±2.0 ^c	23.2±1.5 ^d
Glc-Gluten	14.3±1.0 ^a	17.2±0.8 ^b	78.9±6.3 ^c	56.1±8.6 ^d

^{a-d} Values marked with different letters are significantly different (P < 0.05). ND: not detected

Formation of furosine was also monitored in these model systems in order to understand if decreased content of dicarbonyl compounds was a result of protein glycation under the gastrointestinal conditions. Considering the results given in Table 2.7, initial furosine content was found to be 14.3 nmol in the “Glc-Gluten” model system whereas no furosine was detected in the “Glc-Ovalbumin” model system initially. Furosine contents of both “Glc-Ovalbumin” and “Glc-Gluten” model systems significantly increased during gastric and duodenal phases. Furosine, acid derivative of N-ε-fructoselysine, is used to evaluate the extent of early stage of Maillard reaction. Glucosamine is formed primarily as a result of interaction of glucose with lysine, which then constitutes to N-ε-fructoselysine

[189]. Increase in furosine content of the model systems at the end of duodenal phase indicated that intestinal conditions favoured protein glycation. This might be due to higher accessibility to amino groups as a result of proteolytic activity besides alkaline pH. On the other hand, observed decrease in furosine content during colon stage, might also be associated with the progress of the glycation reaction in forward direction under colonic conditions.

2.3.3 Investigation of GO-scavenging effects of various sulphur compounds, wine and broccoli extracts under simulated physiological conditions

Carbonyl scavenging potential has been proposed as a key mechanism for the mitigation of glycation. For this reason, several phytochemicals were tested for their carbonyl scavenging activities so far and these phytochemicals were mostly tested in model systems containing dicarbonyl compounds under physiological conditions (pH 7.4 and 37°C) [51, 53, 190, 191]. Besides the published literature about the effect of phenolic compounds on carbonyl scavenging, information about the effect of some of the sulphur compounds under physiological conditions is lack. From the results discussed above, we claimed that cysteine, as a sulphur containing amino acid, could bind carbonyl group during simulated *in vitro* gastrointestinal digestion. Sulphur-containing compounds are consumed with so many foods and might be able to scavenge dicarbonyl compounds under simulated physiological conditions, as well. Bisulphites are one of the additives used to trap undesirable carbonyl compounds in brewery [192]. Besides, since intake of cruciferous vegetables is linked with a decreased risk of several cancers [193-196], there is an increased interest on their consumption. And there is still no information about if bioaccessible part of these vegetables could scavenge dicarbonyl compounds in the body. This study aimed to investigate the GO scavenging abilities of different sulphur containing sources under physiological conditions.

To test this, different sources were reacted with GO at blood pH up to 2 hours. Firstly, PMS was tested. For this purpose, GO was reacted with different concentrations of PMS. The decrease in GO content of “GO” model system provided by its self-degradation was found to be 0.99 % under these conditions. However, decrease in GO concentration significantly changed when it was together with half amount of bisulphite in “GO-0.5PMS” model system at the end of

2 hours reaction time ($p < 0.05$). Initial and remaining GO concentrations in different model systems are given in Table 2.9.

Table 2.9. Initial and remaining amounts of GO in different model systems after reacted at 37°C, 7.4 pH for 2 hours

	GO (μmole)	
	<i>initial</i>	<i>2 hours</i>
GO	10.88 $\pm$ 0.15 ^a	10.77 $\pm$ 0.00 ^a
GO-0.5PMS	10.77 $\pm$ 0.25 ^a	9.64 $\pm$ 0.10 ^b
GO-PMS	10.77 $\pm$ 0.29 ^a	7.94 $\pm$ 0.38 ^d
GO-2.5PMS	10.76 $\pm$ 0.22 ^a	6.12 $\pm$ 0.06 ^g
GO-Wine	10.77 $\pm$ 0.16 ^a	8.83 $\pm$ 0.44 ^c
GO-Wine-PMS	10.77 $\pm$ 0.21 ^a	6.59 $\pm$ 0.33 ^f
GO-Cys	10.78 $\pm$ 0.04 ^a	7.04 $\pm$ 0.02 ^e
GO-Met	10.78 $\pm$ 0.14 ^a	10.66 $\pm$ 0.07 ^a

^{a-g} Values marked with different letters are significantly different ($p < 0.05$)

Elimination of GO was found to be 10.51% and this ratio significantly increased by increasing PMS concentration ($p < 0.05$). Elimination of GO in “GO-PMS” model system composed of equimolar amounts of GO and PMS was observed as 26.24% and 2.5 fold increase of PMS content led to 43.18% elimination of GO in “GO-2.5PMS” model system. Decrease in GO content strongly correlated with increasing concentrations of PMS ($r=0.974$). In a recent study, scavenging ability of polysulphides including sodium disulphide, sodium trisulphide and disodium tetrasulphide against neuronal carbonyl stress induced by MGO was investigated. They found that polysulphides could scavenge MGO and thus protect the formation of MGO-modified proteins in brain cells [197]. Additionally, it was found that sodium hydrogen sulphide could scavenge MGO in vascular muscle cells [198].

Moreover, wine was tested to understand whether sulphite compounds in wine could scavenge GO. For this reason, “GO-Wine” model system composed of 10 μmoles of GO with 5 mL of wine was reacted up to 2 hours after pH was adjusted to 7.4. At the end of 1st hour, 18.01% of GO disappeared and it remained constant until the end of reaction. Since sulphites in wine also bind to the carbonyl

compounds present in wine [192], only free sulphites can act on GO under reaction conditions. Furthermore, it was reported that resveratrol; one of the main phenolic compounds in wine, could scavenge MGO and mitigates glycation [54]. Additionally, amino groups of free amino acids in wine might lead to the reaction with GO, as well. For this reason, disappearance of GO in “GO-Wine” system might be due to scavenge by resveratrol, free amino acids and free sulphites in wine. Resveratrol and free sulphite concentration of red wines was reported to be $2.4 \mu\text{g mL}^{-1}$ and $46 \mu\text{g mL}^{-1}$, respectively [199, 200]. According to Lehtonen [201], amino acid content of red wines ranged between $393\text{-}1050 \mu\text{g mL}^{-1}$ and from the amino acids, proline was the most abundant ($380\text{-}790 \mu\text{g mL}^{-1}$). Accordingly, contribution of resveratrol in GO scavenging is considered as minor. The reason for decrease in GO might be provided by amino acids depending on the amount of amino acids having highly reactive amino and sulfhydryl groups as well as free sulphites in wine. In order to understand the effect of sulphite, additional PMS was added to reaction medium, as well. In addition to free sulphites in wine, 7 μmoles of PMS was added. As given in Table 2.9, presence of 7 μmoles of sulphite compounds resulted in 20.81% additional disappearance namely 38.82% decrease in GO concentration in “GO-Wine-PMS” model system at the end of 2nd hour.

As it is known, carbonyl-bisulphite adducts can be formed rapidly in neutral solutions, therefore this decrease might be attributed to formation of carbonyl-bisulphite adducts. These results indicated that PMS; an additive commonly used in wines, could scavenge GO under these conditions and its free form in wines might be a potential dicarbonyl scavenger in the body.

It is known that dicarbonyl compounds are capable of reacting with amino acid residues in proteins leading to the formation of advanced glycation end products in the body [42]. Among the amino acids, cysteine and methionine are the sulphur containing ones. Since the position of sulphur atom in these two amino acids is different, they were tested for their GO scavenging ability. Equimolar addition of cysteine to GO in “GO-Cys” model system caused to $34.63 \pm 0.21\%$ decrease in GO concentration whereas methionine addition did not cause to significant change in GO in “GO-Met” model system ($p > 0.05$). Considering their molecular structures, cysteine bears sulphur as a side group (thiol group) while sulphur is

attached to two carbon atoms in methionine backbone. It is reported that thiol compounds are highly reactive in reacting with carbonyl compounds [202]. Accordingly, cysteine could easily scavenge GO under physiological conditions provided by its thiol group, and the position of sulphur in methionine limits its reactivity.

As it is known, broccoli sprouts are one of the important sulphur sources in daily diet. Since bioaccessible compounds of broccoli such as glucosinolates come across with dicarbonyl compounds in blood following the absorption from the small intestine, its carbonyl scavenging ability might be of importance. For this reason, broccoli sprouts subjected to different cooking processes were digested firstly. Bioaccessible fraction of broccoli sprouts were then reacted with 10 μ moles of GO in different model systems namely “GO-BB”, “GO-SB” and “GO-RB” corresponding to GO with boiled broccoli, steamed broccoli and raw broccoli, respectively. As shown in Figure 2.5, bioaccessible extracts of broccoli samples interacting with GO caused to significant decreases in GO content ($p < 0.05$). GO itself decreased $0.99 \pm 0.23\%$ whereas this ratio varied between 11.70-54.48% in the presence of different broccoli extracts. RB sprouts were found to be less effective in GO scavenging leading to $11.70 \pm 2.31\%$ decrease in “GO-RB” model system. On the other hand, $41.30 \pm 3.51\%$ and $54.48 \pm 1.67\%$ of GO was scavenged in the presence of GO together with boiled and steamed broccoli extracts in “GO-BB” and “GO-SB” model systems, respectively.

It was reported that bioavailable extract of broccoli contains free soluble phenolics and glucosinolates [203]. Glucosinolates are the compounds constituting to sulphur containing compounds in broccoli. Accordingly, the difference in carbonyl scavenging behaviour of broccoli extracts might be due to different glucosinolate concentrations as a result of different cooking applications.

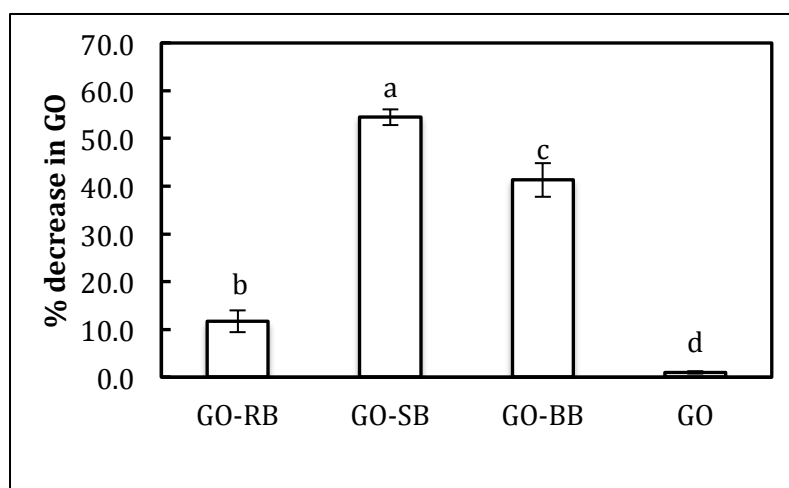


Figure 2.5. Decrease in GO concentrations after reaction in simulated physiological conditions for 2 hours in different model systems composed of GO and bioaccessible broccoli fractions namely "GO", "GO-RB", "GO-SB" and "GO-BB"

To test this, glucosinolates were monitored initially and in broccoli digests. From the glucosinolates, GR is the most abundant glucosinolate in broccoli sprouts constituting 25.2% of total glucosinolates [203]. In both total and extracted ion chromatograms (Figure A3.a, Figure A3.b and Figure A3.c) most intense peak had the same retention time with $[M+H]^+$ ion having m/z of 436 in all broccoli samples and this indicated that GR is dominant among the glucosinolates, as expected. Accordingly, peak areas of initial and bioaccessible GR contents of broccoli sprouts were recorded and given in Figure 2.6.

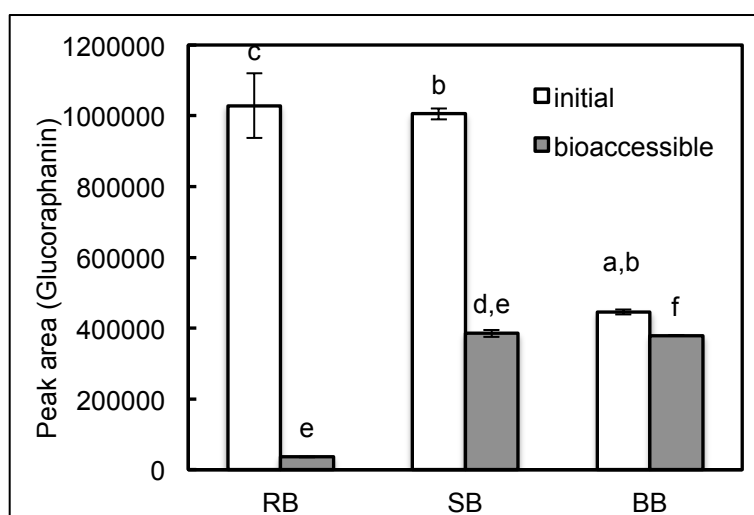


Figure 2.6. Peak area of glucoraphanin (GR) contents of broccoli samples subjected to different cooking treatments and their bioaccessible fractions

Initial GR content of raw and steamed broccoli sprouts were significantly higher than boiled ones ($p < 0.05$). Similarly, Cuomo et al [204] reported that initial GR levels of boiled broccoli samples were the lowest whereas steamed and raw samples contained higher amounts of GR. They concluded that it was due to partial loss of GR to boiling water. Additionally, Jones et al [205] reported that remaining GR in broccoli samples were higher in steamed samples than boiled samples.

Within digestion, GR contents of all broccoli samples decreased significantly ($p < 0.05$). These results are in accordance with those previously published by others. Similarly, Rodriguez-Hernandez et al [206] found that bioavailable GR in raw-freeze dried broccoli samples decreased within digestion. In addition Vallejo et al [203] investigated the change of glucosinolates during digestion and they concluded that bioavailable part of glucosinolates in broccoli was significantly lower than its initial content.

In contrast to higher initial contents, raw broccoli sprouts lost the most part of (96.48%) its bioaccessible GR content. This was provided by the presence of active myrosinase enzyme that might lead to conversion of GR to other compounds during digestive process. Similarly, GR loss during digestion was found to be 61.71% in steamed broccoli sprouts. This might be attributed to partial inactivation of myrosinase enzyme during steaming ($95^{\circ}\text{C} \times 5 \text{ min}$). Lowest GR loss as a result of digestion was observed as 15.02% in boiled broccoli sprouts, as expected. Boiling of broccoli ($95^{\circ}\text{C} \times 30 \text{ min}$) caused to inactivation of myrosinase enzyme preventing the degradation of GR during digestion.

However, bioaccessible GR content of steamed and boiled broccoli samples were significantly higher than raw ones ($p < 0.05$). In the study of Cuomo et al [47] in which bioaccessibility of GR in broccoli sprouts was investigated, it was reported that steaming caused to highest GR bioaccessibility and it was followed by boiling.

According to the results, GR contents of bioaccessible fraction of broccoli samples were found to be linked with the decrease in GO content. Higher bioaccessible GR caused to higher elimination of GO. This indicated that GR in broccoli sprouts could have scavenging ability of GO. In a recent study, it was confirmed that sinigrin, a glucosinolate in cruciferous vegetables, could retard glycation due to its highly carbonyl trapping potential [207].

2.3.4 Investigation of the reactions of HMF during *in vitro* digestion of biscuits

Since HMF is formed in Maillard reaction and dehydration of sugars during caramelization, biscuits are one of the major dietary sources of HMF. Commercial baby, regular and twice baked biscuits were selected as bakery products to be used for *in vitro* multistep enzymatic digestion.

Initially, HMF concentrations were found as 3.86 ± 0.03 , 3.95 ± 0.13 and 55.88 ± 0.32 $\mu\text{g/g}$ in baby, regular baked and twice baked biscuits, respectively. HMF content of twice-baked biscuits was found to be significantly higher than the others ($p < 0.05$). This difference might arise from the difference in thermal process conditions applied for baking of the biscuits.

Surface browning is an important feature of biscuits as it indicates the degree of thermal load. Therefore, colour analyses were performed in the biscuits to determine the difference in heating conditions. a^* values were found to be 0.60 ± 0.03 , 2.78 ± 0.13 and 3.06 ± 0.16 for baby, regular and twice-baked biscuits, respectively. It is a fact that a^* value of bakery products increases with the progress of baking. Observed colour data indicated that baby biscuits were the biscuits subjected to the least thermal treatment whereas twice-baked biscuits were exposed to the highest thermal load as expected.

Samples were taken at the end of each stage of digestion process in order to monitor the changes in the HMF content of biscuits during gastrointestinal digestion. Table 2.10 shows the amounts of HMF in the digested biscuits after gastric, duodenal and colon phases of the digestion process.

At the end of gastric phase, significant ($p < 0.05$) increase was observed in HMF content of all biscuit digests but the highest increase was recorded in the digests of baby biscuits. HMF levels increased by 14.4, 1.3 and 5.5 times in the baby, twice baked and regular biscuits digests, respectively.

Table 2.10. Changes in the concentrations of HMF in different biscuits during the stages of *in vitro* digestion process

	HMF, µg/g			
	<i>initial</i>	<i>gastric phase</i>	<i>duodenal phase</i>	<i>colon phase</i>
Baby	3.86±0.03 ^a	55.48±1.81 ^c	5.17±0.13 ^a	4.73±0.92 ^a
Twice-baked	55.88±0.32 ^c	70.76±0.92 ^d	41.64±0.08 ^e	42.04±1.31 ^e
Regular	3.95±0.13 ^a	21.76±0.52 ^b	4.15±0.14 ^a	4.03±1.31 ^a

^{a–e} Values marked with different letters for each type of biscuit are significantly different (p < 0.05)

According to a study of Rufian-Henares and Delgado-Andrade [173] it was reported that there was a significant increase in HMF content of soluble fraction of digested breakfast cereals that might be due to the release of protein-bound Amadori products and their conversion to HMF during digestive process. In biscuits, baking leads to the formation of intermediates together with HMF. HMF is formed dominantly through dehydration of sugars in low-moisture foods heated at elevated temperatures. Therefore, 3-DG is one of the key precursors responsible for HMF formation in biscuits. As it is known, 3,4-dideoxyglucosone (3,4 DG) is another sugar dehydration product of glucose through the removal of one molecule of water from 3-DG [56]. During baking, formation of HMF through Schiff base of amino acids with glucose is also possible. All these intermediates may be considered as potential precursors of HMF during the gastric phase. To confirm this, the changes in intermediates in biscuits during gastric phase were monitored. Analyses of sugar dehydration products as well as HRMS scan analyses of these precursors were carried out with the biscuits and the digests at the end of gastric phase.

Firstly, concentrations of 3-DG and 3,4-DG were obtained from both undigested and digested biscuits. It was found that 3-DG concentration in baby biscuits was comparably higher than the other biscuits initially whereas lowest concentration was observed in twice-baked biscuits. As it was mentioned before, HMF content of twice-baked biscuits was significantly higher than the others (p < 0.05). Baby biscuits are subjected to moderate baking conditions to be absent of toxic compounds formed during thermal process and these conditions might lead to accumulation of intermediates in high amounts. On the other hand, twice-baked

biscuits which are subjected to 2-fold thermal treatment, contained lower amounts of intermediates in contrast to higher amounts of HMF.

Within the gastric phase, concentration of 3-DG and 3,4-DG decreased in all biscuits, as expected. As given in Table 2.11, 29.01% of 3-DG disappeared in baby biscuits whereas percentage decrease was found to be 17.96% and 19.04% in the twice-baked and regular biscuits, respectively. Besides, percentage decrease in 3,4-DG content of baby biscuits was significantly higher compared to others ($p < 0.05$). Mass balance of gastric digestion was calculated as 104.59% for baby, 99.84% for twice-baked and 98.00% for regular biscuits. These results indicated that sugar dehydration products accumulated in biscuits depending on baking conditions and are converted to HMF under gastric conditions.

As mentioned before, Schiff base is formed in consequence of the reaction of amino acids and sugars leading to the formation of 3-DG through the removal of $R-NH_2$ group [56]. In the biscuits, Schiff bases were also monitored and the presence of Schiff bases of glucose with arginine, cysteine, lysine, phenylalanine, tryptophan and tyrosine was confirmed (Figure A.4a and Figure A.4b). Despite some of the Schiff bases disappeared and some of them slightly decreased at the end of gastric phase, there was no increase in 3-DG or 3,4-DG concentration ($p > 0.05$) (Table 2.11). This might also play role in the formation of HMF through the conversion of accumulated Schiff bases to 3-DG and 3,4-DG and then their further conversion to HMF under acidic gastric conditions. Figure 2.7 gives the proposed mechanism of HMF formation through sugar dehydration products during gastric phase of the digestion process.

Following the gastric phase, significant portion of HMF disappeared in all biscuits ($p < 0.05$) (Table 2.10). Compared to initial contents, almost equal HMF levels were revealed at the end of *in vitro* digestion of regular and baby biscuits.

In twice-baked biscuits, HMF concentration at the end of digestion was significantly lower than initial concentration ($p < 0.05$). Excluding the intermediate steps of digestion, the results are consistent with the reported by Rufian-Henares and Delgado-Andrade [173]. Similarly, they reported that HMF content of breakfast cereals showed stability during *in vitro* digestion even transformation of HMF was possible.

Nevertheless, it is known that HMF contains a furan ring, a reactive carbonyl group, and an allylic hydroxyl group that may undergo further reactions with food components [57].

As shown in Figure 2.7, HMF might form Schiff bases and Michael adducts with amino or thiol compounds during the further stages of digestive process. HMF might react with nucleophilic amino and thiol groups of amino acids, which accumulate as a result of protein hydrolysis during gastric digestion. Thus, formation of HMF adducts with amino acids might cause to disappearance in HMF content of biscuits.

To test this, model systems composed of amino acids and HMF were subjected to digestion, as well. As given in Table 2.12, no change in HMF content of “HMF-Lys” and “HMF-Cys” model systems was observed in gastric phase ($p > 0.05$). However, significant decrease started with the duodenal phase and progressed with the colon phase ($p < 0.05$). Disappearance of HMF was observed as 24.38% in the presence of equimolar amount of cysteine; meanwhile it reached to 63.57% in the presence of 7-fold cysteine. On the other hand, there was a significant decrease in control “HMF” model system at the end of duodenal phase ($p < 0.05$). According to the results of the study of Nikolov and Yaylayan [33] in HMF-glycine model systems, presence of amino compounds in reaction medium limited the decomposition of HMF. Considering this, decrease in “HMF” model system might be due to interactions between HMF and the side chains of enzymes as a source of proteins during gastrointestinal digestion.

Table 2.11. Changes in the peak area of the dehydration products and Schiff bases of glucose with different amino acids during gastric phase of *in vitro* digestion

Compound	Baby biscuit			Twice baked biscuit			Regular biscuit		
	<i>initial peak area</i> ($\times 10^5$)	<i>gastric peak area</i> ($\times 10^5$)	<i>decrease %</i>	<i>initial peak area</i> ($\times 10^5$)	<i>gastric peak area</i> ($\times 10^5$)	<i>decrease %</i>	<i>initial peak area</i> ($\times 10^5$)	<i>gastric peak area</i> ($\times 10^5$)	<i>decrease %</i>
<i>Sugar dehydration products</i>									
3,4-DG	11.59 $\pm$ 0.01 (7.98 $\pm$ 0.01 $\mu\text{g g}^{-1}$)	8.86 $\pm$ 0.00 (6.10 $\pm$ 0.00 $\mu\text{g g}^{-1}$)	23.58 $\pm$ 0.00	11.24 $\pm$ 0.07 (7.75 $\pm$ 0.51 $\mu\text{g g}^{-1}$)	11.21 $\pm$ 0.07 (7.76 $\pm$ 0.05 $\mu\text{g g}^{-1}$)	0.31 $\pm$ 0.00	16.49 $\pm$ 0.06 (11.36 $\pm$ 0.40 $\mu\text{g g}^{-1}$)	15.15 $\pm$ 0.05 (10.43 $\pm$ 0.37 $\mu\text{g g}^{-1}$)	8.14 $\pm$ 4.59
3-DG	30.93 $\pm$ 0.02 (146.64 $\pm$ 0.14 $\mu\text{g g}^{-1}$)	21.96 $\pm$ 0.01 (104.17 $\pm$ 0.07 $\mu\text{g g}^{-1}$)	29.01 $\pm$ 4.04	20.59 $\pm$ 2.60 (84.16 $\pm$ 17.95 $\mu\text{g g}^{-1}$)	18.00 $\pm$ 2.16 (69.04 $\pm$ 1.14 $\mu\text{g g}^{-1}$)	17.96 $\pm$ 4.10	22.35 $\pm$ 0.86 (100.79 $\pm$ 5.04 $\mu\text{g g}^{-1}$)	19.65 $\pm$ 0.69 (81.59 $\pm$ 2.88 $\mu\text{g g}^{-1}$)	19.04 $\pm$ 4.05
<i>Schiff base</i>									
glucose-arginine	4.62 $\pm$ 0.23	2.59 $\pm$ 1.18	44.01 $\pm$ 2.80	6.78 $\pm$ 0.34	3.32 $\pm$ 2.01	51.17 $\pm$ 2.44	42.59 $\pm$ 2.13	30.15 $\pm$ 7.34	29.22 $\pm$ 3.54
glucose-cysteine	27.09 $\pm$ 1.35	ND	100 $\pm$ 0.00	2.56 $\pm$ 0.13	ND	100 $\pm$ 0.00	32.73 $\pm$ 1.64	ND	100 $\pm$ 0.00
glucose-lysine	5.13 $\pm$ 0.25	ND	100 $\pm$ 0.00	34.43 $\pm$ 1.72	3.32 $\pm$ 1.79	90.35 $\pm$ 0.48	45.66 $\pm$ 2.28	9.78 $\pm$ 2.072	78.57 $\pm$ 1.07
glucose-phenylalanine	905.81 $\pm$ 45.29	703.11 $\pm$ 122.19	22.38 $\pm$ 3.88	475.57 $\pm$ 23.78	344.45 $\pm$ 77.63	27.57 $\pm$ 3.62	396.35 $\pm$ 19.82	430.62 $\pm$ 29.24	+8.65 $\pm$ 7.43
glucose-tryptophan	1812.31 $\pm$ 90.61	1918.20 $\pm$ 113.74	+5.87 $\pm$ 5.29	1699.81 $\pm$ 84.98	1530.93 $\pm$ 123.86	9.92 $\pm$ 4.50	204.77 $\pm$ 10.24	191.52 $\pm$ 12.26	6.47 $\pm$ 4.68
glucose-tyrosine	467.06 $\pm$ 23.35	298.01 $\pm$ 98.73	36.19 $\pm$ 3.19	835.39 $\pm$ 41.77	335.74 $\pm$ 28.89	59.80 $\pm$ 2.01	498.73 $\pm$ 24.94	281.77 $\pm$ 126.05	43.50 $\pm$ 2.82

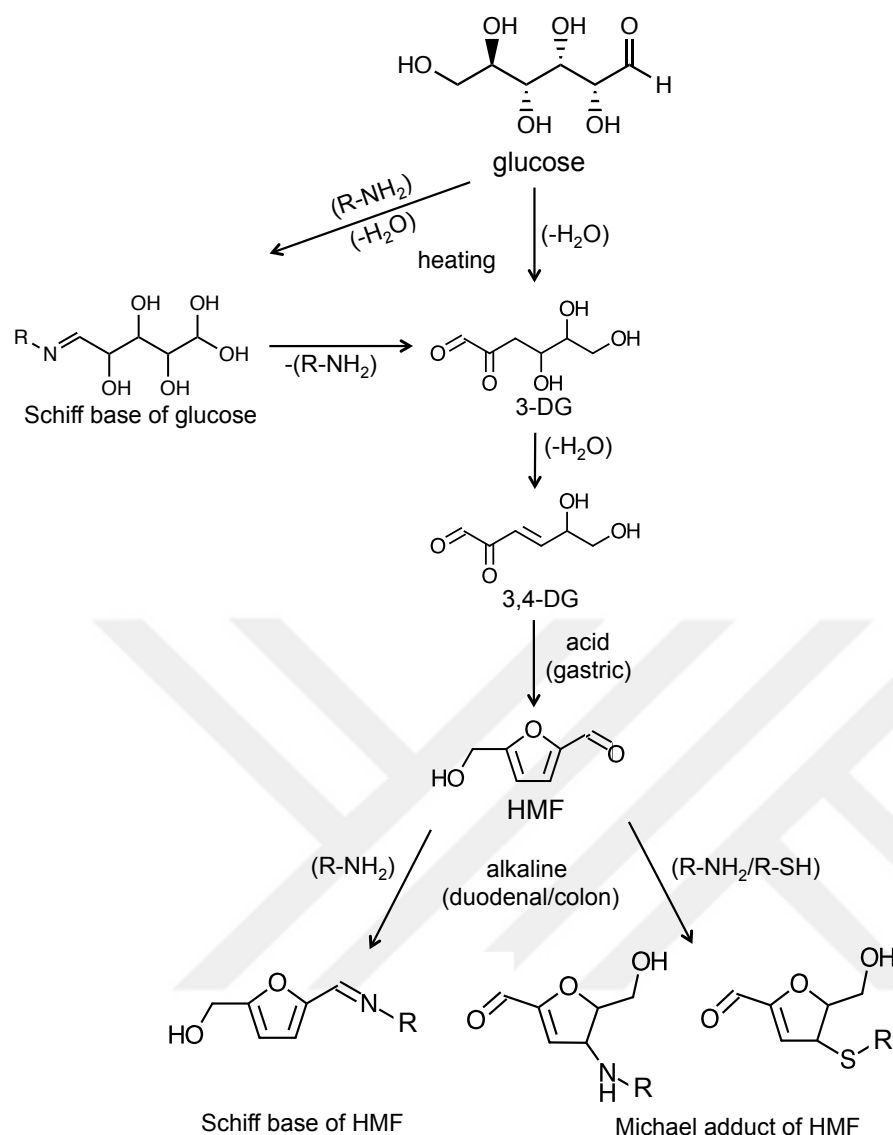


Figure 2.7. Proposed scheme for the conversion of intermediates to HMF and the reactions of HMF with amino and sulfhydryl groups during digestion

To exclude this, a model system containing HMF alone was also subjected to digestion in the absence of enzymes namely “HMF w/o Enzyme”. Results of the “HMF w/o Enzyme” digests revealed that the decrease in “HMF” model system digests might originate from the interactions of HMF with amino acid residues of enzymes under basic intestinal conditions.

Table 2.12. Changes in the amounts of HMF in different model systems during the stages of *in vitro* digestion process

Model system	HMF, μmole			
	<i>initial</i>	<i>gastric phase</i>	<i>duodenal phase</i>	<i>colon phase</i>
HMF w/o Enzyme	9.95 $\pm$ 0.06 ^a	10.09 $\pm$ 0.02 ^a	9.47 $\pm$ 0.07 ^c	9.23 $\pm$ 0.14 ^c
HMF	9.90 $\pm$ 0.06 ^a	9.94 $\pm$ 0.02 ^{a,b}	8.24 $\pm$ 0.08 ^d	8.09 $\pm$ 0.08 ^d
HMF-Lys	10.01 $\pm$ 0.06 ^a	10.12 $\pm$ 0.03 ^{a,b}	8.25 $\pm$ 0.15 ^d	7.44 $\pm$ 0.04 ^f
HMF-Cys	10.09 $\pm$ 0.11 ^a	9.74 $\pm$ 0.25 ^a	7.84 $\pm$ 0.10 ^{d,e}	7.63 $\pm$ 0.12 ^e
HMF-7Cys	9.36 $\pm$ 0.24 ^c	9.19 $\pm$ 0.81 ^f	3.04 $\pm$ 0.31 ^g	3.41 $\pm$ 0.28 ^g

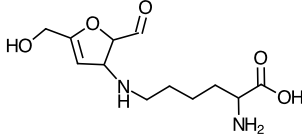
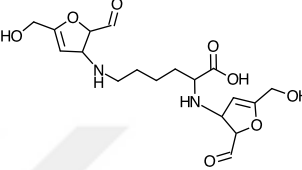
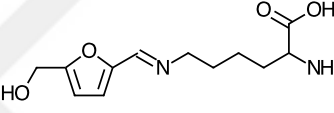
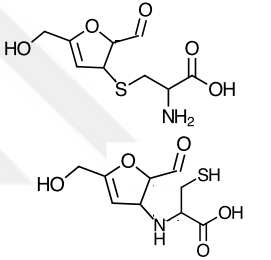
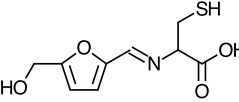
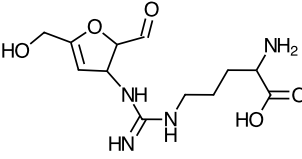
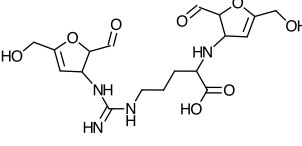
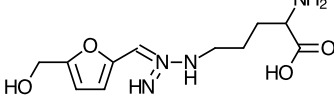
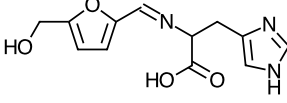
a–h Values marked with different letters for each type of model system are significantly different (p<0.05)

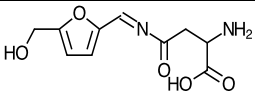
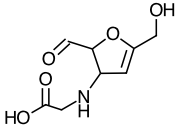
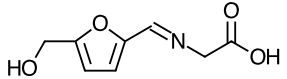
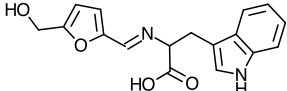
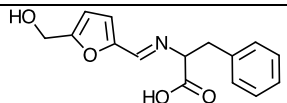
The model system results indicated that HMF reacted with amino and sulfhydryl groups under intestinal conditions. HRMS analyses were done to confirm the presence of possible adducts formed in both HMF-amino acid model systems and biscuits, Michael adducts of HMF with both lysine and cysteine was observed in the model system digests. In the digested biscuits, presence of Michael adducts of HMF with lysine, cysteine, arginine and glycine was confirmed with high mass accuracy (Δ <2 ppm). Additionally, since some of the amino acids have more than one amino or/and sulfhydryl groups, Michael adducts with multiple HMF might also be formed. Michael adduct 2, which is formed of two moles of HMF with lysine and arginine was confirmed in the digests of all biscuits (Figure A4.c-g). In a study of Zou et al [59], reactivity of HMF with amino acids including cysteine, lysine and glycine was investigated in different HMF-amino acid mixtures heated at temperatures ranging between 40 and 160. They reported that 91% of the added HMF (315.3 $\mu\text{g/mL}$) disappeared by the addition of 25 $\mu\text{mol/mL}$ of cysteine at 40°C due to Michael type addition of amino or sulfhydryl groups to HMF.

As mentioned above, HMF might be added to amino acids through Schiff base formation. For this reason, Schiff bases of amino acids were monitored, as well. By scan HRMS analysis, presence of Schiff bases of HMF with lysine, cysteine, arginine, phenylalanine, asparagine, glycine, histidine and tryptophan was

confirmed in the biscuit digests with very high mass accuracy ($\Delta < 1$ ppm) (Figure A4.c-g).

Table 2.13. Chemical structures of the amino acid adduct of HMF in the digests of biscuits and HMF-amino acid model systems as confirmed by scan HRMS analysis

Amino Acid	Adduct	Theoretical Mass	Observed Mass	Δ (ppm)	Molecular Structures
Lysine	Michael adduct 1	273.1445	273.1445	0.0000	
	Michael adduct 2	399.1762	399.1766	1.0021	
	Schiff base	255.1339	255.1340	0.3920	
Cysteine	Michael adduct 1 or Michael adduct 1'	248.0587	248.0588	0.4031	
	Schiff base	230.0482	230.0483	0.4347	
Arginine	Michael adduct 1	301.1506	301.1507	0.3321	
	Michael adduct 2	427.1823	427.1823	0.0000	
	Schiff base	283.1401	283.1401	0.0000	
Histidine	Schiff base	300.0979	300.0977	-0.6664	

Asparagine	Schiff base	241.0819	241.0818	-0.4148	
Glycine	Michael adduct 1	202.0710	202.0711	0.4949	
	Schiff base	184.0604	184.0604	0.0000	
Tryptophan	Schiff base	313.1183	313.1180	-0.9581	
Phenylalanine	Schiff base	274.1074	274.1073	-0.3648	

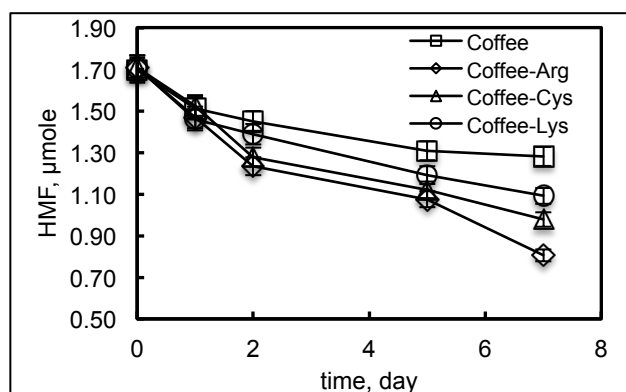
According to the results of the study of Nikolov and Yaylayan [208], formed Schiff bases of HMF and amino acids are prone to decarboxylate and finally two isomeric decarboxylated Schiff bases are formed through oxazolidine-5-one intermediate. However, presence of decarboxylated forms of confirmed Schiff bases could not be observed in digests of the biscuits. Table 2.13 gives the observed masses and chemical structures of amino acid-HMF adducts of the digested biscuits and model systems.

2.3.5 Kinetic evaluation of the reactions of HMF with amino acids

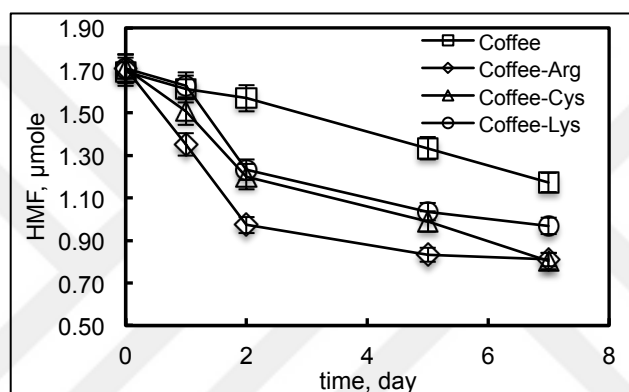
After both elimination and formation reactions of HMF during digestion were confirmed, this study aimed to investigate the reaction kinetics and temperature dependence of HMF with amino and thiol groups of HMF. For this, model systems simulating both low and high moisture conditions were prepared.

Changes in the concentrations of HMF were monitored in both high and low moisture model systems kept at 5, 25 and 50°C during the reaction period up to 7 days. Initial HMF content of roasted coffee was found to be 1.706 ± 0.061 μ moles. At the end of 7th day, 24.5% of HMF was depleted in coffee stored at 5°C, whereas percentage depletion increased in the presence of amino acids. It was found that, 52.8%, 42.7% and 36.1% of HMF was depleted with the addition amino acids in “Coffee-Arg”, “Coffee-Cys” and “Coffee-Lys” model systems, respectively (Figure 2.8a). As temperature is increased to 25°C, elimination ratio gradually increased (Figure 2.8b). When the temperature is 50°C, this ratio reached to 39.7%, 57.6% 58.0% and 62.1% in “Coffee”, “Coffee-Arg”, “Coffee-Cys” and “Coffee-Lys” model

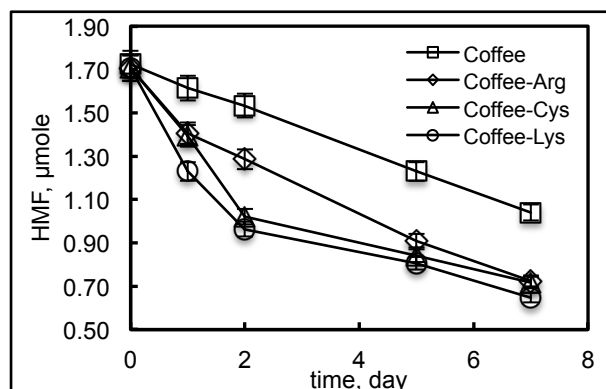
systems, respectively (Figure 2.8c)



(a)



(b)



(c)

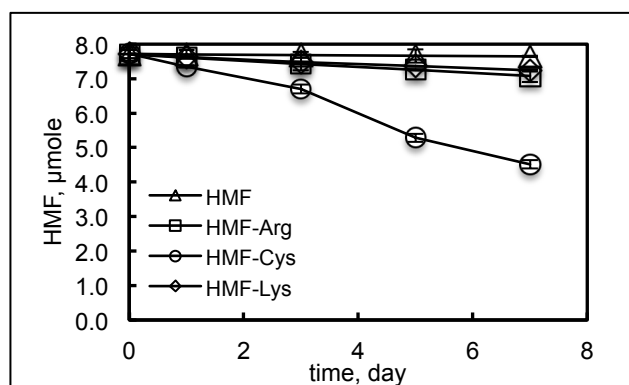
Figure 2.8. Changes in the concentration of HMF in "Coffee", "Coffee-Arg", "Coffee-Cys" and "Coffee-Lys" model systems at different temperatures; (a) 5°C, (b) 25°C and (c) 50°C

In high moisture model systems, at the end of reaction period (7 days) at 5°C, self-degradation of HMF was found to be 0.9% and as in HMF model systems, the elimination ratio increased in the presence of amino acids (Figure 2.9a). Addition

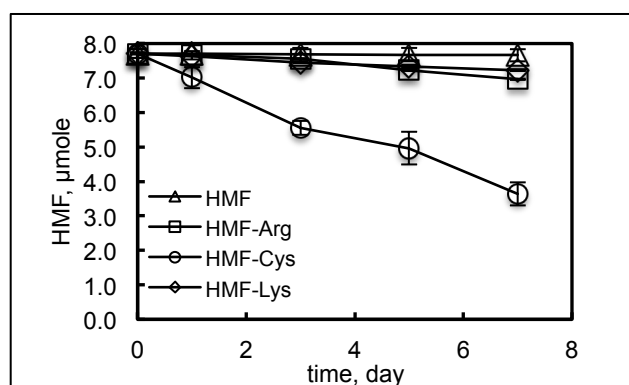
of cysteine caused to 41% depletion even at low temperature, 5°C, whereas almost all HMF was eliminated at the end of reaction period at 50°C (97%). Percentage depletion in HMF was observed respectively as 9.7%, 52.8% and 6.5% for “HMF-Arg”, “HMF-Cys” and “HMF-Lys” model systems reacted at 25°C for 7 days (Figure 2.9b) and reached to 31.9%, 97.2 and 10.2% in the same model systems by increasing temperature to 50°C (Figure 2.9c).

In a similar study, Zou et al [59] investigated the reactivity of HMF with selected amino acids and the results showed that cysteine (25 $\mu\text{mole/mL}$) depleted 91% of HMF (315.3 $\mu\text{g/mL}$) in HMF-cysteine model system after heating at 40 °C.

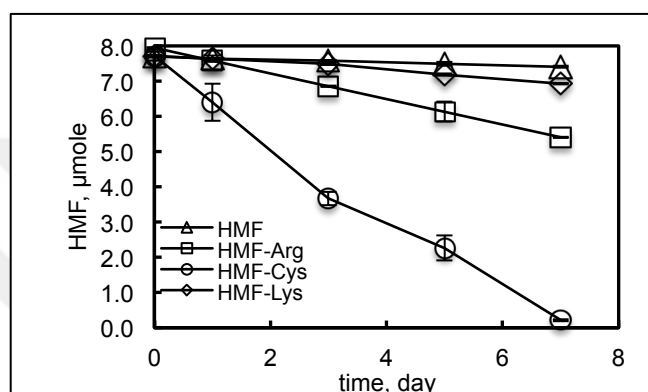
From the results obtained in the model systems, it is clearly seen that HMF degrades itself in some extent but the addition of amino acids cause to the reaction of HMF with amino acids. It is known that, nucleophilic groups ($-\text{SH}$, $-\text{NH}_2$) of amino acids side chains are easily added to β -carbon of HMF through Michael type addition. Besides, if the addition occurs between amino group of amino acids and carbonyl group of HMF, reaction proceeds through elimination of one molecule of water resulting the formation of Schiff base. The results of the study of Nikolov and Yaylayan [33] indicated that presence of amino compound in reaction medium limited the decomposition of HMF. Carbonyl group of HMF was blocked due to Schiff base formation with amino compounds thus dimerization of HMF was inhibited. They additionally indicated that dimerization was mostly prevented when the glycine/HMF ratio was high. For these reasons, elimination of HMF through its self-degradation might be considered as minor compared to its reactions with amino or thiol groups in the presence of amino acids.



(a)



(b)



(c)

Figure 2.9. Changes in the concentration of HMF in "HMF", "HMF-Arg", "HMF-Cys" and "HMF-Lys" model systems at different temperatures; (a) 5°C, (b) 25°C and (c) 50°C

During the reaction of HMF-amino acid model systems, it was expected that the HMF would react with nucleophilic groups of amino acids forming HMF-amino acid adducts. Since amino acids bear more than one nucleophilic groups and HMF has several reactive sites, multi-molecular additions between HMF and amino acids are possible. As mentioned above, Schiff base might be one of the possible adducts formed in HMF-amino acid model systems. Adducts formed in model systems were analysed by HRMS by performing full scan; m/z ranged between 50 and 600. In order to confirm the molecular structures of the adducts, their observed masses were compared with corresponding theoretical masses. Presence of HMF-amino acid adducts were confirmed in high and low moisture model systems with very high mass accuracy ($\Delta < 1$ ppm) but only data recorded for high moisture model systems was illustrated in Figure A5.

Figure A5.a illustrates TIC and EIC recorded for "HMF-Arg" model system kept at 50°C for 7 days. The results of HRMS analyses indicated the presence of $[M+H]^+$

ion having m/z of 301.1506 ($\Delta=0.0633$ ppm) confirming the formation of Michael adduct 1 having $C_{12}H_{20}N_4O_5$ molecular formula as a result of addition of one molecule of HMF to arginine in the reaction mixture. In addition, the presence of $[M+H]^+$ ion having m/z of 283.1401 ($\Delta=0.0208$ ppm) indicating the formation of Schiff base of HMF with arginine having $C_{12}H_{18}N_4O_4$ molecular formula was confirmed. However, presence of Michael adduct 2 ($C_{18}H_{26}N_4O_8$) formed of two moles of HMF with arginine whose $[M+H]^+$ ion having m/z of 427.1823 was not confirmed in “HMF-Arg” model systems. Presence of $[M+H]^+$ ion having m/z of 475.2622 ($\Delta=0.4608$ ppm) which is Adduct 3 formed of addition of an arginine molecule to Michael adduct 1 of HMF with arginine having molecular formula $C_{18}H_{34}N_8O_7$ was also confirmed.

Similarly, TIC and EIC recorded for “HMF-Cys” model system kept at 50°C for 7 days was illustrated in Figure A5.b. Cysteine bears $-NH_2$ and $-SH$ groups which might result in formation of two different Michael adduct structures having same molecular formula ($C_9H_{13}NO_5S$) with the addition of cysteine to HMF. Presence of $[M+H]^+$ ion having m/z of 248.0587 ($\Delta=0.1688$ ppm) revealing the formation of Michael adduct 1 and 1' was confirmed. Michael adduct 2 is the adduct formed as a result of addition of two moles of HMF to cysteine whereas Adduct 3 and 3' corresponds to the adduct formed of in consequence of addition of a cysteine to Michael adduct 1 and 1'. Presence of $[M+H]^+$ ions having m/z of 374.0906 ($\Delta=0.9233$ ppm) representing the formation of Michael adduct 2 ($C_{15}H_{19}NO_8S$) was also confirmed in “HMF-Cys” model system. Nevertheless, $[M+H]^+$ ion having m/z of 369.0785 indicating the presence of Adduct 3 and 3' ($C_{12}H_{20}N_2O_7S_2$) could not be found in “HMF-Cys” model system. Additionally, presence of $[M+H]^+$ ion having m/z of 230.0482 ($\Delta=0.0736$ ppm) indicating the Schiff base ($C_9H_{11}NO_4S$) formation was confirmed, as well.

Lysine bears α and ϵ - NH_2 groups that might be added to one or two moles of HMF forming Michael adducts 1 and 2 having molecular structures $C_{12}H_{20}N_2O_5$ and $C_{18}H_{26}N_2O_8$, respectively. As shown in Figure A5.c, recorded TIC and EIC data for “HMF-Lys” model system kept at 50°C for 7 days confirmed the presence of $[M+H]^+$ ions having m/z of 273.1445 ($\Delta=0.2326$ ppm) and 399.1762 ($\Delta=0.8053$ ppm) corresponding to these two Michael adducts. Likewise in other model

systems, presence of $[M+H]^+$ ion having 255.1339 ($\Delta=0.5101$ ppm) indicating the formation of Schiff base of HMF with lysine ($C_{12}H_{18}N_2O_4$) was revealed in “HMF-Lys” model system. In the meantime, $[M+H]^+$ ion having 419.2500 could not be observed thus it can be concluded that addition of one mole of lysine to the Michael adduct 1 of HMF with lysine ($C_{18}H_{34}N_4O_7$) did not take place in “HMF-Lys” model system.

In the study of Zou et al [59] the reactions of HMF with cysteine or lysine were confirmed and adducts were identified in HMF-amino acid model systems reacted at 80°C by HPLC-MS-MS. Similar to our results, they confirmed the presences of Michael adduct 1 and Schiff base in HMF-cysteine and Schiff base in HMF-lysine model systems. Similarly, Hidalgo et al [24] reported a rapid reduction of acrylamide upon heating in the presence of N-acetyl-cysteine or lysine as a consequence of the Michael type addition of the nucleophilic groups to the β -carbon of acrylamide. Moreover, it was reported that disappearance of acrylamide in acrylamide/glycine mixtures due to its reactions with amino groups was observed not only at high temperatures but also at low temperatures such as 37°C [26]

In order to determine the rate constants, the reaction of HMF with amino acids was considered to proceed as;



A represents HMF, B represents amino acid with amino and/or thiol compounds, and C represents HMF-amino acid adducts. k is the rate constant for the reaction of HMF with amino or thiol compounds. The differential rate equation for the mechanism of the formation of HMF-amino acid adducts could be described as follows;

$$\frac{d[A]}{dt} = -k [A] [B] \quad (2)$$

In both model systems, amino acid concentration is excess in reaction medium compared to HMF. Pseudo-first order, although the actual reaction mechanism is bimolecular, can happen when one of the reactants is excess (van Boekel, 2008). For the reaction given in Eq.1, B is excess and the rate constant $k' = k [B]$ is constant as long as $[B]$ does not change significantly and thereby reaction is

considered as pseudo-first order. The Eq.2 given becomes as follows;

$$\frac{d[A]}{dt} = -k [A] [B] = -k' [A] \quad (3)$$

Then the reaction rate constants were estimated for temperatures 5, 25 and 50°C by solving the differential equation derived for the reaction mechanism. Table 2.13 gives the reaction rate constants for the reactions of HMF with arginine, cysteine and lysine. From the calculated pseudo first order reaction rate constants, rate constant for the reaction of HMF with cysteine was found to be significantly the highest. Since cysteine bears reactive -SH group, it has strong avidity for the double bond of conjugated vinyl compounds [23]. This result is in accordance with those found by a previous study. Zou et al [209] reacted one mmole of cysteine (which contains α -NH₂ and -SH groups), glycine (which only contains α -NH₂), 2-mercaptoethanol (which only contains -SH group), and ethylamine (which only contains -NH₂) with 0.1 mmole of HMF at 160°C for 15 min. They reported that HMF was depleted by 94.9% after the addition of cysteine and cysteine depleted most of the HMF dominantly by reacting with HMF through Michael addition.

Reaction rate constant of cysteine was followed by arginine and lysine, respectively under high moisture conditions (HMF-amino acid model systems). Compared to these rate constants, reaction rate constant of arginine and lysine increased under the low moisture conditions of Coffee-amino acid model systems.

Table 2.14. Kinetic rate constants of reactions of HMF with amino acids in both high moisture ("HMF-Arg", "HMF-Cys" and "HMF-Lys") and low moisture ("Coffee-Arg", "Coffee-Cys" and "Coffee-Lys") model systems

temperature (°C)	Model systems		
	$k'(\times 10^{-1} \text{ day}^{-1})$	$k'(\times 10^{-1} \text{ day}^{-1})$	$k'(\times 10^{-1} \text{ day}^{-1})$
<i>high-moisture</i>	HMF-Arg	HMF-Cys	HMF-Lys
50	0.55±0.03	4.65±0.23	0.160±0.10
25	0.15±0.01	1.03±0.05	0.090±0.05
5	0.12±0.01	0.79±0.04	0.088±0.04
<i>low-moisture</i>	Coffee-Arg	Coffee-Cys	Coffee-Lys
50	1.18±0.06	1.23±0.06	1.22±0.06
25	1.01±0.05	1.03±0.05	0.84±0.04
5	0.97±0.05	0.61±0.03	0.58±0.03

As given in Table 2.14, the rate constants of the reactions of HMF with amino or thiol groups in model systems increased by increasing temperature. Accordingly, Zou et al [59] heated HMF with cysteine, glycine and lysine at different temperatures for 15 min, and they reported that the reaction of HMF with amino acids was temperature dependent.

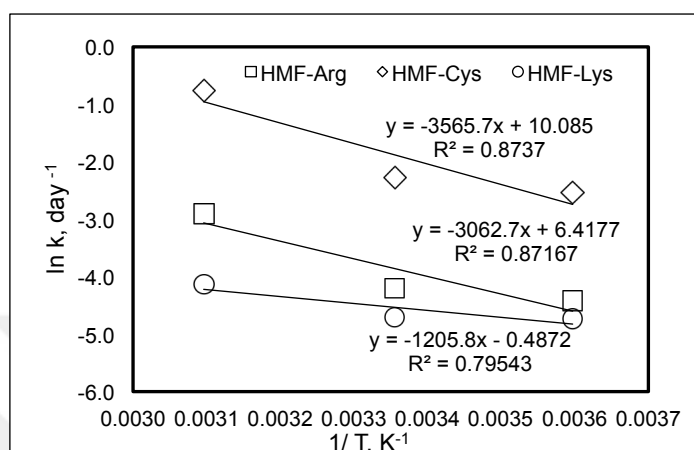
The effect of temperature on the rate constant k is expressed by the Arrhenius law as follows;

$$k = k_{\text{ref}} \exp \left[\frac{E_a}{R} \left(\frac{1}{T_{\text{ref}}} - \frac{1}{T} \right) \right] \quad (4)$$

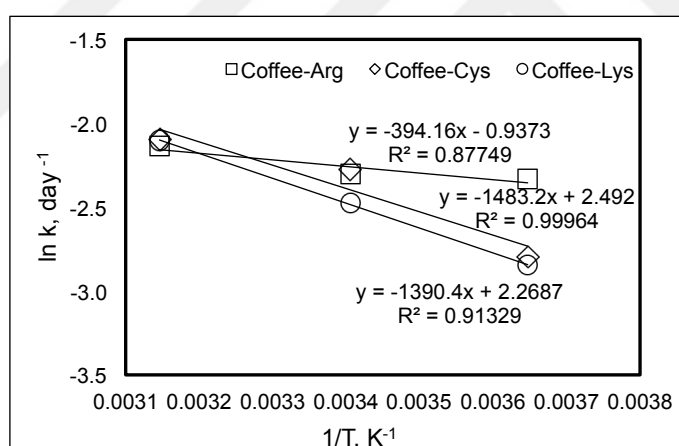
where k_{ref} is the rate constant at the reference temperature (T_{ref}), E_a is the apparent activation energy of for the reaction of amino acids with HMF (J mol^{-1}), and R is the gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$).

The plots of $1/T$ versus $\ln k$ were shown in Figure 2.10 for the reaction of HMF in both high and low moisture model systems. From the slopes of linear fitting lines, the activation energies were calculated as 25.457, 29.639 and 10.018 kJ mol^{-1} for

the reactions of HMF with arginine, cysteine and lysine in high moisture model systems ("HMF-Arg", "HMF-Cys" and "HMF-Lys"), respectively. Similarly, the activation energies were calculated as 3.275, 11.55 and 12.33 kJ mol⁻¹ for the reactions of HMF with arginine, cysteine and lysine in low moisture model systems ("Coffee-Arg", "Coffee-Cys" and "Coffee-Lys"), respectively.



(a)



(b)

Figure 2.10. Plots of $1/T$ versus $\ln k$ of model systems; (a) "HMF-Arg", "HMF-Cys" and "HMF-Lys" (b) "Coffee-Arg", "Coffee-Cys" and "Coffee-Lys"

In a similar study in which temperature dependence of acrylamide elimination in the presence of cysteine was examined, activation energy was calculated as 24.4 kJ mol⁻¹ for the reaction of acrylamide with cysteine [25]. Additionally, activation energy of the reaction of acrylamide with an amino compound, butylamine, was reported to be 44 kJ mol⁻¹ [26].

The activation energies of chemical reactions in foods are generally between 50 and 150 kJ mol⁻¹ [210]. Accordingly, temperature dependence of the reactions of HMF with amino or thiol groups is relatively low. Despite the temperature dependence is low, this reaction takes place even at low temperatures. Particularly, compared to reactions of HMF with arginine and cysteine, reactions of HMF with lysine are less affected by the temperature increase. Considering the reaction conditions, comparably higher activation energies were found in high moisture conditions than low moisture conditions. This means that limited water in reaction system restricted the temperature dependence of the reaction.

2.4 CONCLUSION

The simulated digestion conditions favoured the Michael addition of amino acids to acrylamide. Owing to its two nucleophilic groups (–SH, –NH₂), cysteine becomes highly reactive toward acrylamide, especially under the simulated duodenal conditions. As a result, acrylamide levels of baked or fried products decrease significantly during *in vitro* enzymatic digestion process. However, the intermediates present in fried potatoes act as precursors increasing acrylamide levels under gastric conditions.

The results revealed that the dicarbonyl compounds present in foods may react with free amine and thiol groups of amino acids and proteins under the simulated conditions of gastrointestinal digestion. Scan HRMS analyses of the digests of biscuit samples confirmed the formation of lysine, cysteine, arginine and histidine adducts of MGO and 3-DG. The formation of these adducts might lead to a decrease in the amounts of available dicarbonyl compounds in the gastrointestinal system. In addition to reactions of MGO and 3-DG with amino and thiol groups of amino acids, different sulphur sources could have ability of GO-scavenging under physiological conditions. Bisulphites in wine, cysteine and broccoli sprouts were found to be effective whereas methionine did not cause to significant change ($p > 0.05$) in GO scavenging in body conditions. GO scavenging of broccoli sprouts might be attributed to their GR contents. Even raw and steamed broccoli had initial GR content in higher amounts than boiled broccoli, bioaccessible portion of GR is important for the body. For this reason, considering the higher bioaccessible GR contents, steaming and boiling might be the preparation techniques that should be preferred for cooking broccoli sprouts, from the carbonyl scavenging point of view.

Furthermore, HMF results of the digested biscuits revealed that gastric conditions were favourable for the conversion of intermediates accumulated during baking of biscuits to HMF. On the other hand, HMF was observed as highly reactive toward amino and sulfhydryl groups through Michael type addition as well as Schiff base formation under intestinal conditions. In addition to digestion conditions, HMF reacted with nucleophilic amino and/or thiol groups of given amino acids resulting in the formation of several adducts through Michael type addition and Schiff base formation in low and high moisture HMF-amino acid model systems. Among the amino acids, cysteine was found to be highly reactive toward HMF owing to its thiol group. The reaction of HMF with cysteine was found to take place even at temperatures as low as 5°C. Increasing the temperature also increased the reaction rates obeying the Arrhenius law. From the calculated activation energies, it was concluded that limited amount of water in the reaction medium restricted slightly the temperature dependence of the reaction. Considering the temperature range covered in the present study, the results suggest that the reactions of HMF with amino acids cannot be avoided under *in vitro* conditions during the storage of thermally processed foods, as well as under the *in vivo* conditions of human body.

3. CHAPTER 3

REACTIVITY OF POTATO STARCH AS POLYMERIC FOOD COMPONENTS DURING *IN VITRO* DIGESTION

3.1 INTRODUCTION

Starch is a polymeric food component and one of the main carbohydrates in human nutrition. It is mostly found in corn, potatoes, wheat and rice. As it was mentioned before, the effect of carbohydrates on blood glucose level is expressed as GI. Since the GI of potato is quite high after baking, boiling and frying, glycaemic access of glucose from cooked potato is of importance [147]. There are several studies reporting the effect of different cooking methods on digestible starch portions and GI of potatoes. Thed and Philips [211] determined the resistant starch contents of baked and deep fried potatoes. They reported that baking and deep-frying formed 6.2% and 9.1% resistant starch in potatoes, respectively. In another study, the GI values of baked potatoes, chips and French fries were found as 67.8, 74.3 and 56.6, respectively [153]. Previous studies provide only a limited insight into the formation of digestible starch content of cooked potatoes. However, knowledge on the effect of shape and dimension on the formation of RDS and SDS fractions of baked and fried potatoes is still lacking.

In addition to cooking, digestion behavior of starch is also affected by the presence of other food components including fibers, proteins, oils or several polyphenol compounds. These components might be present together with starch in food matrix, or might be added. Affecting digestion behavior of starch is provided through different mechanisms such as formation of new complexes resistant to digestion or behaving like a physical barrier against the amylolytic enzymes. There are several studies determining the effects of these compounds on GI, but the study about the effect of consumption of these compounds together with potato in the same plate is lack.

For this purposes, firstly, it is aimed to investigate the effect of surface to volume (S/V) ratio as shape factor, as well as cooking method (baking and frying) on the amounts of RDS and SDS formed in potatoes. Secondly, the effect of consuming other foods together with potato on digestion behaviour of potato starch was aimed in this chapter.

3.2 EXPERIMENTAL

3.2.1 Chemicals and consumables

Potassium chloride, sodium chloride, magnesium chloride, ammonium bicarbonate, potassium dihydrogen phosphate was purchased from Merck (Darmstadt, Germany). D-glucose, pepsin (≥ 250 U/mg solid) from porcine gastric mucosa and pancreatin (4 x USP) from porcine pancreas were purchased from Sigma Aldrich (Deisenhofen, Germany). Sulphuric acid (95-98%) was purchased from JT Baker (Deventer, Holland). Potassium hexacyanoferrate, zinc sulfate, disodium hydrogen phosphate anhydrous, and sodium dihydrogen phosphate dihydrate were purchased from Merck (Darmstadt, Germany). The Carrez I and Carrez II solutions were prepared by dissolving 15 g of potassium hexacyanoferrate and 30 g of zinc sulfate in 100 ml of water, respectively.

Syringe filters (nylon, 0.45 μm), Oasis HLB (1 mL, 30 mg) solid-phase extraction cartridges were supplied by Waters (Millford, MA, USA). Shodex Sugar SH-1011 (300 mm $\times$ 8 mm i.d., 6 μm) column was supplied by Shodex (Tokyo, Japan).

3.2.2 Preparation of cooked potatoes

Potato tubers (Agria variety) were obtained from local supermarket in Ankara. After washing and peeling, potato tubers were cut into thin strips (6 x 6 x 60 mm), thick strips (12 x 12 x 60 mm), discs (diameter of 60 mm, thickness of 3 mm), and cubes (9 x 9 x 9 mm). Surface area (S), volume (V) and surface to volume (S/V) ratios of potatoes subjected to frying and baking are given in Figure 3.1.

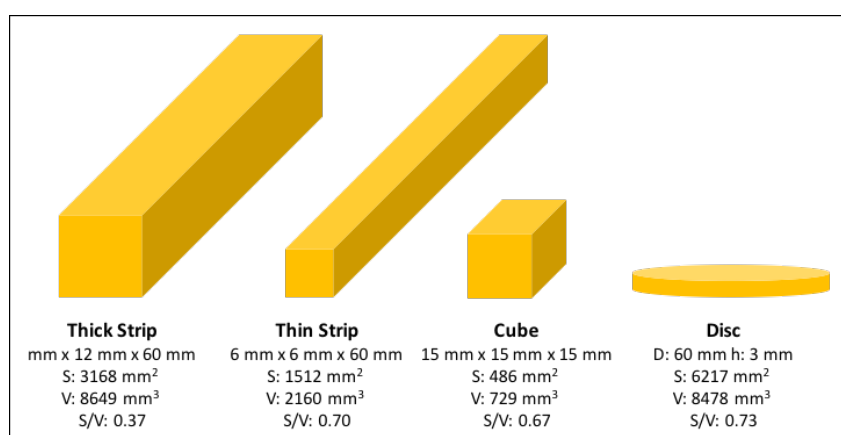


Figure 3.1. Shape, dimension, surface area, volume and calculated surface to volume ratios of potatoes subjected to frying (170°C x 5 min) and baking (200°C x 25 min)

Potatoes cut into different shapes were divided into 20 g lots. Each lot was fried at 170°C for 5 min in oil or baked at 200°C for 25 min in oven. These baking and frying conditions were used to produce well-cooked potatoes. Three grams of cooked potato samples were dried in an oven set at 105°C to a constant weight to determine their moisture contents. After cooking, potatoes were quickly frozen at -80°C in a short while. Then, they were freeze-dried and ground prior to the *in vitro* digestion process. Frying and baking were repeated in triplicate.

3.2.3 Preparation of binary and ternary systems of potato with other foods

For preparation of binary and ternary systems resembling mixed meals, strip potatoes were baked at 200°Cx30 min. 2.5 g of lyophilized potato with 2.5 mL of deionized water was used for “Potato” system. 1.25 g of wheat bran, meat, olive oil or wine was added to 2.5 g of potato and 1.25 mL of water for the preparation of “Potato-Wbran”, “Potato-Meat”, “Potato-Oil” and “Potato-Wine” binary systems, respectively. Ternary systems were prepared with 2.5 g of potato and 1.25 g of each food. For “Potato-Wine-Wbran” and “Potato-Oil-Wbran” model systems; 1.25 g of wheat bran and wine or olive oil was added to 2.5 g of potato. Similarly, 1.25 g of wine and oil was added to 2.5 g of potato for “Potato-Oil-Wine” ternary system.

3.2.4 *In vitro* measurement of the digestible starch fractions

An *in vitro* digestion procedure explained in Chapter 2 was used to digest freeze-dried cooked potatoes. Aliquots of the digests during duodenal phase were withdrawn from the flask at the end of 30 and 120 min of for the determination of glucose concentration. *In vitro* digestion of the samples was repeated in triplicate.

3.2.5 Analysis of glucose in the digests

Aliquots of the digests withdrawn from the flask were freeze-dried prior to extraction. Five hundred mg of dried powder was triple extracted with hot water. Both extraction and analysis of glucose by using HPLC-RID were performed according to the procedure published by Kocadagli et al [212].

For the determination of RAG, SAG, RDS, SDS and AS contents, aliquots withdrawn at the 30 min and 120 min of duodenal phase of *in vitro* digestion were analysed for glucose. Initial free glucose (FG) concentrations of cooked potatoes were also determined. The FG, RAG, SAG, RDS, SDS and AS contents were calculated as g per 100 g (in dry basis) using the following equations [126];

$$FG = G_0 \quad (1)$$

$$RAG = G_{30} \quad (2)$$

$$SAG = G_{120} - G_{30} \quad (3)$$

$$RDS = (RAG - FG) \times 0.9 \quad (4)$$

$$SDS = (SAG - FG) \times 0.9 \quad (5)$$

$$AS = RDS + SDS \quad (6)$$

where G_0 is initial concentration of glucose in freeze-dried cooked potato; G_{30} and G_{120} are concentrations of glucose in the freeze-dried digests withdrawn at the 30 min and 120 min of duodenal phase.

In order to obtain the information about the effect of other foods on GI relatively, concentration of glucose released during digestion was calculated as follow:

$$G_p = G_{p120} - G_{p0}$$

G_{p0} refers to initial glucose concentration of potato whereas G_{p120} refers to the concentration of glucose at the end of digestion of potato. The differences in glucose concentration of given systems were compared to glucose released from potato (G_p) during digestion.

3.2.6 Statistical analysis

The data were subjected to analysis of variance (one-way ANOVA) by using SPSS 17.0 statistical package. Duncan test was applied to the data in order to evaluate the statistical significant differences between mean values. Difference between results were found to be significant when $p < 0.05$

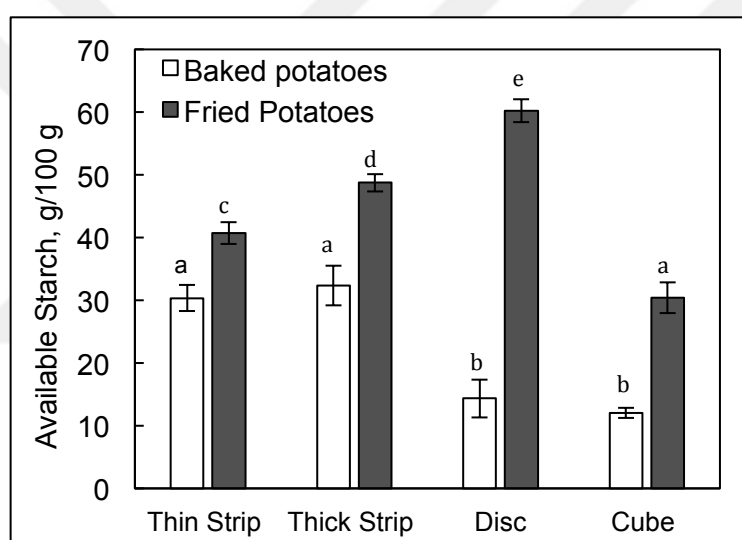
3.3 RESULTS AND DISCUSSION

3.3.1 Investigation of the effects of shape and processing on digestibility of potato starch

The rate of starch digestion was expressed as the percentage of starch hydrolysed during *in vitro* digestion process. The amounts of starch hydrolysed at the end of 30 and 120 min of simulated duodenal phase of digestion represented RDS and SDS contents, respectively. Meanwhile the residual starch that could not be digested at the end of 120 min was considered as indigestible [213]. Therefore, the sum of RDS and SDS contents was taken into account to indicate the amount of available starch (AS) formed in cooked potatoes.

The amounts of AS formed in potatoes during baking and frying are shown in Figure 3.2. Comparing to baking, frying formed significantly higher ($p<0.05$) amounts of AS in potatoes. The AS levels of baked potatoes were found to range between 12.02 ± 0.78 g/100 g (for cube shape) and 32.35 ± 3.16 g/100 g (for thick strip shape); whereas those of fried potatoes were found to range between 30.42 ± 2.47 g/100 g (for cube shape) and 60.25 ± 1.85 g/100 g (for disc shape).

Not only cooking method, but also the shape factor also caused significant differences ($p<0.05$) on the amounts of AS formed in potatoes. Potatoes baked in strip shape were found to contain the highest AS amounts among the baked potatoes. However, potatoes baked in disc shape were found to contain the highest AS amounts among the fried potatoes.



Different letters on the bars indicate statistically significant differences ($p<0.05$)

Figure 3.2. The amounts of RDS and SDS formed in potatoes baked (at 200°C for 25 min) or fried (at 170°C for 5 min) in different shapes and sizes (the values in dry matter basis).

Thed and Philips [211] reported that frying of frozen French fries and baking of whole potatoes led to 64.6% and 65.3% of AS formation, respectively. The amounts of AS in whole oven baked potatoes; French fries and commercial potato crisps were reported as 52.70%, 62.60%, and 65.15% respectively [153]. In addition, it was reported that the AS contents of fried sweet potatoes varied between 68-80% for different cultivars [214].

For a fixed shape (strip, disc or cube), any differences in the AS content of cooked potatoes might be due to differences in the thermal load of potatoes during baking

and frying processes. It is a fact that frying of foods takes less time than baking, because of faster rise of temperature inside the food during frying due to higher convective heat transfer coefficient of frying oil. Hence, frying ($170^{\circ}\text{C} \times 5 \text{ min}$) and baking ($200^{\circ}\text{C} \times 25 \text{ min}$) applications applied here were considered as rapid and slow cooking methods, respectively. It is thought that exposure of potatoes to high temperatures for longer time could be the reason to make significant part of starch indigestible in baked potatoes. Kale et al [215] previously reported that increase in baking time and temperature in breads resulted the modification of starch thus lowering digestible starch content.

The amounts of RDS and SDS formed in potatoes baked (at 200°C for 25 min) or fried (at 170°C for 5 min) in different shapes are given in Table 3.1. The RDS levels of fried potatoes were significantly higher ($p < 0.05$) than those of baked potatoes for all shapes except thick strip. The RDS levels of baked potatoes were found to range between $3.12 \pm 2.70 \text{ g/100 g}$ (for disc shape) and $20.52 \pm 3.00 \text{ g/100 g}$ (for thick strip shape); whereas those of fried potatoes were found to range between $14.36 \pm 1.98 \text{ g/100 g}$ (for cube shape) and $35.64 \pm 0.66 \text{ g/100 g}$ (for disc shape). The SDS levels of baked potatoes were found to range between $6.53 \pm 0.33 \text{ g/100 g}$ (for cube shape) and $20.28 \pm 3.68 \text{ g/100 g}$ (for thin strip shape); whereas those of fried potatoes were found to range between $7.10 \pm 2.32 \text{ g/100 g}$ (for thin strip shape) and $26.67 \pm 0.35 \text{ g/100 g}$ (for thick strip shape). Odenigbo et al [214] reported similar results for different cultivars of sweet potatoes fried in strip shapes at 180°C for 5 min. For different cultivars, RDS and SDS levels of fried sweet potatoes ranged between 18-24 and 17-22, respectively.

Table 3.1. The amounts in dry matter basis of rapidly digestible starch and slowly digestible starch formed in potatoes baked (at 200°C for 25 min) or fired (at 170°C for 5 min) in different shape

	RDS (g/100 g)	SDS (g/100 g)	SDS/AS (%)	Moisture (%)
<i>Baked potatoes</i>				
thin strip	10.07±0.45 ^a	20.28±3.68 ^{a, b}	67	70.06±0.45 ^a
thick strip	20.52±3.00 ^b	11.83±3.32 ^{c, d}	37	69.7±1.61 ^a
disc	3.12±2.70 ^c	11.22±3.40 ^{c, d, e}	78	40.03±0.87 ^b
cube	5.49±1.23 ^c	6.53±0.33 ^e	54	57.95±0.15 ^c
<i>Fried potatoes</i>				
thin strip	33.61±1.21 ^d	7.10±2.32 ^{d, e}	17	59.72±0.56 ^d
thick strip	22.06±2.44 ^b	26.67±0.35 ^f	55	73.44±0.17 ^e
disc	35.64±0.66 ^e	24.61±3.03 ^{b, f}	41	56.88±1.53 ^c
cube	14.36±1.98 ^f	16.06±2.95 ^{c, d}	53	65.32±0.15 ^f

*Values indicated with different letters in each column are significantly different ($p < 0.05$).

Foods having low levels of RDS are of importance, because consumption of RDS in high amounts is correlated with increased risk of certain chronic diseases such as diabetes and coronary heart diseases [133]. On the other hand, consumption of SDS provides controlled and slow increase in blood glucose levels. Therefore, foods having high levels of SDS are important in controlling the glycaemic response of body. According to European Food Safety Authority (EFSA), carbohydrate-rich foods containing at least 40% of its available starch as SDS, are considered as high-SDS foods. Comparing with low-SDS foods, the results of the studies presented that the consumption of high-SDS foods has been related with lower blood glucose and insulin responses [216].

As given in Table 3.1, the SDS/AS ratios of baked potatoes were found to be higher than 40% for all shapes except thick strip (37%). Similarly, the SDS/AS ratios of fried potatoes were found to be higher than 40% for all shapes except thin strip (17%). These results indicate that both baked and fried potatoes can be generally considered as high-SDS foods, with the exceptions of thick strip baked potatoes and thin strip fried potatoes.

As described above, cutting into different shapes prior to cooking strongly influenced the amount of digestible starch, as well as its rapidly and slowly digestible fractions, in both baked and fried potatoes. In order to understand relationship between the S/V ratio as shape factor and the amounts of RDS and SDS formed in potatoes were analysed by means of linear regression for baking and frying separately. Figure 3.2 shows the plots of S/V ratio vs RDS and S/V vs SDS for baked and fried potatoes.

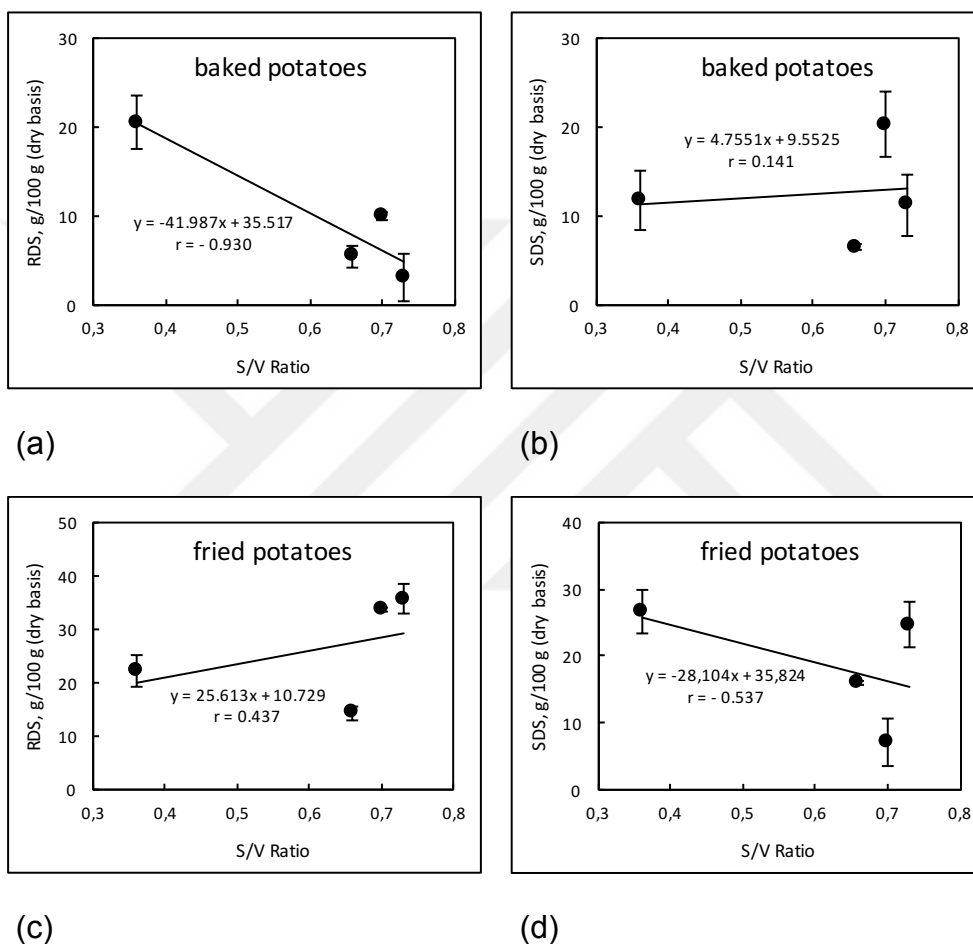


Figure 3.3. The scatter diagrams indicating possible relationship between shape factor (the S/V ratio) and the amounts of RDS and SDS formed in baked and fried potatoes (a) plot of S/V ratio vs RDS content of baked potatoes, (b) plot of S/V ratio vs SDS content of baked potatoes, (c) plot of S/V ratio vs RDS content of fried potatoes, (d) plot of S/V ratio vs SDS content of fried potatoes

For baked potatoes, a strong negative correlation ($r=-0.930$) was observed between the S/V ratio and the amount of RDS formed. This meant that the amount of RDS formed in baked potatoes decreased as the S/V ratio of cut potatoes prior to cooking increased. However, there was only a weak positive correlation ($r=0.141$) between the S/V ratio and the amount of SDS formed in baked potatoes.

For fried potatoes, moderate positive correlation ($r=0.437$) was observed between the S/V ratio and the amount of RDS formed. Contrary, there was moderate negative correlation ($r=-0.537$) between the S/V ratio and the amount of SDS formed in fried potatoes.

As mentioned earlier, baking and frying can be considered as kinds of slow and rapid cooking methods, respectively. Since the temperatures applied are high ($>150^{\circ}\text{C}$), both cooking methods cause the formation of a crust layer due to rapid evaporation of moisture from the outer zones of potato pieces. The temperature of potato pieces rapidly increases to the boiling temperature of water onset of cooking. The thermal energy transferred from oven air in baking process or from oil in frying process is consumed in order to evaporate water from potatoes until critical moisture content is attained. In the meantime, water vapour leaving out the potato system prevents the temperature of potato exceed the boiling temperature of water. In general, only the outer zones of potato slices reach temperatures exceeding 100°C during the later stages of cooking process. The crust layers of potato slices are exposed to elevated temperatures for longer time in baking than frying. The results of present study claim that longer exposure to elevated temperatures during baking could be one of the reasons for the formation of lower amounts of digestible starch in cooked potatoes by transforming starch into its resistant form. In addition, since starch gelatinization is a reaction requiring water in liquid state, rapid evaporation of water from the outer zones of potato slices prevents or limits starch gelatinization due to lack of enough water in crust layers. To investigate the distribution of total digestible starch in cooked potatoes, crust layers of cooked potatoes were separated from the inner parts prior to *in vitro* digestion. The crust layer of baked potatoes was found to contain 9.96 ± 1.49 g/100 g of AS. However, the inner parts contained significantly higher amounts (72.83 ± 3.64 g/100 g) of AS.

Although the processing conditions were fixed during baking and frying, changing the shapes and sizes of cut potato slices caused considerable differences in the final moisture contents of baked and fried potatoes. In order to understand relationship between the moisture content and the amounts of RDS and SDS formed in cooked potatoes were analysed by means of linear regression for baked and fried potatoes separately. Figure 3.3 shows the plots of moisture content vs

RDS and moisture content vs SDS for baked and fried potatoes.

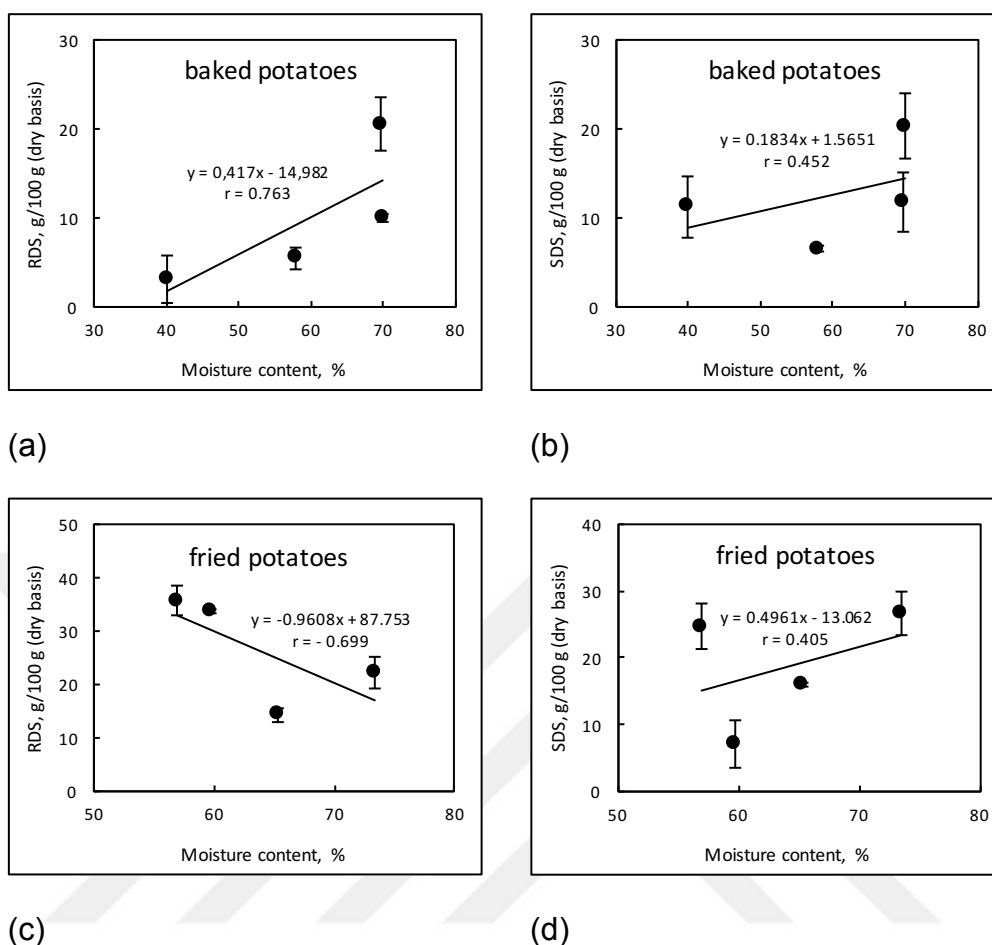


Figure 3.4. The scatter diagrams indicating possible relationship between moisture content and the amounts of RDS and SDS formed in baked and fried potatoes (a) plot of moisture content vs RDS content of baked potatoes, (b) plot of moisture content vs SDS content of baked potatoes, (c) plot of moisture content vs RDS content of fried potatoes, (d) plot of moisture content vs SDS content of fried potatoes

For baked potatoes, strong positive correlation ($r=0.763$) was observed between the moisture content and the amount of RDS formed. Similarly, moderate positive correlation ($r=0.452$) was observed between the moisture content and the amount of SDS formed in baked potatoes. These results indicate that higher the final moisture content means higher amounts of RDS and SDS formed for baked potatoes. For fried potatoes, strong negative correlation ($r=-0.699$) was observed between the moisture content and the amount of RDS formed. Contrary, there was moderate positive correlation ($r=0.405$) between the moisture content and the amount of SDS formed in fried potatoes. It is known that the SDS content of starchy foods is influenced by the gelatinization process. Exposure to heat,

pressure and/or moisture cause to decrease in SDS content through its conversion into RDS [132].

3.3.2 Investigation of the effects of different foods on digestibility of potato

As it is mentioned before, presence and/or addition of some food components affects digestibility of starch, thus GI of potatoes. For this purposes, different binary and ternary systems of potato were tested. As it is known, hydrolysis of starch during digestion results in formation of glucose ($G_{120}-G_0$). Accordingly, consumption of different foods together with potato will lead to the change in release of glucose. The differences in glucose concentration of given systems were compared to glucose released from potato (G_p) during digestion. Calculated difference in systems per difference in potato provides information about the effect of these systems on GI relatively.

Olive oil, wine, low-fat minced meat, wheat bran with baked strip potato and their combinations resembling mixed meals were tested in this study. In all systems, baked potatoes were used in equal quantities (2.5 g). In control system namely “Potato”, additional foods were replaced by water.

Relative increase in glucose levels of all systems is given in Table 3.3. At the end of digestion, released glucose from both binary and ternary systems were found to be significantly lower than released from potato ($p < 0.05$). The released glucose from baked potato was considered as 100%. The highest decrease in the released glucose from binary systems was provided by wine addition. Presence of wine in half amount of potato (potato : wine; 2:1 (w/w)) caused to $20.60 \pm 6.72\%$ increase in glucose in “Potato-Wine” system compared to “Potato” system. Similarly, presence of oil, wheat bran and meat in half amounts of potato led to 75.44%, 17.25%, and 27.47% decrease in relative glucose release of “Potato-Wine”, “Potato-Wbran” and “Potato-Meat” systems compared to “Potato” system, respectively.

Table 3.2. Relative glucose released at the end of digestion in different systems resembling mixed meals

	$G_{120}-G_0 / G_p$ (%)
Potato	99.90±6.05 ^a
<i>Binary systems</i>	
Potato-Oil	24.53±2.82 [†]
Potato-Wine	20.60±6.72 ^{†,g}
Potato-Wbran	82.67±4.06
Potato-Meat	72.46±2.36
<i>Ternary systems</i>	
Potato-Oil-Wine	22.64±1.66 ^{†,g}
Potato-Oil-Wbran	55.19±2.99 ^d
Potato-Wine-Wbran	24.96±0.16 ^g

Although it was reported that amylose could form amylose-lipid complexes that are resistant to digestion [217, 218], formation of these complexes depends on some factors. It was stated that complexation temperature is important for the enzymatic resistance of these complexes [218]. Additionally, it was indicated that the difference between digestibility of amylose and amylose-lipid complex stems from their hydrolysis rate [217]. These complexes are reported to be slowly hydrolysed. For this reason, formation of these complexes is not possible under simulated digestion conditions. The reason for the decrease in the amount of released glucose from “Potato-Oil” systems might be due to restriction of amylolytic enzymes for acting on gelatinized starch provided by oil. Since oil surrounds the starch, it could act as a barrier and prevents the starch hydrolysis. As it is known, wheat bran, as a dietary fiber, is resistant to digestion. However, it is related with providing slower digestion rate through influencing the viscosity [165]. Wheat bran absorbs water in the reaction media. Since water is needed for the hydrolysis reaction, this could lead to reduction in reaction yield. Therefore, the decrease in digestibility of potato starch in “Potato-Wbran” system might be attributed to absorption of water by wheat bran.

As it was mentioned before, protein-starch interactions could retard the starch digestion rate. Henry et al [219] determined the effect of different toppings on *in vivo* glycaemic response of baked potatoes. The results of the study indicated that

potatoes had the higher GI whereas addition of cheddar cheese lead to be considered as low-GI food with GI value 39. Accordingly, presence of meat together with baked potato restricted the digestibility of potato starch in “Potato-Meat” system.

There have been so many studies about the α -amylase inhibition by various polyphenol compounds [166, 220]. Similarly, presence of polyphenols in some varieties of potatoes could prevent the digestibility of potatoes [168]. Results of the study of Kwon et al [221] indicated that some of the tested red wines could inhibit α -amylase activity. Decrease in digestible starch fraction of “Potato-Wine” system could be attributed to amylase inhibition provided by wine polyphenols.

Since oil, wheat bran or wine addition caused to significant decrease in digestibility of potato, similar decrease in ternary systems is expected. For ternary systems, “Potato-Oil-Wine”, “Potato-Oil-Wbran” and “Potato-“Wine-Wbran” systems were prepared from 2.5 g of baked potato and 1.25 g from each one in order to compare with binary systems.

As given in Table 3.3, relative released glucose levels from potato with wheat bran significantly decreased when they are together with wine or oil ($p < 0.05$). Relative glucose released from “Potato-Wbran” system was calculated to be $82.67 \pm 4.06\%$ whereas it decreased to $55.19 \pm 2.99\%$ and $14.96 \pm 0.16\%$ within the addition of oil or wine in “Potato-Oil-Wbran” or “Potato-Wine-Wbran” systems, respectively. However, because of wine could provide remarkably higher decrease in the levels of glucose released from potato, presence of oil or wheat bran together with wine could not lead to significant change in “Potato-Oil-Wine” or “Potato-Wine-Wbran” systems ($p > 0.05$).

3.4 CONCLUSION

Baking and frying were comparatively investigated for the formation of digestible starch fractions in potatoes cooked in different shapes and sizes. It was shown for the first time that the S/V ratio as a shape factor has strong influences on the amounts of RDS and SDS formed in potatoes during baking and frying. The results confirmed that frying produce significantly higher amounts of digestible starch in potatoes compared to baking. Interestingly, cooking potatoes in cube shape lowers significantly the amount of available starch in both baking and frying processes. Mixed meal resembling binary and ternary systems of potato revealed

that consumption of potato together with olive oil, wine, wheat bran or meat inhibited the digestibility and therefore glucose increased in those model systems in lower extent. This inhibition provided by different mechanisms. From the tested foods, wine was found to be the most effective towards inhibition of digestibility. Ternary systems could exert combined effect of these foods on digestibility of potato. Considering the potato is one of the high-GI foods, consumption of potato together with other foods might be a potential way of lowering its GI in daily practice.



CONCLUSION AND GENERAL DISCUSSION

So far, several studies have been done to investigate the fate of food components during digestion. The main focus of these studies was to determine the difference between ingested and digested fraction. In this thesis, considering the potential reactivity of process contaminants owing to their highly reactive carbonyl group, possible reactions of these contaminants were monitored during each step of digestion individually. As a result, it was shown that their reactions could take place during digestion. The results of **Chapter 2** proposed that gastric conditions could be considered as reactive media for the further conversion of intermediates into process contaminants. Intermediates present in fried potatoes could act as precursors increasing acrylamide content under gastric conditions. Similarly, 3-DG and 3,4-DG in biscuits accumulated as a result of baking were converted to HMF during gastric phase. On the other hand, results of the intestinal phase demonstrated that intestinal conditions could be considered as balancing media provided by the accumulating hydrolysed amino acids. Acrylamide, MGO, 3-DG and HMF were found to be highly reactive toward amino and sulfhydryl groups of amino acids through nucleophilic addition as well as Schiff base formation under intestinal conditions. Reactions of these contaminants with accumulating amino acids lead to a decrease in the amounts of available acrylamide, dicarbonyl compounds and HMF in the gastrointestinal system. However, the balance between the elimination and potential formation of these contaminants during the *in vitro* digestion process suggests that ingested amounts with foods may not directly indicate the net amount available for the body. In addition, these results presented the importance of food composition and process conditions since they directly affect the formation and accumulation of intermediates, which might possess potential threat during digestion.

Results obtained from kinetic evaluation of the reactions between HMF and amino and sulfhydryl groups of amino acids showed that the reactions could take place even at low temperatures under both low and high moisture conditions. Additionally, the results showed that cysteine exert highest reactivity towards tested contaminants due to its thiol group. In addition to cysteine, results proved that sulphur compounds such as potassium metabisulphite and sulphur rich foods including wine and broccoli could provide a reduction in GO, as another reactive

process contaminant, under physiological conditions. The results suggested that GO-scavenging by broccoli was related to bioaccessible glucoraphanin content of ingested broccoli depending on cooking application. These results exhibited the significance of protein and/or sulphur content of foods that might suppress the negative health concerns of process contaminants.

Digestion behaviour of potato starch, as a polymeric food component, was tested in **Chapter 3**. The results conclude that, surface-to-volume ratio could be considered as a shape factor, which strongly affects the digestible fractions of potato during cooking. The results suggested that frying or baking potatoes in cube shape is important considering the lowest available starch content. Moreover, the results revealed that consumption of wine, olive oil, wheat bran or meat together with baked potato inhibited the glucose release compared to potato consumption alone. The results provided practical information on how conditions can be taken under control to keep available starch and rapidly digestible starch contents of cooked potatoes low. In addition, obtained results proposed that mixed meal composed of fiber, lipid, protein or polyphenols might be effective to control glucose levels released through consumption of potatoes.

Overall, ingested amounts of process contaminants with foods may not directly indicate its absorption rate through gastric, duodenal and colonic routes. It should be kept in mind that reactive compounds maintain their reactivities during digestion and consequences should be fully considered.

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ANNEX 1. Supplementary Figures for Chapter 2

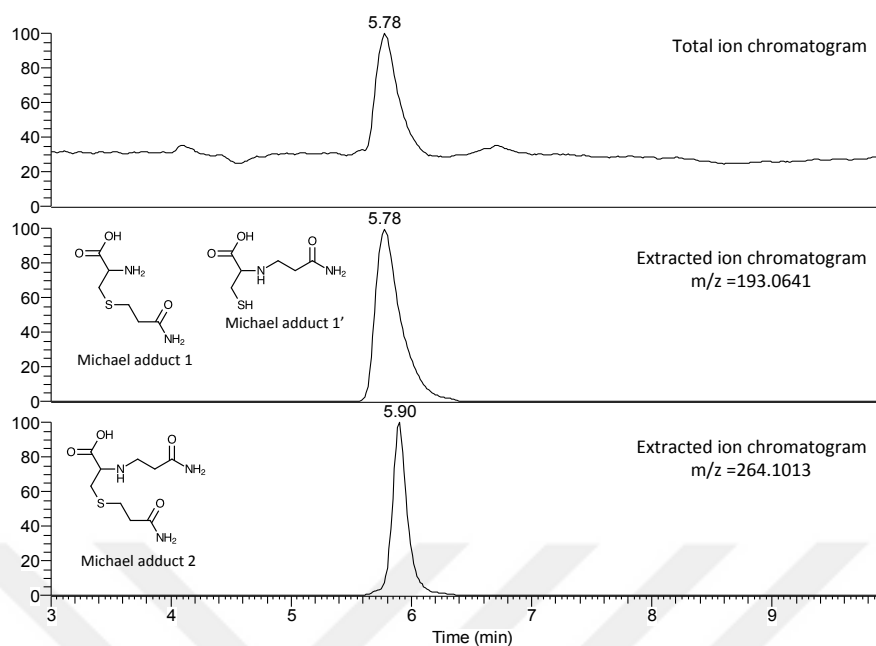


Figure A1.a Total and extracted ion chromatograms of Michael adducts of acrylamide with cysteine in "Acr-Cys" model system

ACR-cysteine adducts

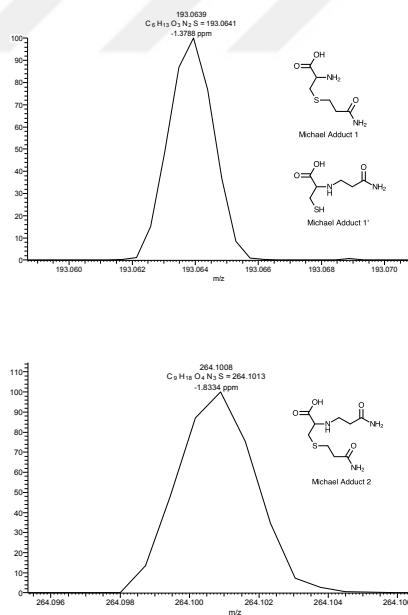


Figure A1.b Mass spectra of confirmed Michael adducts of acrylamide with cysteine in "Acr-Cys" model system

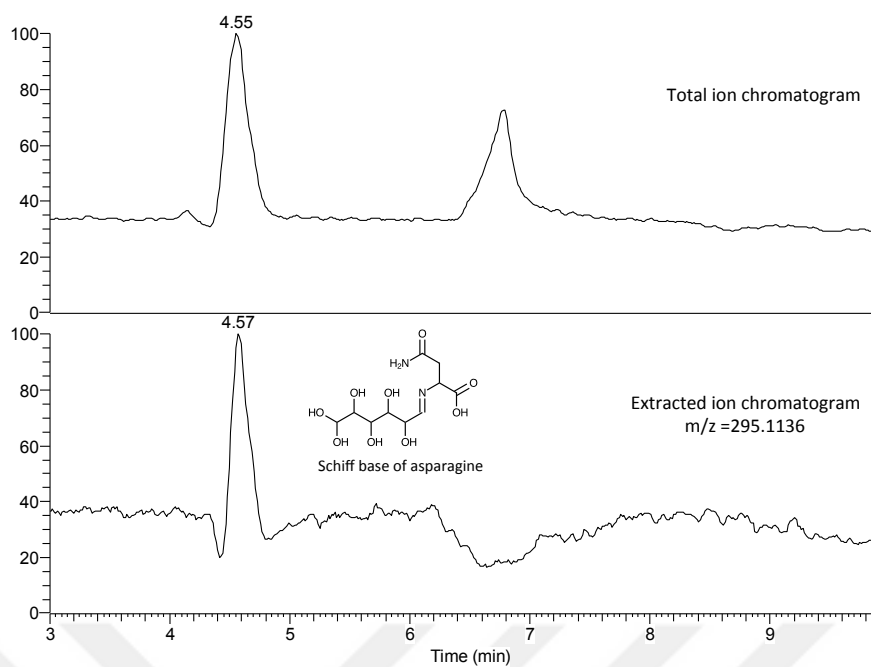


Figure A1.c Total and extracted ion chromatograms of Schiff base of asparagine with glucose in "Asn-Glc" model system.

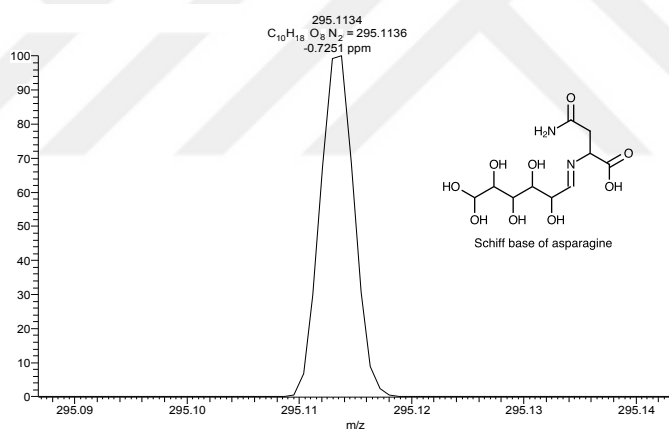
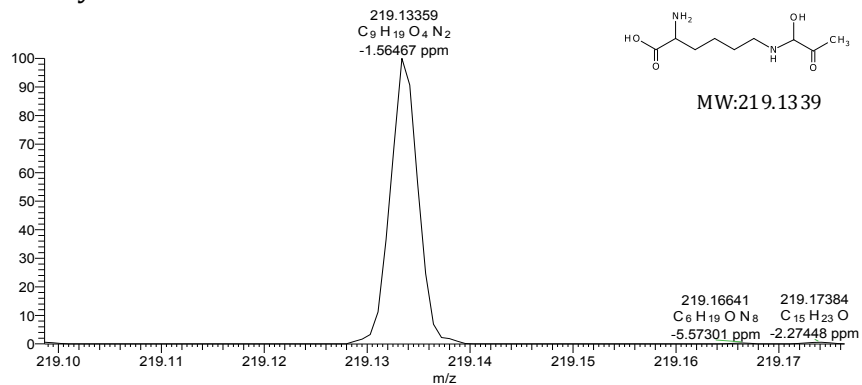


Figure A1.d Mass spectrum of confirmed Schiff base of asparagine with glucose in "Asn-Glc" model

MGO-Lys



MGO-Cys

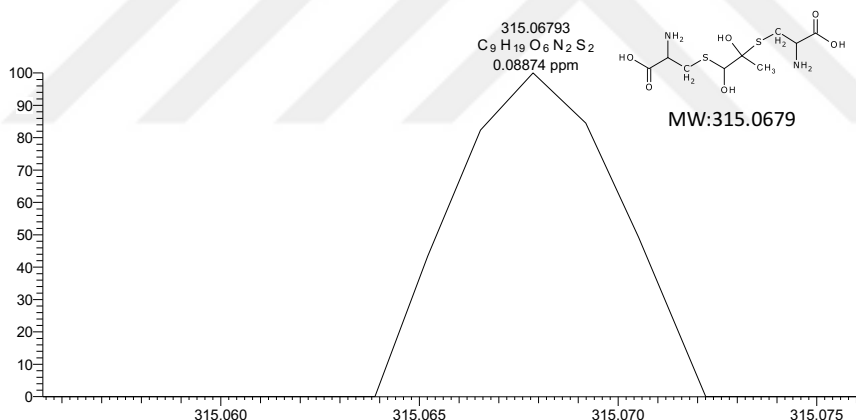
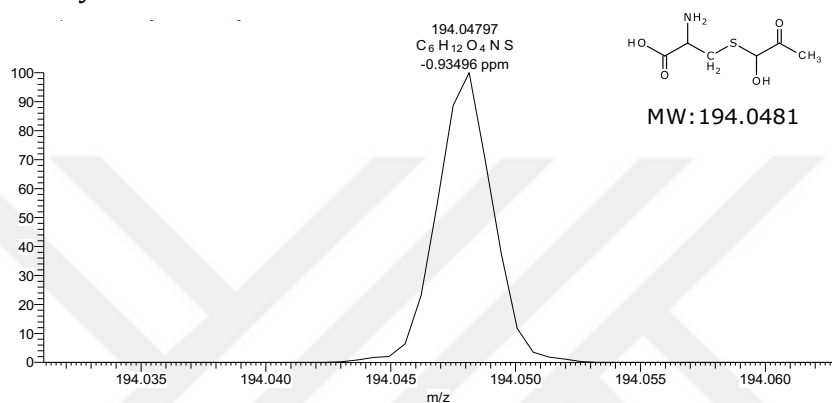
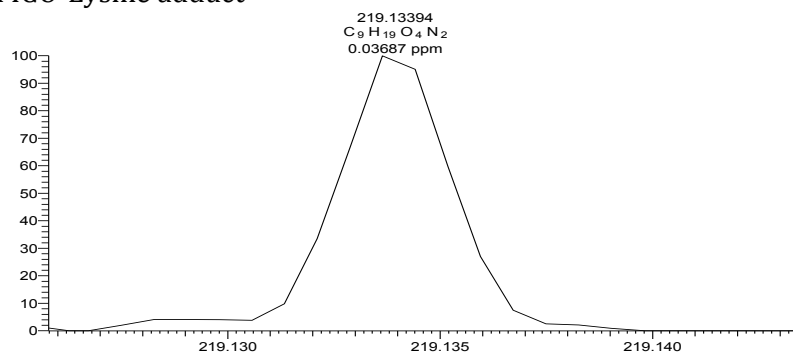
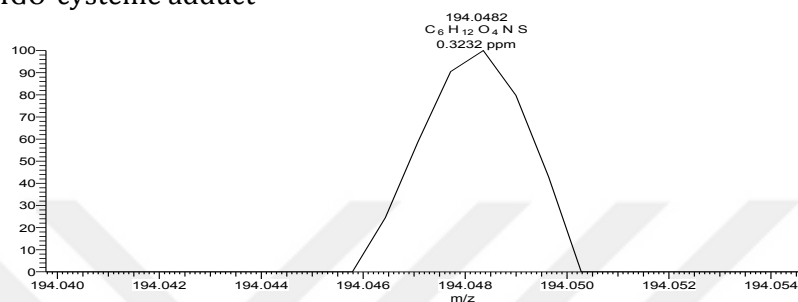


Figure A2.a Mass spectrums of the confirmed adducts of MGO with lysine and cysteine in "MGO-Lys" and "MGO-Cys" model systems

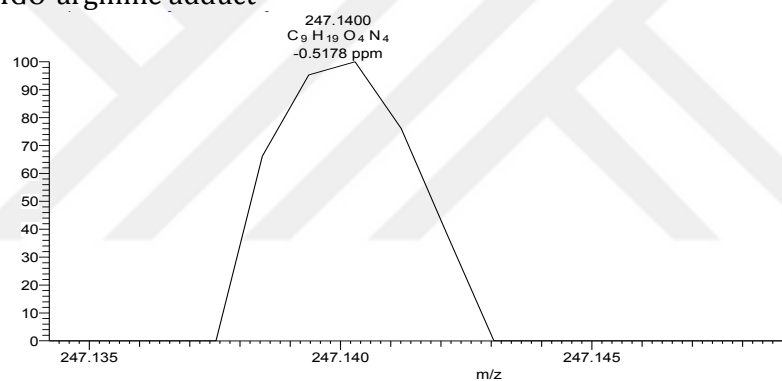
MGO-Lysine adduct



MGO-cysteine adduct



MGO-arginine adduct



MGO-histidine adduct

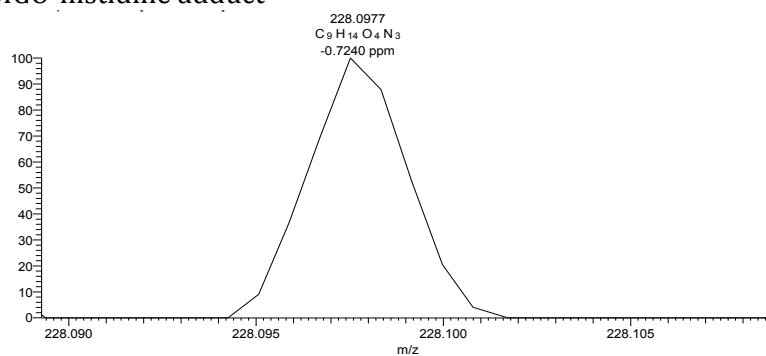
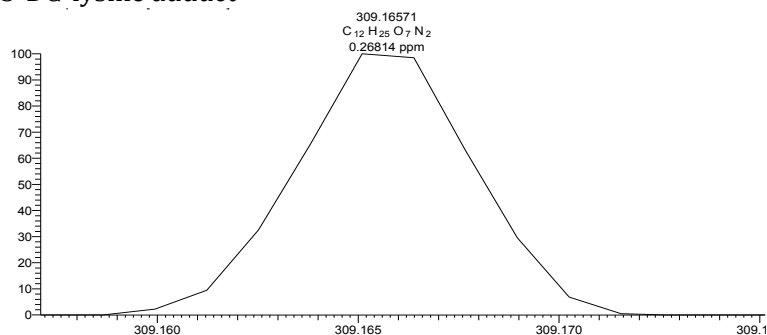
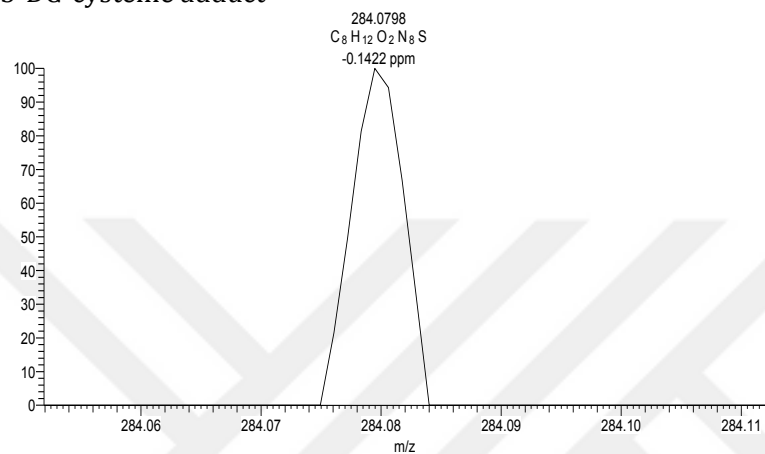


Figure A2.b Mass spectrums of the confirmed adducts of MGO with lysine, cysteine, arginine and histidine in biscuit digests

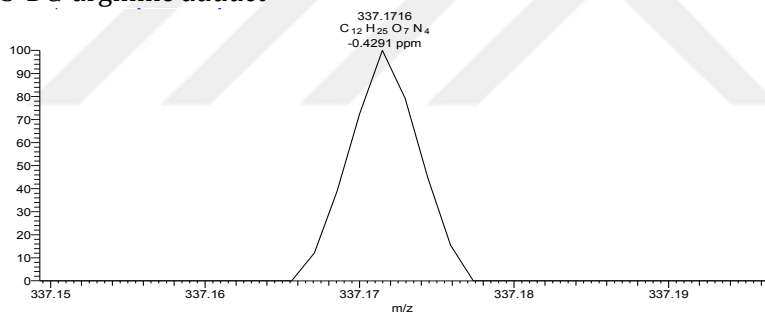
3-DG-lysine adduct



3-DG-cysteine adduct



3-DG-arginine adduct



3-DG-histidine adduct

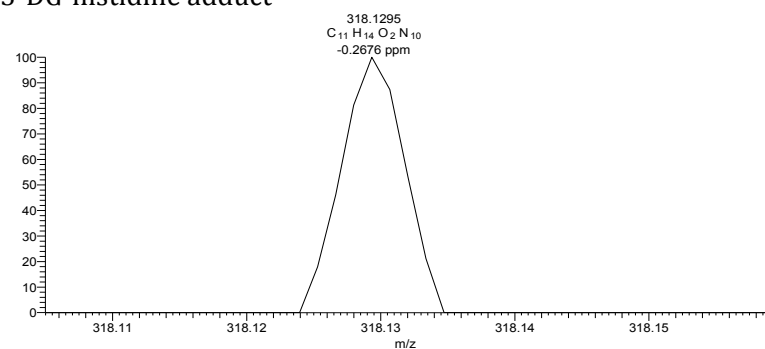


Figure A2.c Mass spectrums of the confirmed adducts of 3-DG with lysine, cysteine, arginine and histidine in biscuit digests

twice-baked biscuits

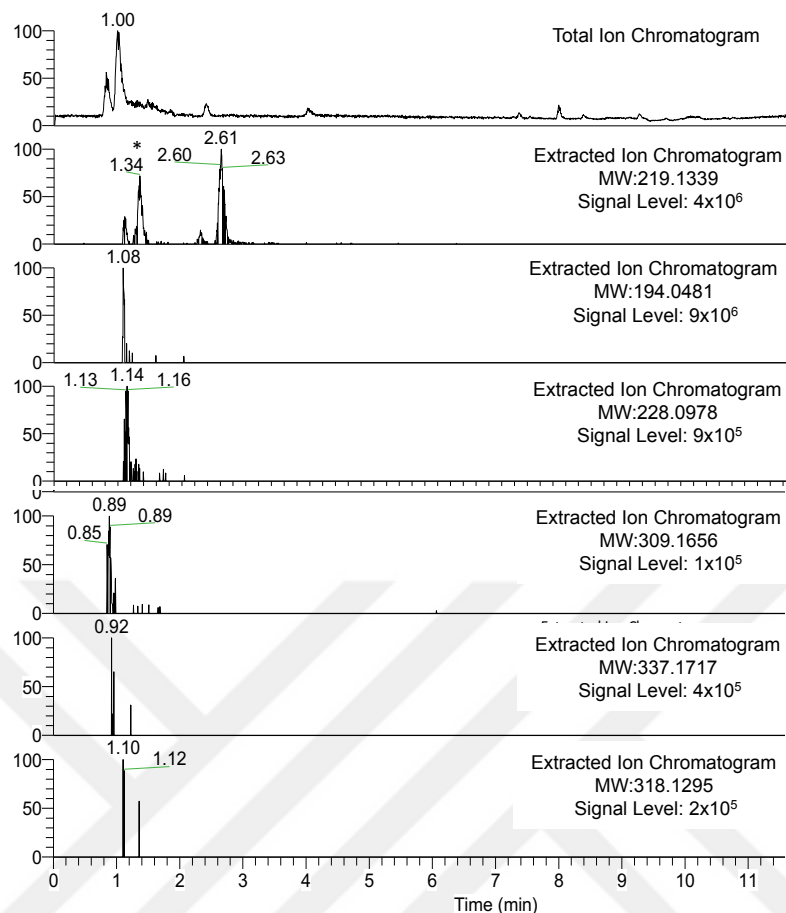


Figure A2.d Total and extracted ion chromatograms of the adducts of MGO and 3-DG with lysine, cysteine, arginine and histidine in twice baked biscuit digests

regular biscuits

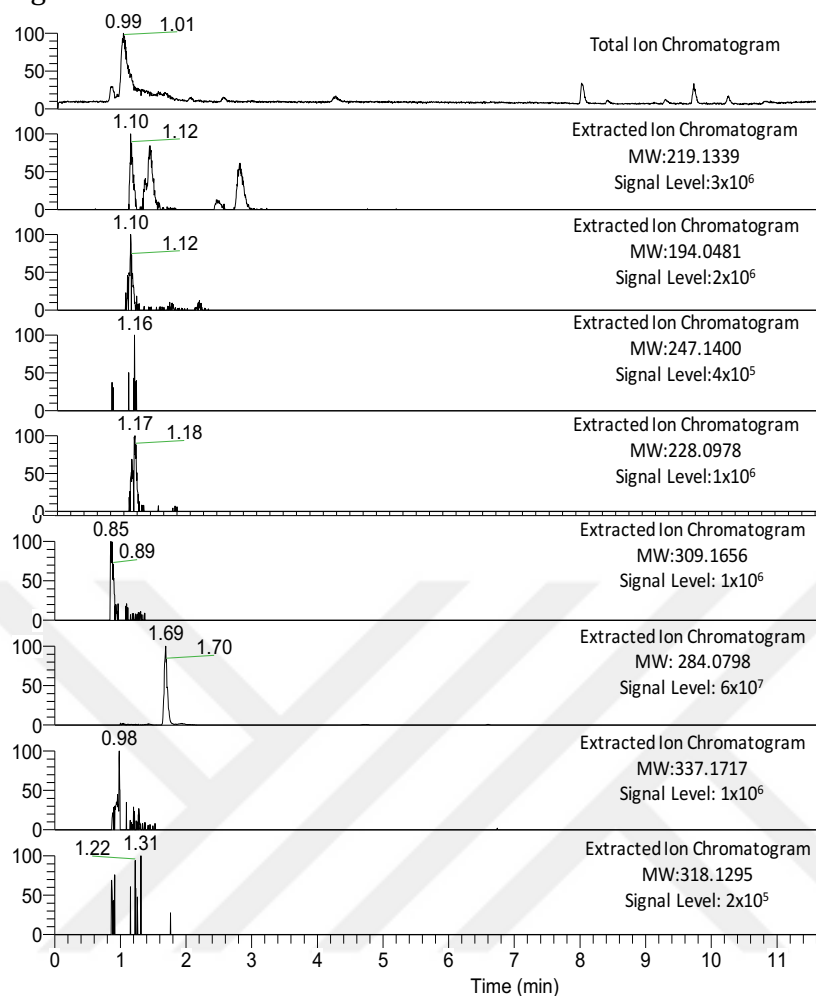


Figure A2.e Total and extracted ion chromatograms of the adducts of MGO and 3-DG with lysine, cysteine, arginine and histidine in regular biscuit digests

baby biscuits

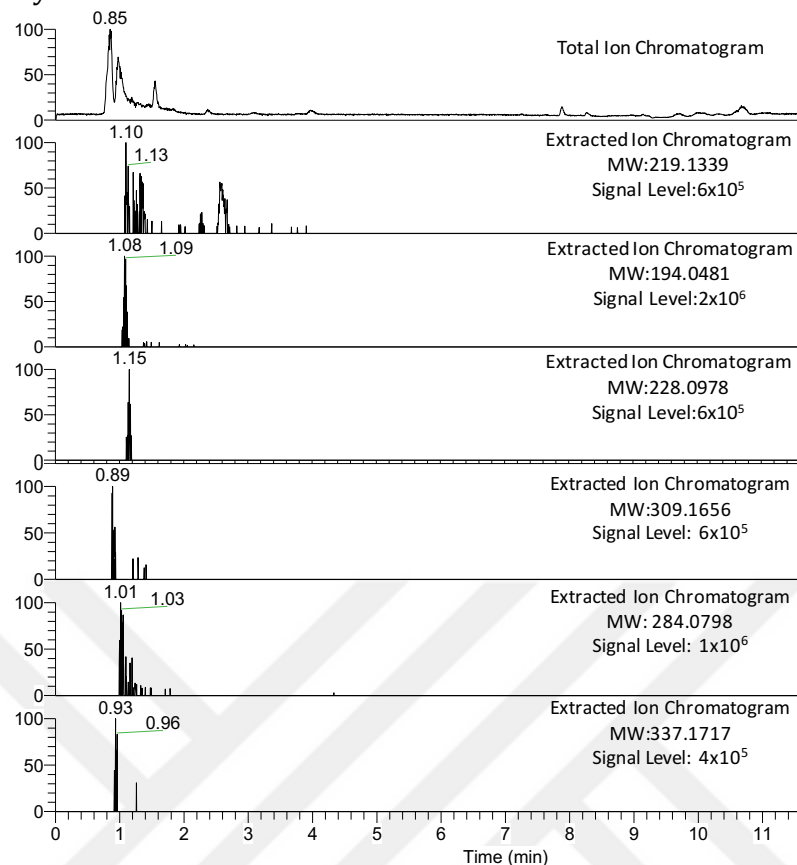
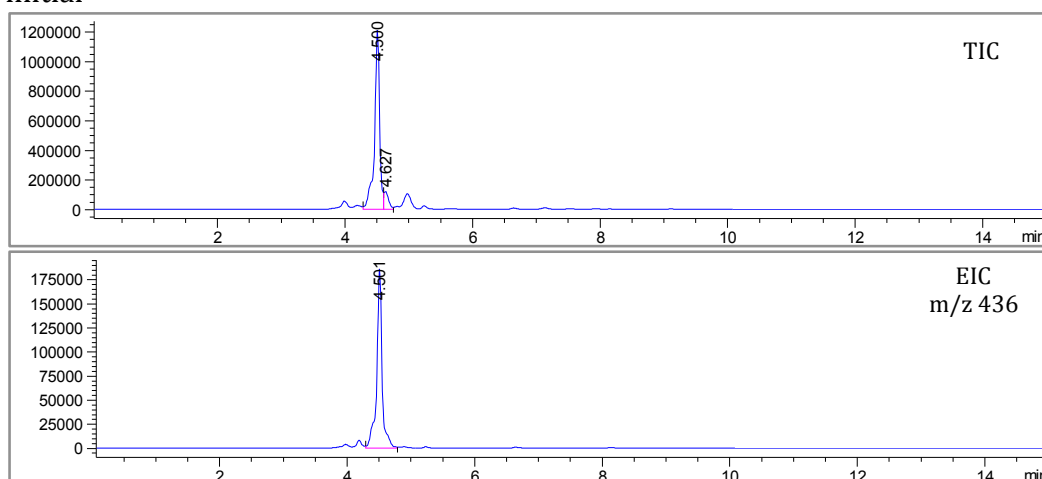


Figure A2.f Total and extracted ion chromatograms of the adducts of MGO and 3-DG with lysine, cysteine, arginine and histidine in baby biscuit digests

Raw broccoli
initial



bioaccessible

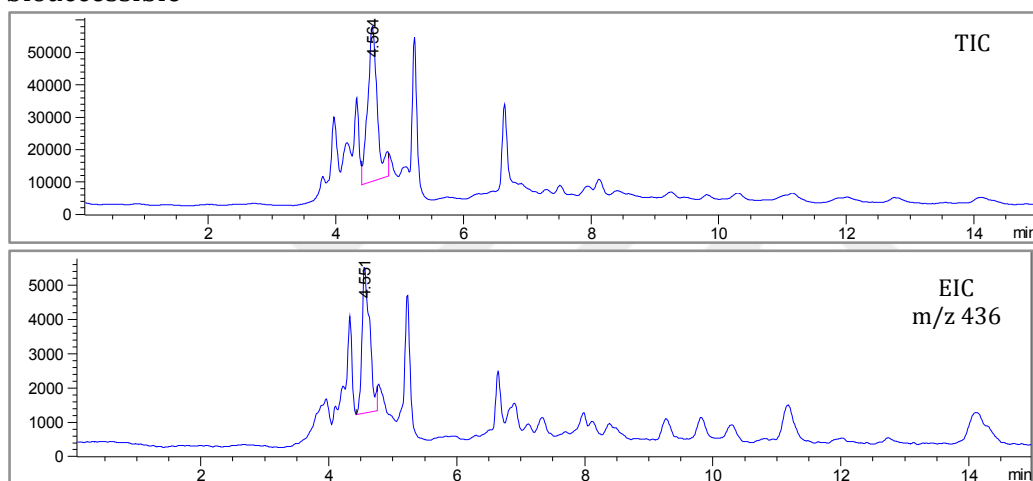
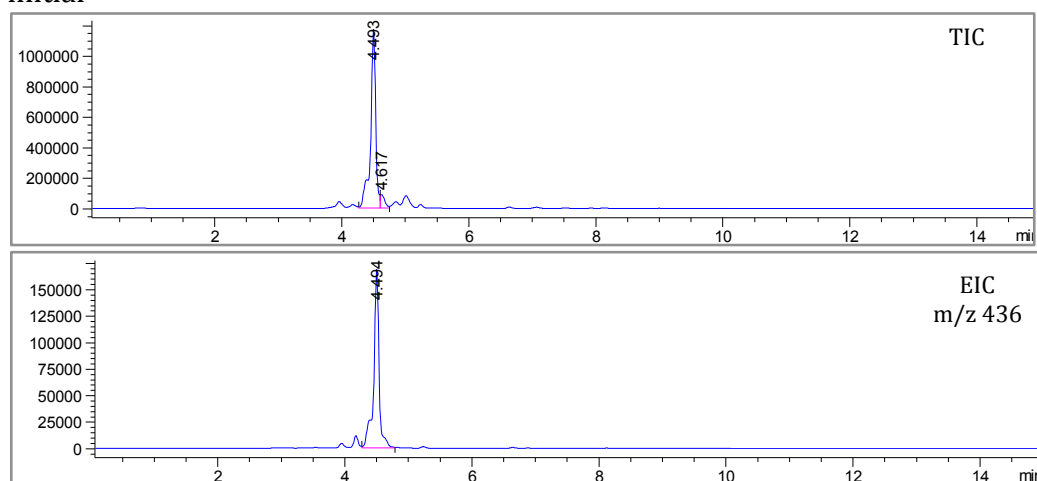


Figure A3. a Total and extracted ion chromatograms of glucoraphanin (m/z 436) of both initial and bioaccessible fractions of raw broccoli

Steamed broccoli
initial



bioaccessible

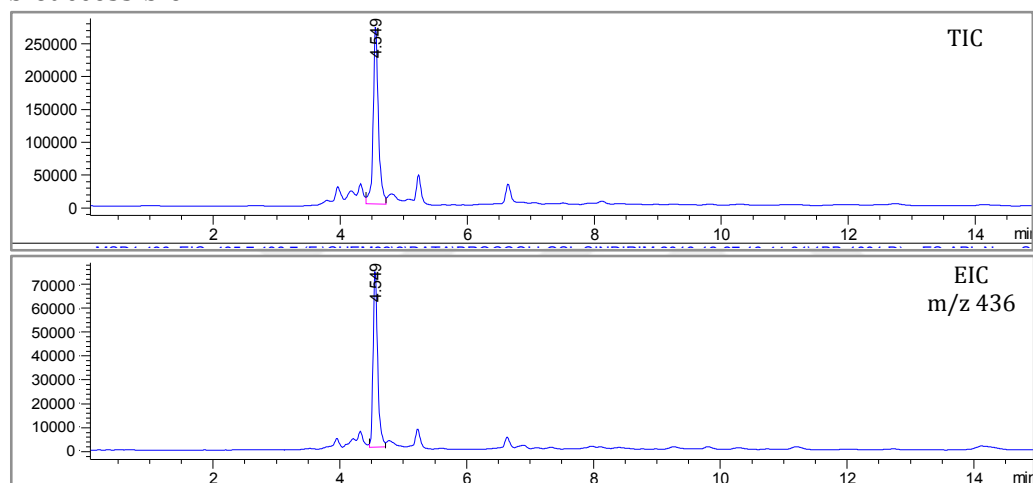
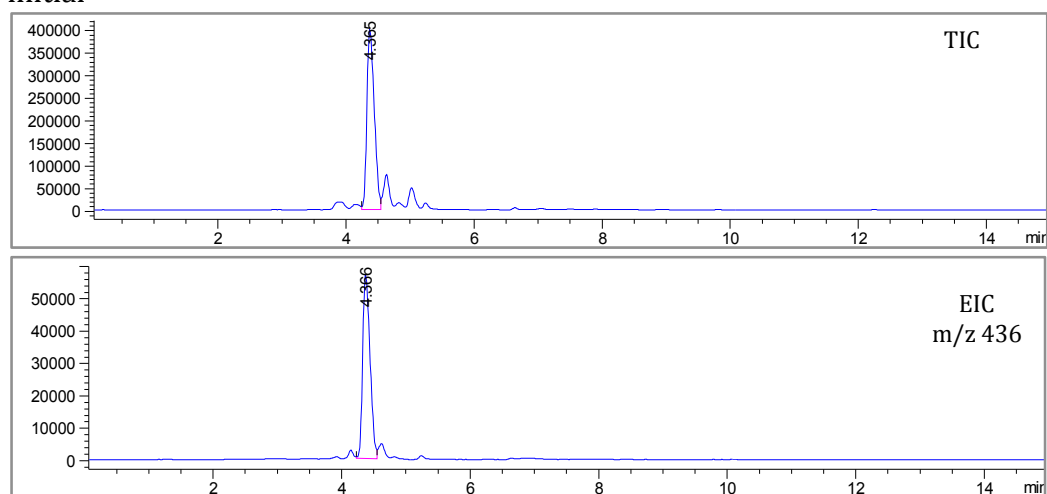


Figure A3. b Total and extracted ion chromatograms of glucoraphanin (m/z 436) of both initial and bioaccessible fractions of steamed broccoli

Boiled broccoli
initial



bioaccessible

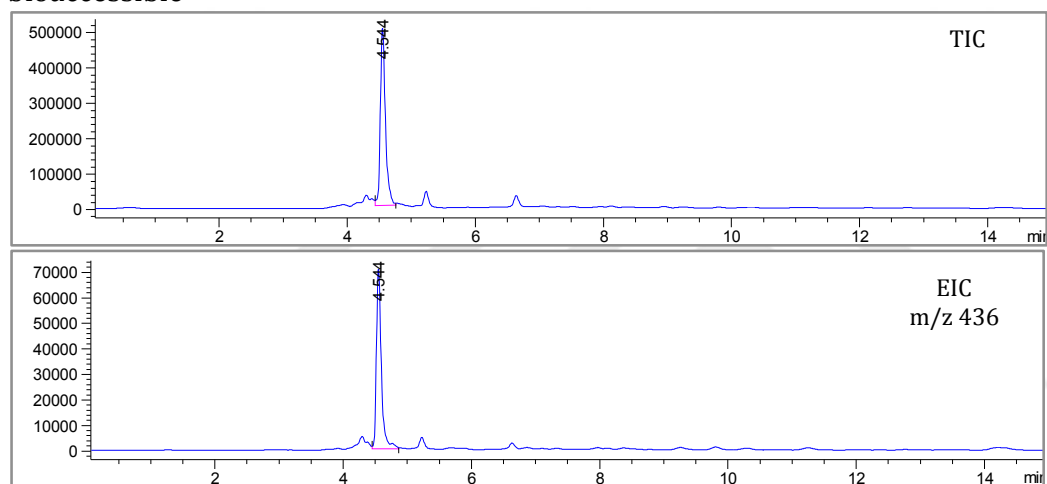


Figure A3. c Total and extracted ion chromatograms of glucoraphanin (m/z 436) of both initial and bioaccessible fractions of boiled broccoli

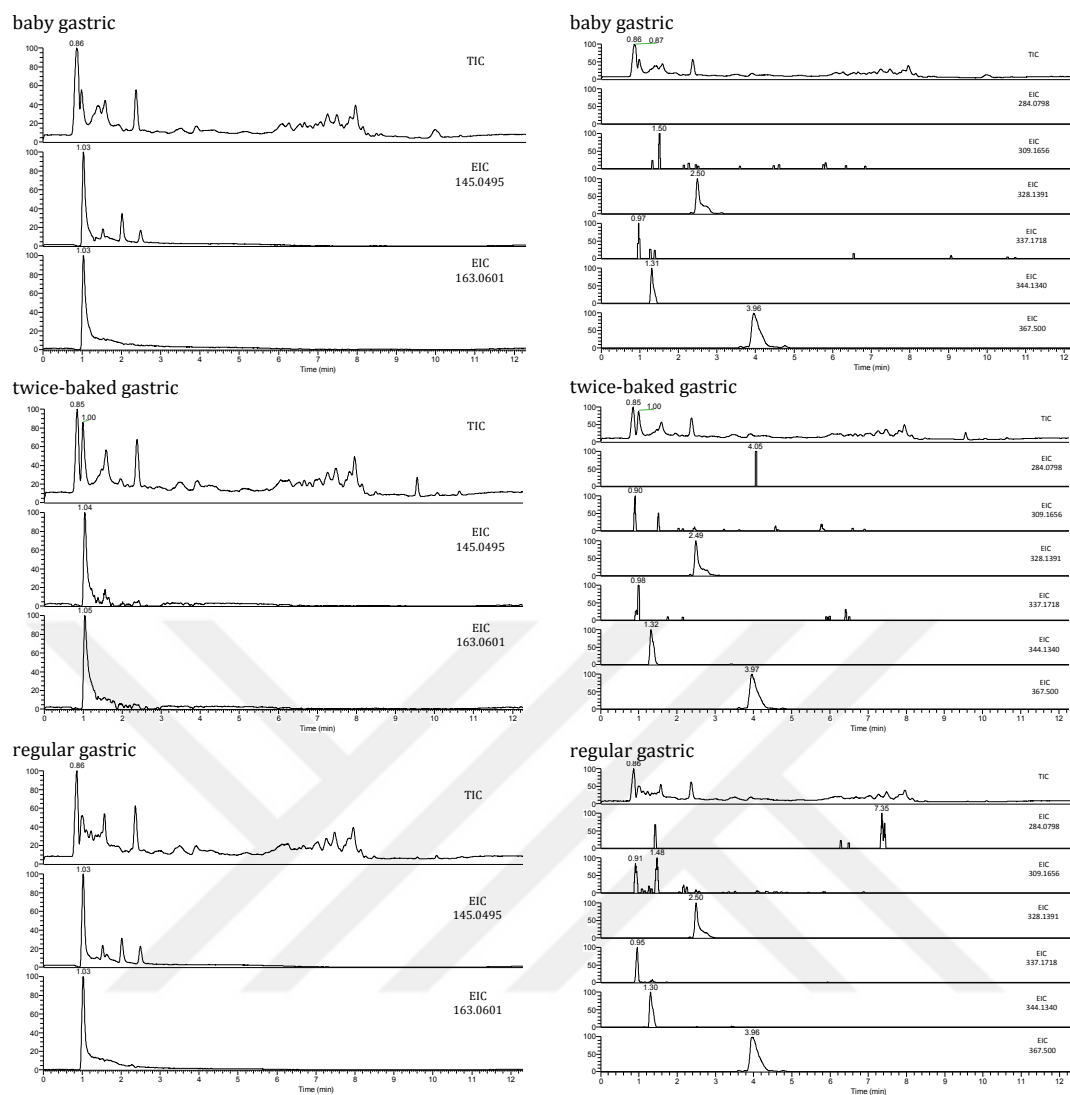
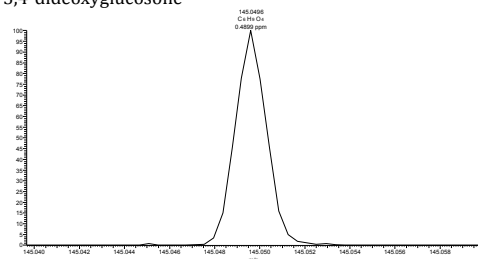


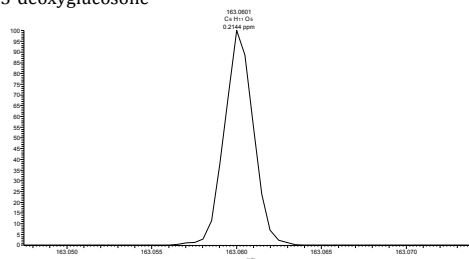
Figure A4. a Total and extracted ion chromatograms for the sugar degradation products and Schiff bases of glucose with amino acids in the digests of biscuits after gastric phase

Sugar dehydration products

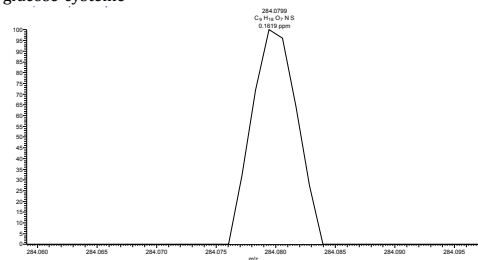
3,4-dideoxyglucosone



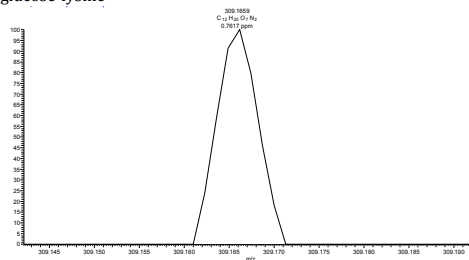
3-deoxyglucosone



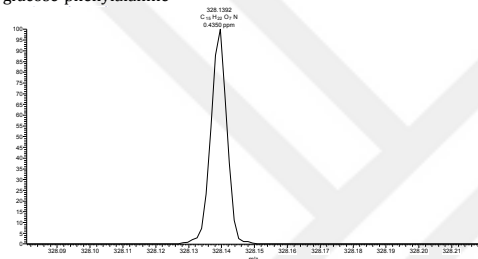
Schiff base glucose-cysteine



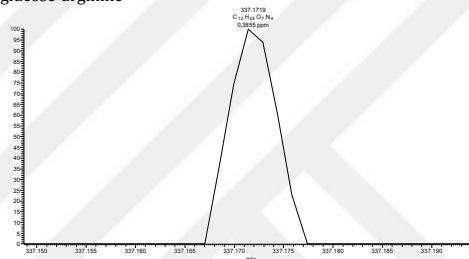
glucose-lysine



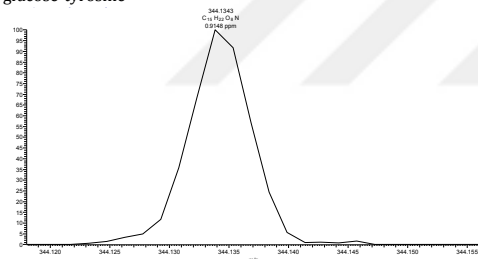
glucose-phenylalanine



glucose-arginine



glucose-tyrosine



glucose-tryptophan

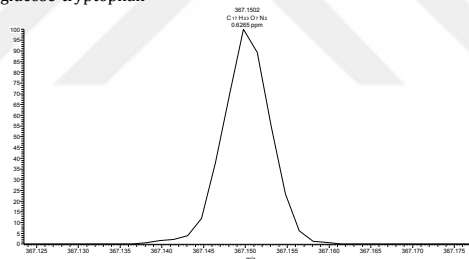


Figure A4. b MS spectrum of the confirmed sugar degradation products and Schiff bases of glucose with amino acids in the digests of biscuits

baby biscuit

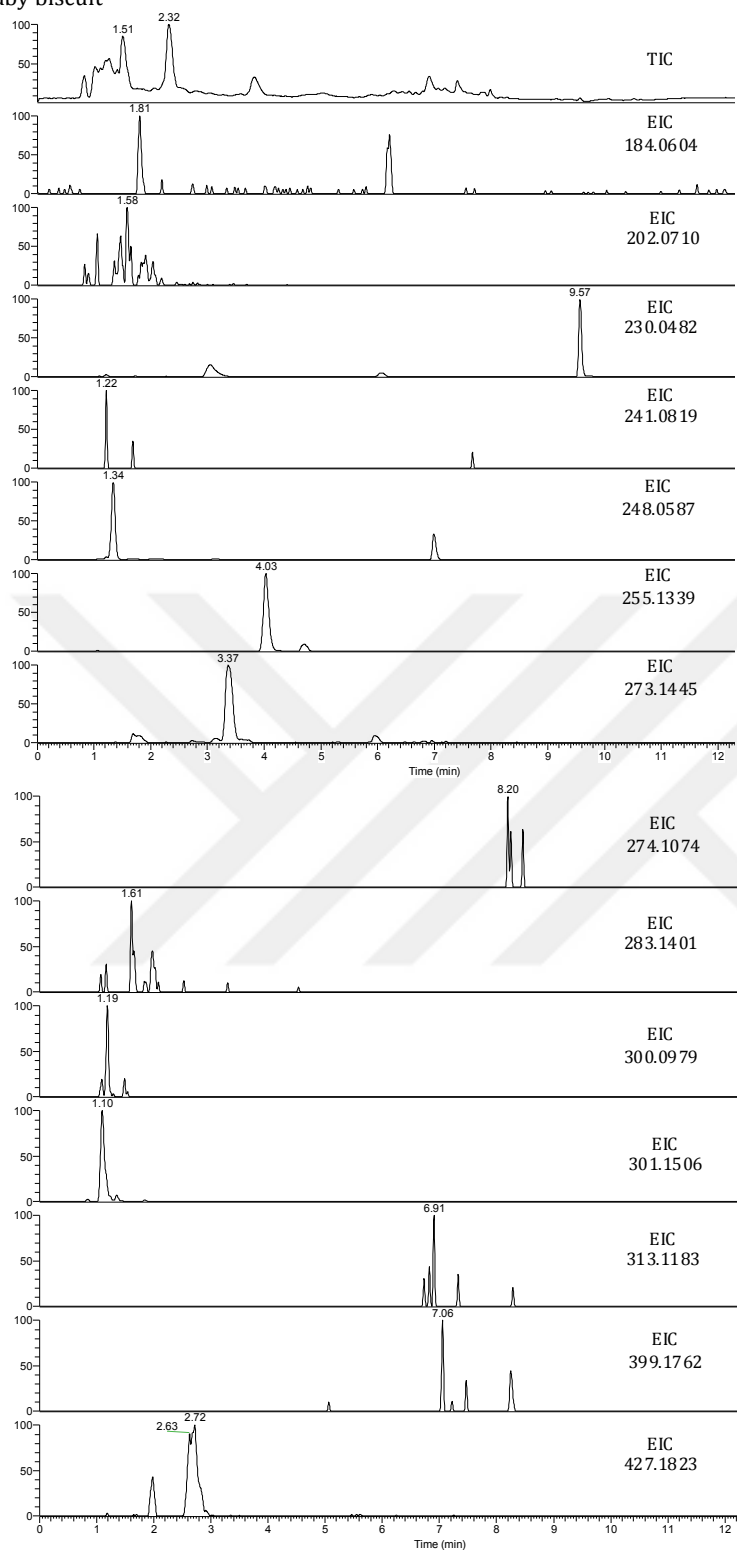


Figure A4. c Total and extracted ion chromatograms for the confirmed adducts of HMF with amino acids in the final digests of baby biscuits

twice-baked biscuits

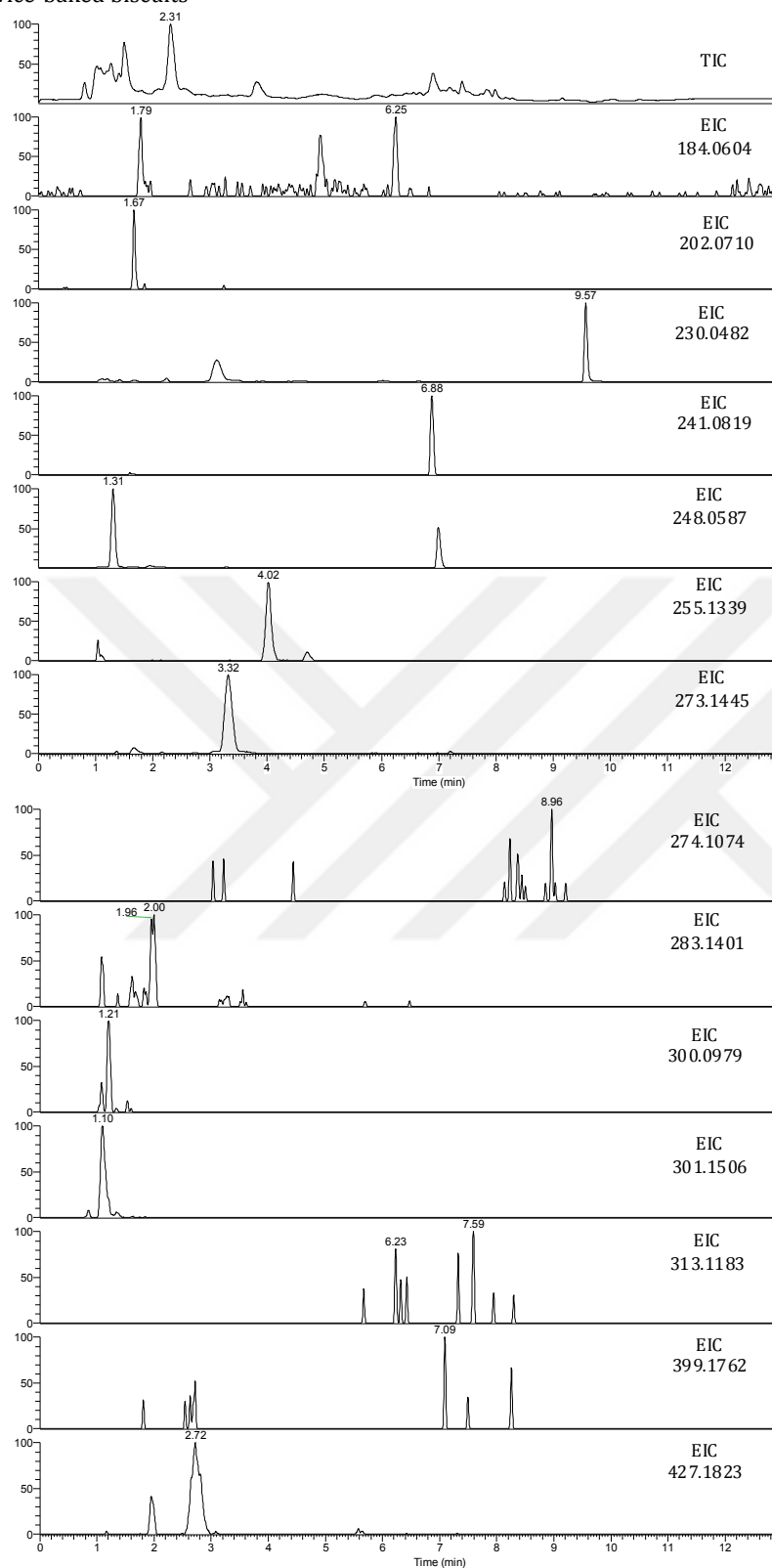


Figure A4. d Total and extracted ion chromatograms for the confirmed adducts of HMF with amino acids in the final digests of twice baked biscuits

regular biscuits

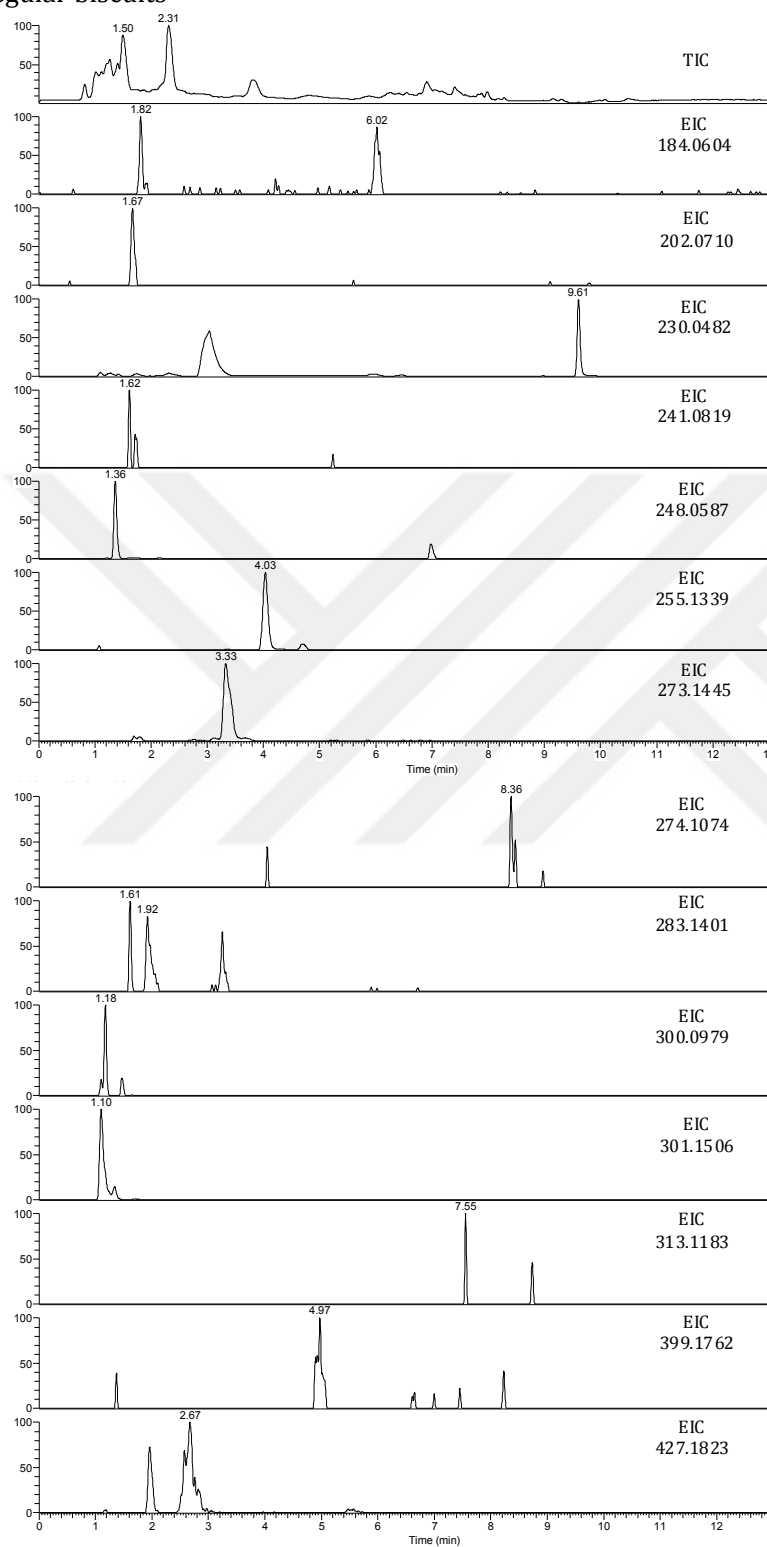


Figure A4. e Total and extracted ion chromatograms for the confirmed adducts of HMF with amino acids in the final digests of regular biscuits

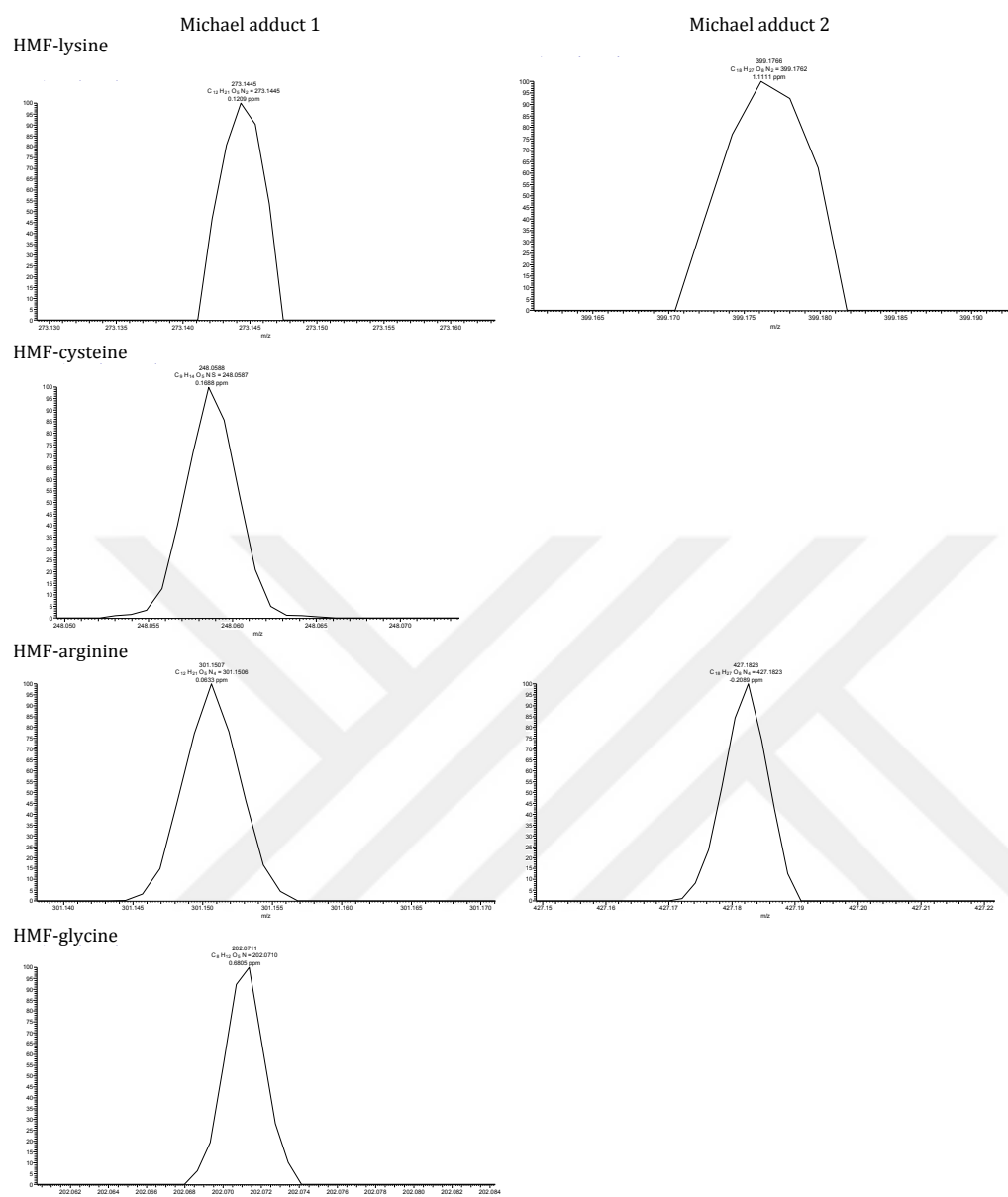


Figure A4. f Total and extracted ion chromatograms for the confirmed Michael adducts of HMF with amino acids in the final digests of different biscuits including baby, twice-baked and regular biscuits

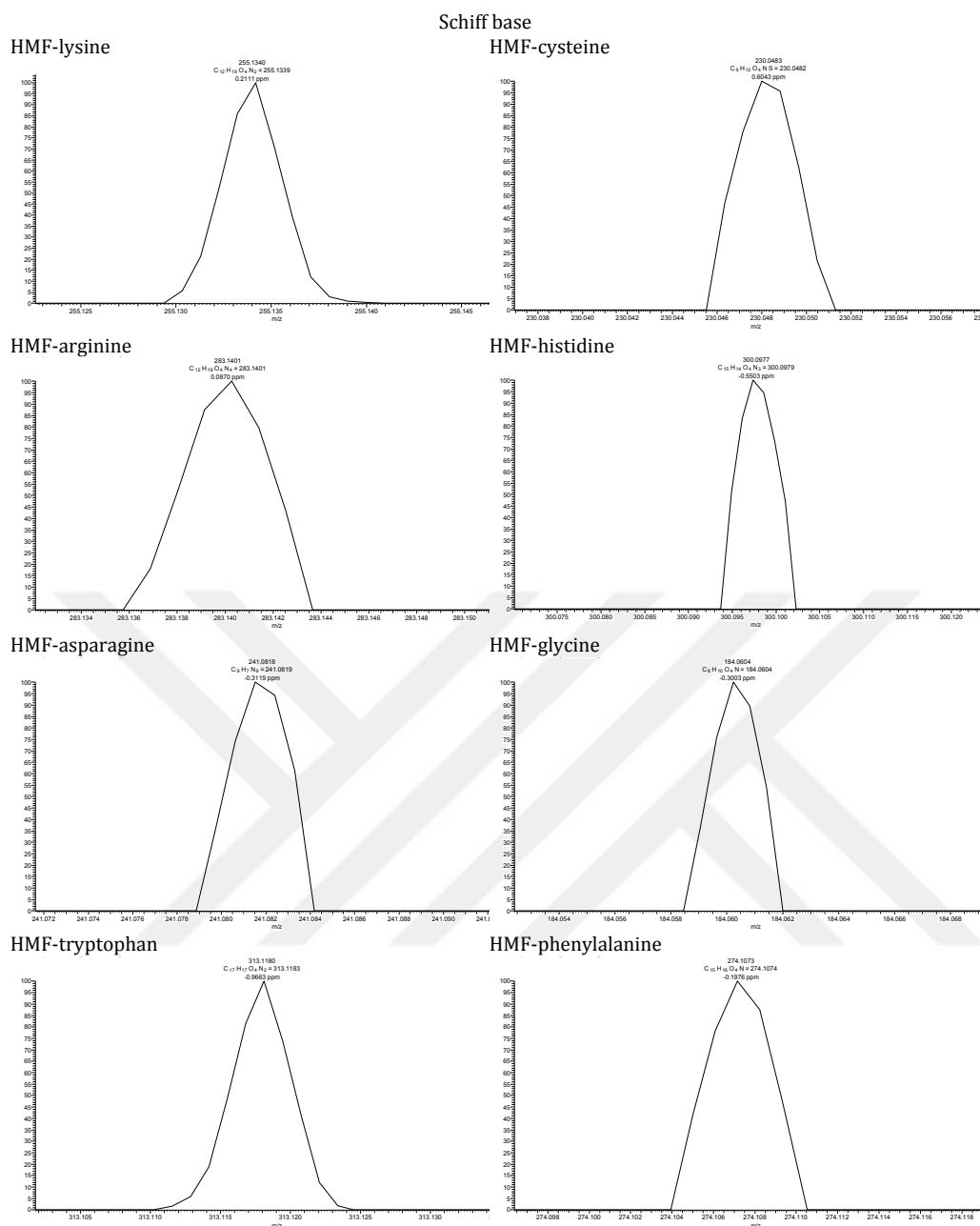


Figure A4. g Total and extracted ion chromatograms for the confirmed Schiff bases of HMF with amino acids in the final digests of different biscuits including baby, twice-baked and regular biscuits

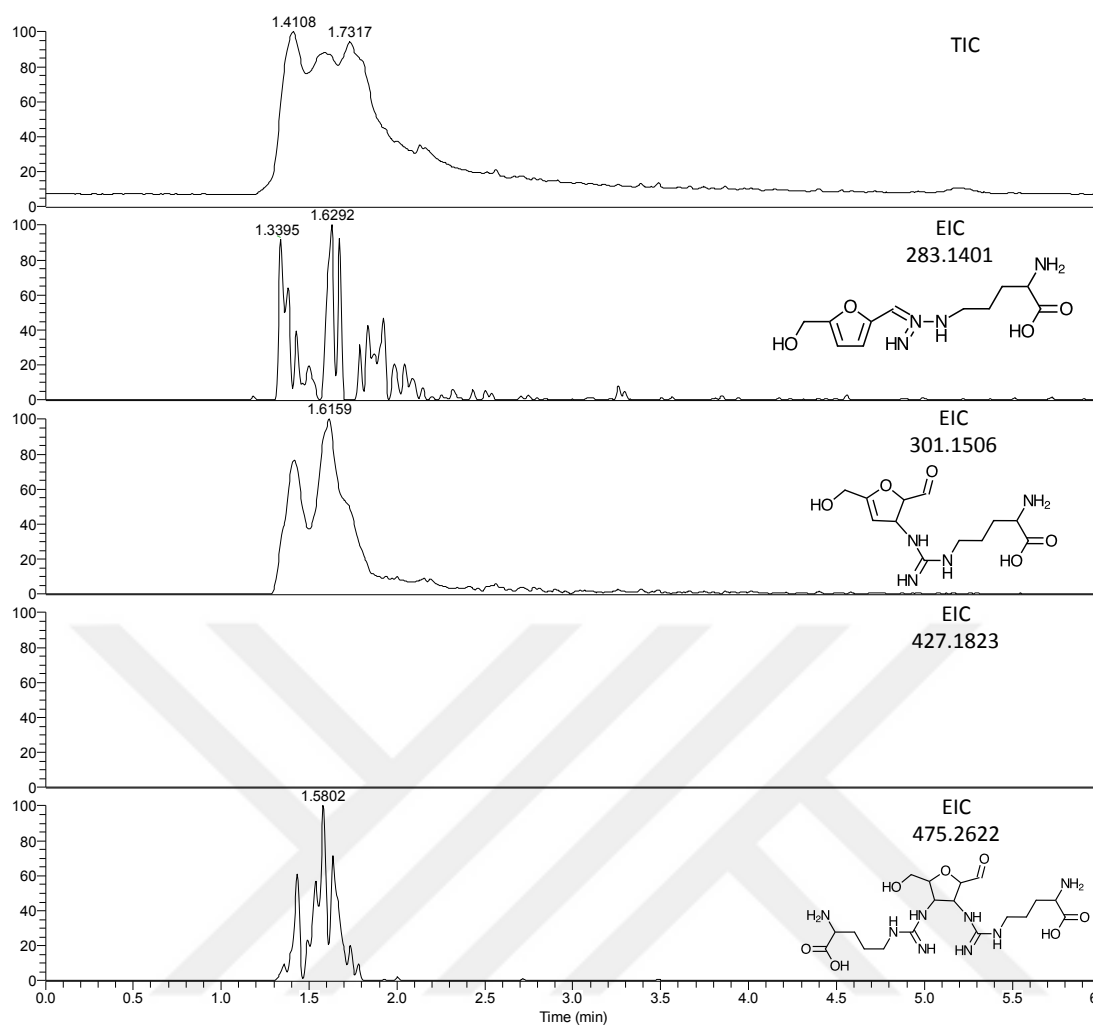


Figure A5. a Total and extracted ion chromatograms of the adducts of HMF with arginine in "HMF-Arg", model system

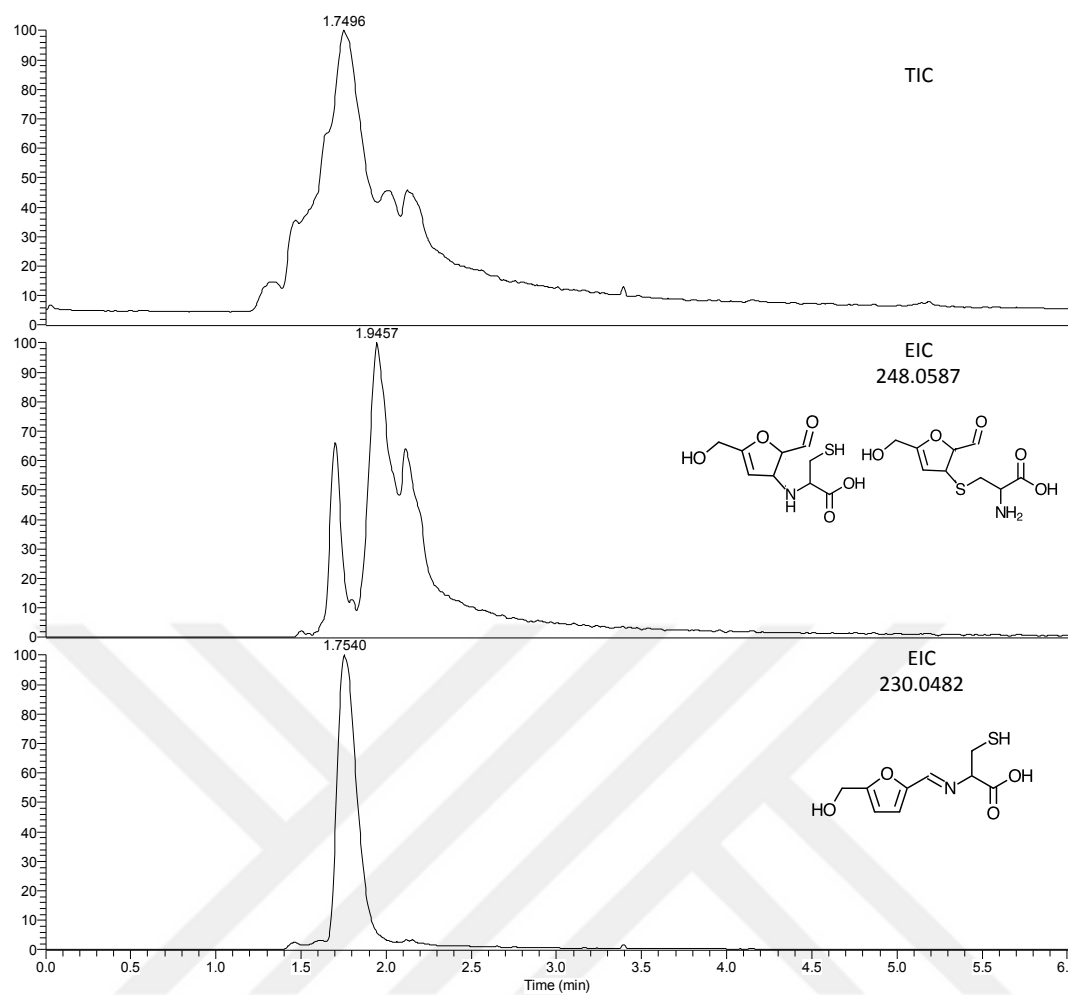


Figure A5. b Total and extracted ion chromatograms of the adducts of HMF with cysteine in "HMF-Cys", model system

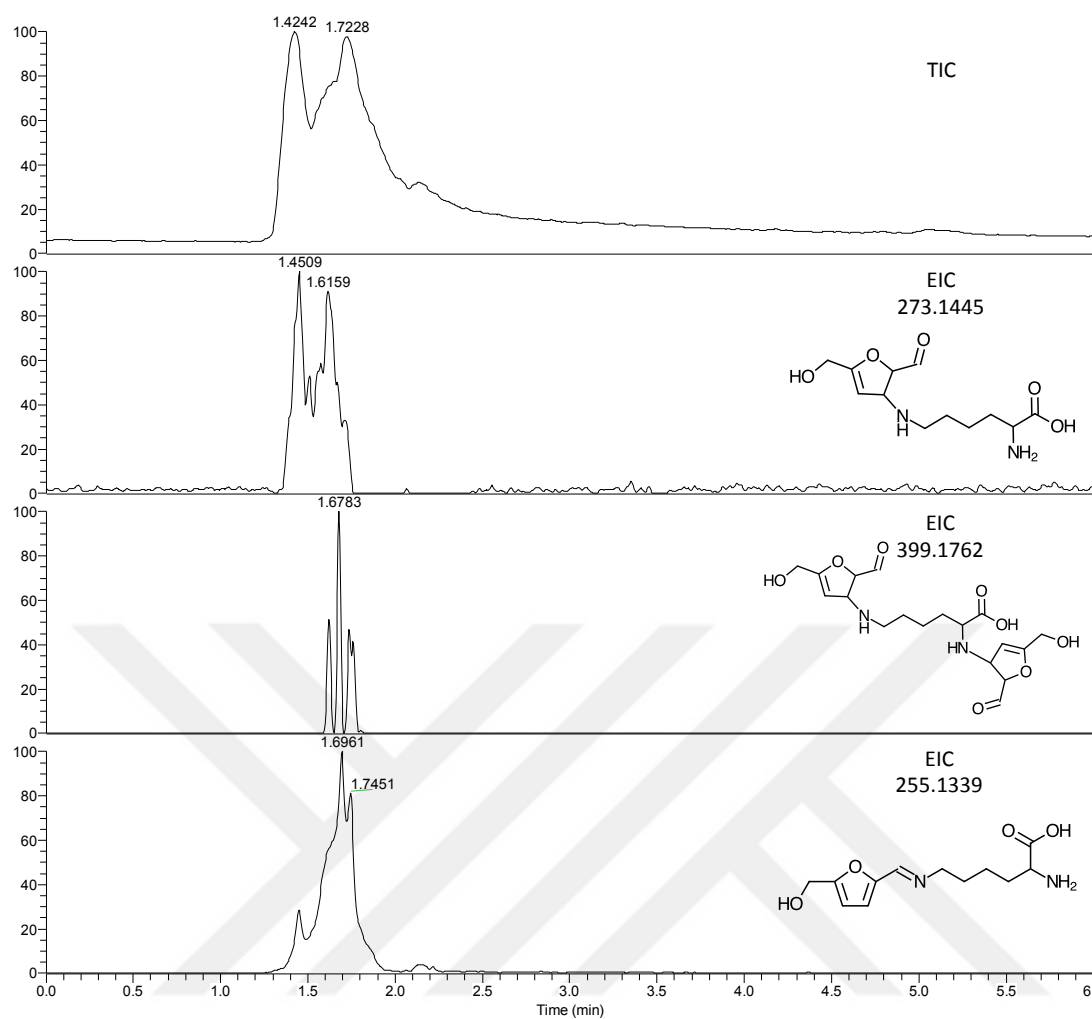


Figure A5. c Total and extracted ion chromatograms of the adducts of HMF with lysine in "HMF-Lys", model system

CURRICULUM VITAE

Credentials

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International projects:

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Publications

Presented within this PhD thesis are indicated with asterisk*

- [1]* Hamzalioglu, A., Gökmen, V., Investigation of glyoxal-scavenging effects of various sulphur compounds, wine and broccoli extracts under simulated physiological conditions, *Journal of Functional Foods* (submitted).
- [2]* Hamzalioglu, A., Gökmen, V., Investigation and kinetic evaluation of the reactions of hydroxymethylfurfural with amino and thiol groups of amino acids, *Food Chemistry* (submitted).
- [3]* Hamzalioglu, A., Gökmen, V., Effects of surface-to-volume ratio on the formation of digestible starch fractions in potatoes during baking and frying, *Journal of Food Engineering* (submitted).
- [4] Tengilimoglu-Metin, M.M., Hamzalioglu, A., Gökmen, V., Kizil, M., Inhibitory Effect of Hawthorn Extract on Heterocyclic Aromatic Amine Formation in Beef and Chicken Breast Meat, *Food Research International*, submitted.
- [5]* Hamzalioglu, A., Gökmen, V., Formation and elimination reactions of 5-hydroxymethylfurfural during in vitro digestion of biscuits, *Food Research International*, doi: 10.1016/j.foodres.2017.05.034, **2017**.
- [6]* Hamzalioglu, A., Gökmen, V., Investigations on the reactions of a-dicarbonyl compounds with amino acids and proteins during in vitro digestion of biscuits, *Food & Function*, 7, 2544-2550, **2016**.
- [7]* Hamzalioglu, A., Gökmen, V., Investigation of the reactions of acrylamide during *in vitro* multistep enzymatic digestion of thermally processed foods, *Food & Function*, 6, 109-114, **2015**.
- [8] Van Der Fels-Klerx, H. J., Capuano, E., Nguyen, H. T., Ataç Mogol, B., Kocadağlı, T., Göncüoğlu Taş, N., Hamzalioglu, A., Van Boekel, M. A. J. S., Gökmen, V., Acrylamide and 5-hydroxymethylfurfural formation during baking of biscuits: NaCl and temperature–time profile effects and kinetics, *Food Research International*, 57, 210-217, **2014**.
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Aragon, I., Online monitoring of acrylamide in biscuits using the front face fluorescence analyzer FLUORALYS, *Aspects of Applied Biology*, 116, 65-77, **2013**.

- [10] Hamzalıoğlu, A., Mogol, B.A., Lumaga, R.B., Fogliano, V., Gökmen, V., Role of curcumin on the conversion of asparagine into acrylamide during heating, *Amino Acids*, 44, 1419–1426, **2013**.
- [11] Fiore, A., Troise, A., Mogol, B.A., Roullier, V., El Mafadi, J., Gourdon, A., Hamzalıoğlu, A., Gökmen, V., Fogliano, V., Controlling Maillard reaction by reactants encapsulation: sodium chloride in biscuits, *Journal of Agricultural and Food Chemistr*, 60 (43), 10808-10814, 2012.
- [12] Hamzalıoğlu, A., Gökmen, V., Role of bioactive carbonyl compounds on the conversion of asparagine into acrylamide during Heating, *European Food Research and Technology*, 235(6) 1093-1099, **2012**.
- [13] Kocadağlı, T., Göncüoğlu, N., Hamzalıoğlu, A., Gökmen, V., In depth study of acrylamide formation in coffee during roasting: Role of sucrose decomposition and lipid oxidation, *Food & Function*, 3(9), 970-975, **2012**.

Book Chapters

- [1] Hamzalıoğlu, A., Gökmen, V., Chapter 18 - Interaction between bioactive carbonyl compounds and asparagine and impact on acrylamide. In *Acrylamide in Food: Analysis, Content and Potential Health Effects*, (Ed: Vural Gökmen), Academic Press, Elsevier, Waltham, MA, USA, **2015**.
- [2] Taş, N.G., Hamzalıoğlu, A., Kocadağlı, T., Gökmen, V., Chapter 17 - Adding Calcium to Foods and Effect on Acrylamide. In *Calcium: Chemistry, Analysis, Function and Effects*, Edited by Victor R. Preedy, The Royal Society of Chemistry, UK, **2014**.
- [3] Kocadağlı, T., Hamzalıoğlu, A., Taş, N.G., Gökmen, V., Bölüm 7 – Gıdalarda Isıl İşlem Sırasında Oluşan Zararlı Bileşikler. *Gıda Kimyası*, 4. Baskı, (Ed: İlbilge Saldamlı), Hacettepe Üniversitesi Yayınları, Ankara, **2014**.

Oral and Poster Presentations

Oral Presentations

Presented within this PhD thesis are indicated with asterisk*

- [1]* Hamzalıoğlu, A. Gökmen, V., (Invited Speaker) Investigation of the effect of different shapes on digestible starch levels in baked or fried potatoes, 13th Congress of Nutrition, 26-28 October 2016, Belgrade, Serbia.
- [2] Hamzalıoğlu, A., Gökmen, V., Bioactive carbonyls promote acrylamide formation in model systems, CEFood 2012: 6th Central European Congress on Food, 23-26 May 2012, Novi Sad, Serbia.
- [3] Hamzalıoğlu, A., Gökmen, V., 2011, Investigation of acrylamide formation in curcumin-asparagine model system, ICEF 2011: International Congress on Engineering and Food, 22-26 May 2011, Athens, Greece

Poster Presentations

- [1]* Hamzalıoğlu, A., Gökmen, V., Effect of *in vitro* gastrointestinal digestion on α -dicarbonyl compounds in biscuits, 12th International Symposium on the Maillard Reaction, Sep 1-4, 2015, Tokyo, Japan
- [2]* Hamzalıoğlu, A., Gökmen, V., Fate of acrylamide during *in vitro* multistep enzymatic digestion of thermally processed foods, 4th International Conference on Food Digestion, March 17-19, 2015, Naples, Italy
- [3] Van der Fels-Klerx, H.J., Nguyen, H.T., Mogol, B.A., Capuano, E., Kocadağlı, T., Göncüoğlu, N., Hamzalıoğlu, A., Gökmen, V., Van Boekel, M.A.J.S., Kinetic modeling of the formation of acrylamide and HMF in biscuits, EuroFoodChem XVII, 7-10 May 2013, Istanbul, Turkey
- [4] Hamzalıoğlu, A., Gökmen, V., Role of bioactive carbonyl compounds on the conversion of asparagine into acrylamide during heating, 2013, EuroFoodChem XVII, 7-10 May 2013, Istanbul, Turkey
- [5] Hamzalıoğlu, A., Gökmen, V., Melting point of carbonyls strongly influence the conversion of asparagine into acrylamide under low moisture conditions, 11th International Symposium on the Maillard Reaction "Centenary of the Maillard Reaction Discovery (1912-2012)", 16-20 September 2012, Nancy, France
- [6] Kocadağlı, T., Göncüoğlu, N., Hamzalıoğlu, A., Gökmen, V., In depth study of acrylamide formation in coffee during roasting, 11th International

Symposium on the Maillard Reaction “Centenary of the Maillard Reaction Discovery (1912-2012)”, 16-20 September 2012, Nancy, France

- [7] Hamzalıoğlu, A., Gökmen, V., Investigation of 3-APA and acrylamide formation in carbonyl-asparagine model systems, RAFA 2011, 1-4 November 2011, Prague, Czech Republic





HACETTEPE UNIVERSITY
GRADUATE SCHOOL OF SCIENCE AND ENGINEERING
THESIS/DISSERTATION ORIGINALITY REPORT

HACETTEPE UNIVERSITY
GRADUATE SCHOOL OF SCIENCE AND ENGINEERING
TO THE DEPARTMENT OF FOOD ENGINEERING

Date: 21/06/2017

Thesis Title / Topic: INVESTIGATION OF THE REACTIVITIES OF MONOMERIC AND POLYMERIC COMPONENTS IN SOME FOODS IN DIGESTION CONDITIONS IN VITRO

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