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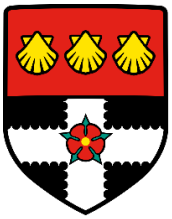

Formation and control of heterocyclic amines and polycyclic aromatic hydrocarbons during meat processing

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Declaration

I confirm that this is my own work and the use of all materials from other sources has been properly and fully acknowledged.

Fei Lu

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-书山有路勤为径，学海无涯苦作舟-

谨以此文献给我的父母，来生我还做你们的女儿

Abstract

Heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) formed during meat processing may pose health risk to the public. This project aimed to investigate the occurrence of HCAs and PAHs in highly consumed cooked meat products including ready-to-eat (RTE) meat, patties and meatballs, and their health risk was also assessed according to the dietary pattern. Different strategies including replacing fat with vegetable oils and adding spices were applied in order to reduce the formation of HCAs and PAHs in final meat products. In addition, inhibitory mechanism of antioxidants in oil and spices on the formation of HCAs and PAHs in meat system were also discussed. In this work, HCAs and PAHs were extracted by solid-phase extraction and analysed by HPLC- Diode array UV/ Fluorescence detector.

For RTE meat in UK, chargrilled chicken had the highest level of HCAs (37.45±4.89ng/g) and PAHs (3.11±0.49ng/g), followed by roasted bacon (HCAs 15.24±1.31ng/g, PAHs 1.75±0.17ng/g) in selected RTE meat products. Increase intake of chargrilled chicken and ham could increase breast cancer and colorectal adenoma risk, but other types of meat had relatively lower health risk.

Replacing pork back fat with vegetable oils including sunflower oil, olive oil and grape seed oil could not only improve fatty acids profile in cooked meat products, but also reduce HCAs, which could be attributed to the existence of tocopherols and polyphenol compounds in the vegetable oils. However, antioxidants in the oils could not reduce the total amount of PAHs effectively, while the complexity of oil decomposition and antioxidants performance at high temperature could partially explain the case.

All 6 spices powder including garlic, onion, red chilli, paprika, black pepper and ginger reduced the formation of total HCAs, while ginger powder achieved the highest inhibition efficiency compared with all other spices. Antioxidant capacity of spices determined their efficiency in prohibiting formation of HCAs and PAHs in great extent, while meat type only affected the formation of HCAs ($p<0.05$), but not PAHs ($p>0.05$). Regression model

suggested that both diallyl disulfide and gallic acid contributed similar inhibitory efficiency on the formation of HCAs and PAHs. Synergistic effect between diallyl disulfide and gallic acid was observed on reducing HCAs ($p<0.05$), but not on PAHs ($p>0.05$).

Abbreviations

4,8-DiMeIQx	2-amino-3,4,8-trimethylimidazo[4,5-*f*]-quinoxaline
AIA(s)	Aminoimidazoazarene(s)
AAPH	2,2'-Azobis(2-methylpropionamidine) dihydrochloride
ABTS·	2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation
AC(s)	Amino-carbolines
ANOVA	Analysis of variance
ARP	Amadori rearrange product
BaA	Benzo[a]anthracene
BaP	Benzo[a]pyrene
BbF	Benzo[b]fluoranthene
BHT	Butylated hydroxytoulene
BSA	Bovine serum albumin
Ch	Chrysene
DAD	Diallyl Disulfides
DAD/FLD	Diode array detection/ fluorescence detector
DNPH	Dinitrophenylhydrazine
EFSA	European Food Safety Authority
FLD	Fluorescence detection
GA	Gallic acid
GAE	Gallic acid equivalent
HCA(s)	Heterocyclic amine(s)
HPLC	High performance liquid chromatography
IARC	International Agency for Research on Cancer
IQ	2-amino-3-methylimidazo[4,5-f]-quinoline
LOD	Limit of detection
LOQ	Limit of quantification
MeIQ	2-amino-3,4-dimethylimidazo[4,5-f]-quinoline
MeIQx	2-amino-3,8-dimethylimidazo[4,5-f]-quinoxaline
MUFA(s)	Monounsaturated fatty acid(s)
MRP(s)	Maillard reaction product(s)

Nd	Not detected
NDNS	National Diet and Nutrition Survey
Nq	Not quantified
ORAC	Oxygen radical antioxidant capacity
PAH(s)	Polycyclic aromatic hydrocarbon(s)
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine
PRS	Propyl sulfonic acid
PUFA(s)	Polyunsaturated fatty acid(s)
ROS	Reactive oxygen species
RSD	Relative standard deviation
RTE	Ready-to-eat
SFA(s)	Saturated fatty acid(s)
SD	Standard deviation
SPE	Solid phase extraction
TBARS	Thiobarbituric acid-reactive substances
TCA	Trichloroacetic acid
TEAC	*Trolox* equivalent antioxidant capacity
TEF	Toxicity Equivalency Factor
TEP	1,1,3,3-tetraethoxylpropane
TPA	Texture profile analysis
TPC	Total phenolic content
UV	Ultraviolet
WHO	World Health Organization

Table of Contents

List of Tables

Lists of Figures:

List of Appendix:

Chapter 1. Introduction and Literature review

1.1 Introduction

Meat is a good source of protein, essential minerals such as iron and zinc, vitamin B and D in daily diet (Ferguson, 2010). Meat consumption increased from 23.1 kg per person per year to 42.20 kg per person per year in the world since 1961 (Sans & Combris, 2015). However, the World Cancer Research Fund (WCRF) report stated that there is a strong link between red meat/ processed meat intake and risk of colorectal cancer. A cohort study indicated that 17-18% increased risk of cancer when people consume 100g red meat or 50g processed meat per day (Thompson, 2015). Cancer Research UK (2015) reported that approximately 21% of colorectal cancer in the UK is associated with consuming red and processed meat. This could be partially attributed to the high level of carcinogenic compounds produced during cooking process, such as heterocyclic amines (HCAs), polycyclic aromatic hydrocarbons (PAHs) and nitrosamines (McAfee et al., 2010). Due to the increase of meat consumption and associated incidence of cancer, the presence and hazard of heat-induced HCAs and PAHs in meat products has become a major concern for both consumers and researchers. Zheng and Lee (2009) indicated that high intake of well-done meat would increase the exposure to HCAs and consequently lead to incidence of colon cancer. Dietary exposure to PAHs from grilled meat has been found linked with elevating risk of lung, breast and gastrointestinal cancer (Jägerstad & Skog, 2005). Thus, it is essential to assess the presence of both HCAs and PAHs when evaluate the relationship between meat intake and cancer risk.

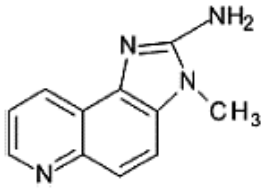
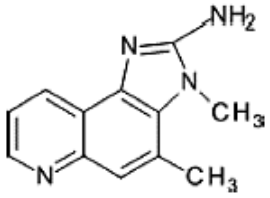
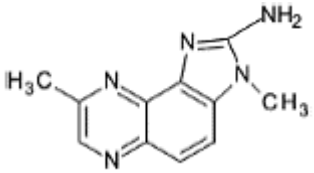
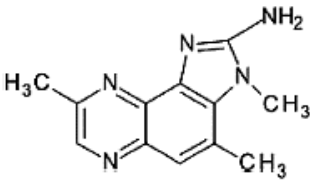
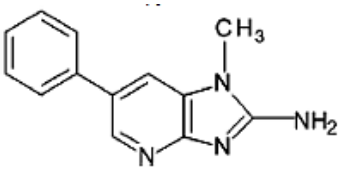
1.2 Formation of HCAs and PAHs

1.2.1 Classification of HCAs

HCAs were firstly discovered in cooked meat products at 1970s, and they are usually generated in heated animal protein-rich foods (Sugimura et al., 2004). There are 2 main types of HCAs, aminoimidazoarenes (AIAs) and amino-carbolines (ACs) (Rahman et al., 2014). ACs are generally formed by pyrolysis of amino acids when cooking temperature is over 300℃, including pyridoindoles such as 2-amino-9H-pyrido[2,3-b]indole (AαC), 3-amino-1,4-

dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) and other carbolines, e.g. 2-amino-5-phenylpyridine (Phe-P-1).

Table 1-1: Chemical structure and carcinogenicity classification of IQ, MeIQ, MeIQx, 4, 8-DiMeIQx and PhIP

Name	Structure	Carcinogenicity[1]
2-amino-3-methylimidazo [4,5-f]quinoline (IQ)		Group 2A
2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ)		Group 2B
2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx)		Group 2B
2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4,8-DiMeIQx)		Group 2B
2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)		Group 2B

[1]Classified by International Agency for Research on Cancer (IARC).

AIAs have been reported generated mainly through Maillard reaction in meat cooked at 150-250℃, which is common temperature used in domestic cooking (Turesky, 2010; Zamora, Alcón, & Hidalgo, 2012). Common AIAs are

2-amino-3-methylimidazo [4,5-f]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8 trimethylimidazo[4,5-f]quinoxaline (4,8-DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). With the accumulating evidence, IARC (1993) has classified IQ as Group 2A (probable human carcinogens), MeIQ, MeIQx, DiMeIQx and PhIP as Group 2B (possible human carcinogens). These compounds are the major research concern because of their potential carcinogenicity. All these polar HCAs contain an imidazole ring with amino and methyl group attached (Table 1-1).

1.2.2 Formation of HCAs

Maillard reaction has been proposed as the main pathway of forming HCAs (Pearson et al., 1992; Turesky, 2010). Main precursors of HCAs are free amino acids, reducing sugar and creatine (Gibis, 2016). Figure 1-1 illustrates that pyrazines and pyridines are intermediates of imidazoquinoxaline and imidazoquinoline in Maillard reaction, respectively. At the early stage, unstable Schiff base could be formed through the reaction between free amino acids and sugar with the loss of water, and convert into N- substituted glycosylamine. At pH of 5.0-6.5 in meat, Schiff base could transform into Amadori rearrange product (ARP), namely, N-substituted-1-amino-1-deoxy-2-ketose (glycol-aldehyde alklyimine, enol type) via Amadori rearrangement and two-carbon fragmentation. Subsequently, glycol-aldehyde alklyimine could undergo biomolecular ring formation to generate dialkyl-dihydro pyrazine and then become dialkyl-pyrazine radicals by losing electron. At the same time, glycolaldehyde alkylimine might also be molecularly rearranged and oxidized into glyoxal monoalkylimine which could further react with amino acids and generate glyoxal. Pyridine radicals are formed via condensation between glyoxal and glyoxal monoalkylimine. The original existing aldehydes and creatinine in meat system could react with pyridine radicals to form imidazoquinoline (IQ and MeIQ), while with dialkyl pyrazine radicals to form imidazoquinoxalines (MeIQx and DiMeIQx).

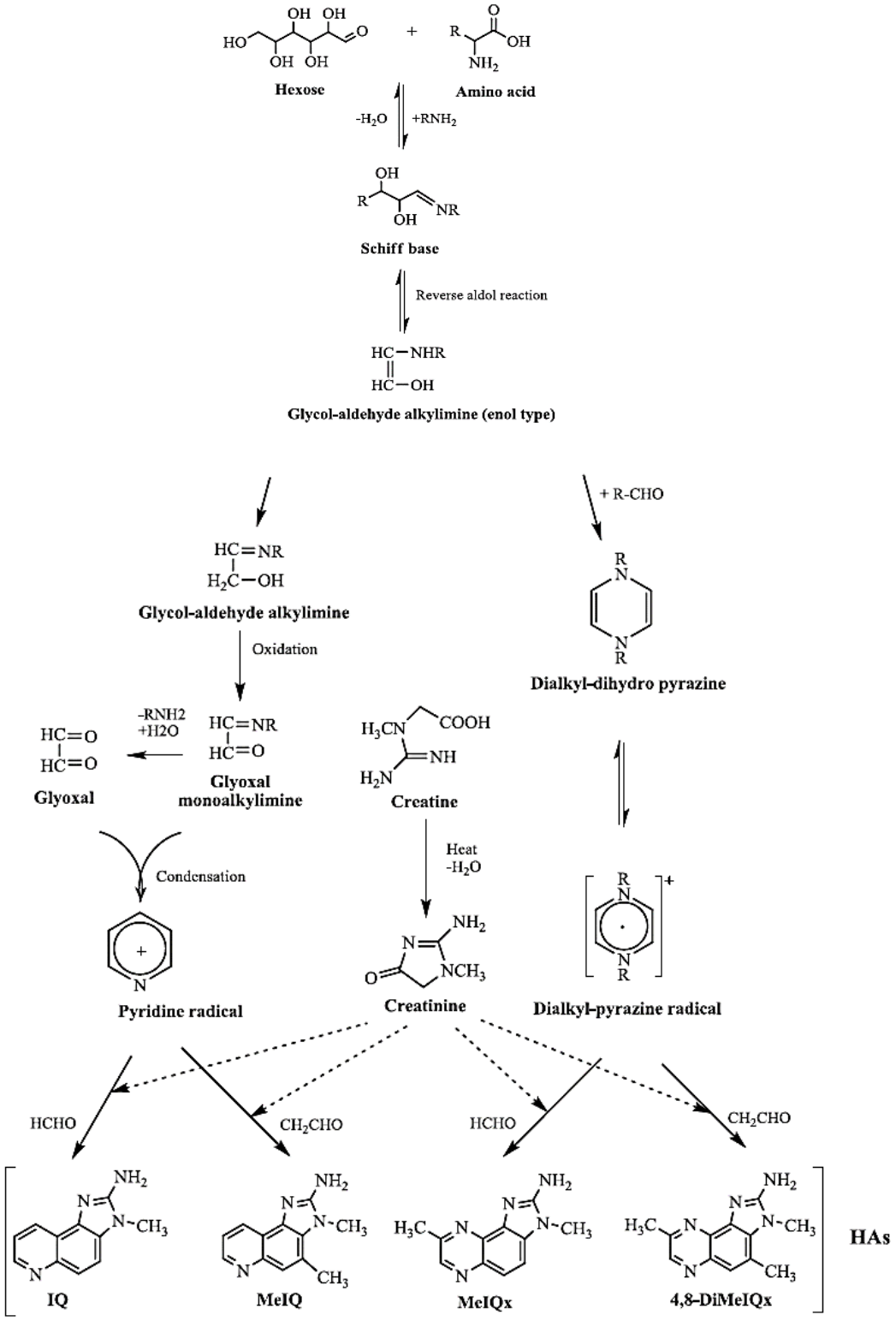


Figure 1-1: Proposed pathway of imidazoquinolines (IQ and MeIQ) and imidazoquinoxalines (MeIQx and 4, 8-DiMeIQx)[1]

[1]Source from Vitaglione & Fogliano (2004).

PhIP can be formed by phenylalanine, creatine and reducing sugars (Murkovic, 2004; Zöchling & Murkovic, 2002). The suggested pathway was showed in Figure 1-2. Firstly, phenylalanine could undergo either thermal degradation or Strecker degradation to generate phenylacetaldehyde, which is a critical intermediate during formation of PhIP. Subsequently, aldol addition product (A in Figure 1-2) could be formed via aldolisation between phenylacetaldehyde and creatinine, and it could be quickly transformed into aldol condensation product with loss of water (B in Figure 1-2). At last, aldol condensation product could further react with amino moiety from phenylalanine or 2-phenylethylamine to generate PhIP. High temperature, prolonged heating time, high cooking loss and high content of creatine are considered as essential factors accelerating the formation of HCAs (Zöchling & Murkovic, 2002).

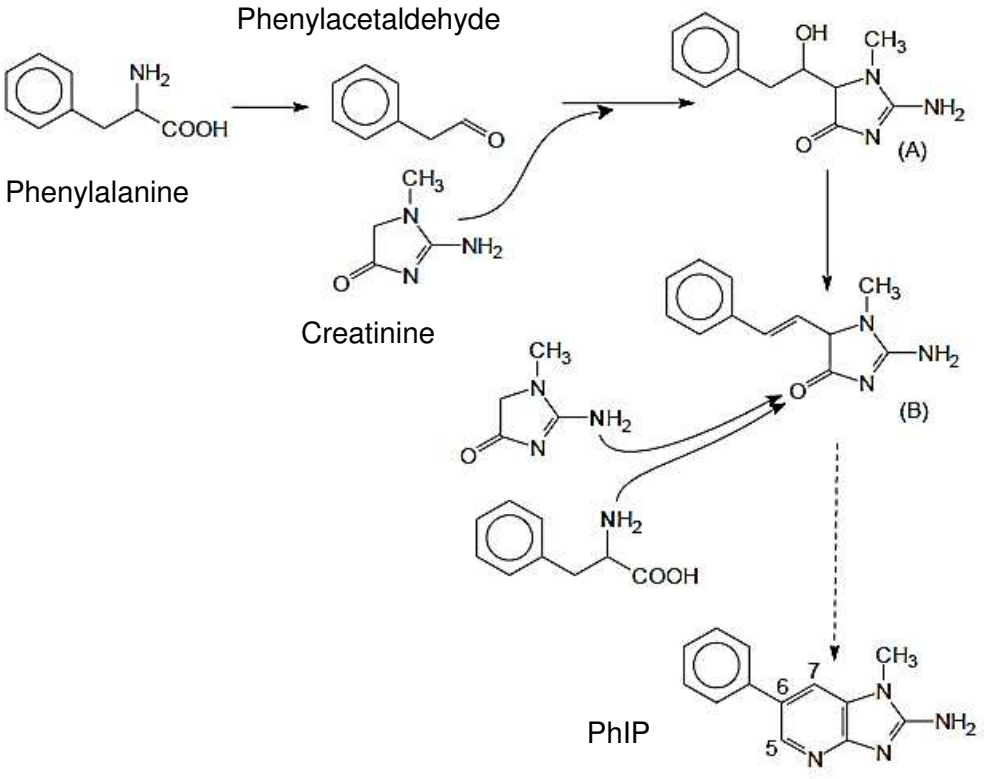


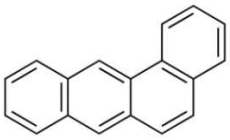
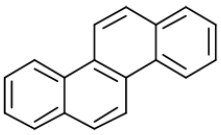
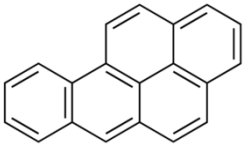
Figure 1-2: Proposed pathway of PhIP in meat products. (A) aldol addition product, (B) aldol condensation product[1]

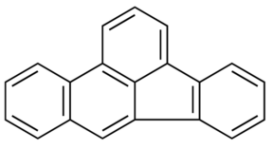
[1]Source from Murkovic (2004).

1.2.3 Classification of PAHs

PAHs are hydrocarbons containing two or more fused benzene rings, such as pyrene, anthracene and naphthalene. 'Light PAHs' is defined as structure of PAHs containing 2-4 benzene rings, while 'heavy PAHs' containing 5 or more fused benzene rings (Farhadian et al., 2010). There are hundreds PAHs existing in air, soil, water and foods. Some PAHs have been reported with potential carcinogenic activity by IARC, such as BaA and BaP have been recognized as probably carcinogenic to humans (2A), Ch and BbF as possibly carcinogenic to humans (2B) (Table 1-2). BaA, BaP and BbF also had sufficienct evidence of carcinogenicity in laboratory animalsb (IARC, 2010). Human can be exposed to PAHs through inhalation, skin absorption and ingestion (PHE, 2008; Purcaro, Moret, & Conte, 2013). The structure, carcinogenicity and toxicity equivalency factor (TEF) of BaA, Ch, BaP and BbF have been summarized in Table 1-2. Except unavoidable occupational exposure to exhaust fume, fume from gas or metal factories, and major route of exposure to PAHs for general public are cigarette smoke and consumption of food containing PAHs.

Table 1-2: Chemical structure, carcinogenicity classification and Toxicity Equivalency Factor of BaA, Ch, BaP and BbF

Name	Structure	Carcinogenicity[1]		Toxicity equivalency factor (TEF)
		Human[2]	Animal[3]	
Benz[a]anthracene (BaA)		2A	SE[2]	0.1
Chrysene (Ch)		2B	LE[3]	0.1
Benzo[a]pyrene (BaP)		2A	SE	1

Benzo[b]fluoranthene (BbF)		2B	SE	0.01

[1] Carcinogenicity of PAH4 classified by IARC (2010).

[2] IARC evaluation: 2A: probably carcinogenic to humans; 2B: possibly carcinogenic to humans.

[3] IARC classification on degree of evidence of carcinogenicity in laboratory animals: sufficient evidence; LE: limited evidence.

1.2.4 Formation of PAHs

The exact mechanism of forming PAHs in processed meat products has not been confirmed yet. However, 3 proposed pathways have been generally accepted. PAHs can be formed through the incomplete combustion or pyrolysis of organic components including fat, protein and carbohydrates at temperature over 200℃, especially above 400℃ (Alomirah et al., 2011); they can be generated in the smoke when lipid dropping onto flame, consequently deposited on food surface. In addition, the incomplete combustion of charcoal also contributes to the development of PAHs (Farhadian et al., 2010; Singh, Varshney, & Agarwal, 2016).

The whole process of forming PAHs contained a series of radical reactions (D'Anna & Violi, 1998; Wang & Frenklach, 1997; Wang, Raj, & Chung, 2013). At high temperature, small molecules such as propane and ethylene, are generated by fragmentation of large organic compounds (pyrolysis). Some key reactions during the formation of benzene ring are listed below, including propargyl recombination (a) and n-C_4H_3 with acetylene (b). The ring is enlarged by adding ethynyl side chain on benzene ring or ring-ring condensation (Figure 1-3). Combination reactions of hydrogen and larger aromatic radicals, including napthyl and phenanthryl radicals lead to grow mass of PAHs (Wang & Frenklach, 1997). Propargyl recombination has been reported as major route, as they are stabilized free radicals, while n-C_4H_3 could easily transform into i-C_4H_3, which had less contribution to forming benzene ring (D'Anna & Violi, 1998).

Benzene formation:

$C_3H_3 + C_3H_3 \rightarrow C_6H_5 + H$ (a)

$n\text{-}C_4H_3 + C_2H_2 \rightarrow C_6H_5$ (b)

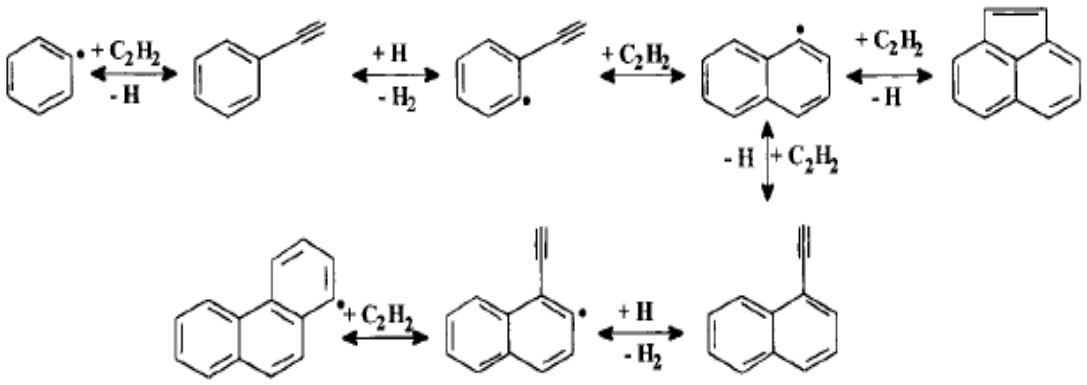


Figure 1-3: proposed pathway of enlarging aromatic ring[1]

[1]Source from D'Anna & Violi (1998).

1.3 Cancer risk of HCAs and PAHs

1.3.1 HCAs and cancer

1.3.1.1 Animal trials

The carcinogenicity of these AIAs on organs/tissues has been tested in long-term animal trials. Rats and mice had been orally exposed to specific amount of IQ, MeIQ, MeIQx and PhIP over several months, and features of their target organs have been observed. Results showed that intake of these HCAs induced tumours in several organs, including liver, intestine, mammary gland and forestomach (Sugimura et al., 2004). Particularly, possibly carcinogen (2A) IQ could also cause hepatocellular adenomas and carcinomas, adenomas and adenocarcinomas of the lung and squamous-cell papilloma and carcinomas of the fore stomach in mice (IARC, 1993). However, results from experimental animal might be limited since amount of human intake of HCAs is not comparable with animal trials. Threshold dose 50 (TD_{50}) is one of the key measurements (in mg/kg body weight/day) used in animal trials to show the dosage level of inducing tumour for half of test animals compared with those with zero dose. TD_{50} of HCAs ranged 0.1-2.2 mg/kg/day in rats and 8.4-

64.6 mg/kg/day in mice (Sugimura et al., 2004). However, these carcinogenic dose data were much higher than human daily intake of HCAs with average 50-1820 ng/ person/day (Layton et al., 1995; Sugimura et al., 2004; Wong et al., 2005). Therefore, animal trials may not be able to explain the risk accurately between HCAs and cancer in human.

1.3.1.2 Epidemiological human studies

Epidemiological studies revealed that there is a positive association between the risk of cancer and frequent consumption of red/processed meat, especially fried, grilled/roasted meat and fish products (Ferguson, 2010). The occurrence of carcinogens in these cooked meat might explain the association between meat intake and cancer risk (Chan et al., 2011; Gibis, 2016). Le Marchand et al. (2002) found out that rectal cancer risk in male could increase 2-3 times with the increasing intake of HCAs (MeIQx, DiMeIQx and PhIP), especially MeIQx ($p<0.05$) from a population-based case-control study in Hawaii area. Consistent results were obtained from a cohort study in European Prospective Investigation into Cancer and Nutrition (EPIC)-Heidelberg, which reported that increasing intake of MeIQx, DiMeIQx and PhIP could increase the risk of colorectal adenomas ($p<0.05$) (Barbir et al., 2012). Similarly, Fu et al. (2011) concluded a significant positive association between high intake of HCAs (MeIQx, DiMeIQx and PhIP) and colorectal polyp (adenomas) ($p<0.05$).

Increasing intake of HCAs is also associated with increasing risk of breast, lung and prostate cancer. Breast Cancer UK (2017) pointed out that breast cancer is the most common cancer in women in the UK, 1 in 8 women would be diagnosed breast cancer in lifetime. Stefani et al. (1997) conducted a hospital-based case-control study and found that intake of HCAs (IQ, MeIQx and PhIP) generated in cooked meat might be strongly related to the increasing risk of breast cancer. Lam et al. (2009) conducted a population-based case-control study in Italy and stated that intake of MeIQx and PhIP was positively associated with the risk of lung cancer with the Odds Ratio (OR) 1.4-1.5 (95% CI: 1.2-1.8 and 1.2-1.7) ($p<0.001$).

1.3.2 PAHs and cancer

Although plenty of PAHs can be found in the environment (water, air and soil), food is another main source of exposure to PAHs. Dietary PAHs exposure from food, including grilled/ barbequed/smoked meat, cereal and vegetables grown in soil provides 90% of total PAHs exposure for non-smoking and non-occupational exposure in general public (EPA, 2012). Intake of PAHs ranged from ng/person/day to mg/person/day (Alomirah et al., 2011; IARC, 2010; Purcaro et al., 2013). Although European Food Safety Authority (EFSA) reported that PAH4 (Sum of BaP, Ch, BaA and BbF) could be the marker for total PAHs, BaA and BaP are the PAHs with the most potent carcinogenicity (Group 2A) found in meat products (Table 1-2). The reactive metabolite of BaP, BaP-7, 8-diol-9, 10-epoxide, has the highest ability of inducing tumours by generating adducts with bio protein or DNAs (Purcaro et al., 2013).

1.3.2.1 Animal trials

IARC (2010) reported that there was sufficient evidence to show the carcinogenicity of BaA and BaP to experimental animals. Oral exposure to BaP increased the incidence of tumours at alimentary tract and forestomach in rats & mice with the dosage 0.2-10 mg/kg/day (EPA, 2012). Long-term inhale exposure to BaP (10mg/m^3) in Syrian golden hamsters also induced respiratory tract cancer and gastrointestinal tract tumours (WHO, 2000). Exposure to BaP could also induce skin tumours in mice, rats, rabbits, and guinea pigs (EPA, 2012). The analysis has been focused on chronic carcinogenicity bioassays in several strains of mice following repeated dermal exposure (2- or 3-times/week exposure) to BaP during the animals' lifetime. New-born mice obtaining intraperitoneal injections of 2.8 μmol BaA for 6 weeks (3 times/week) showed significantly increase incidence and number of pulmonary tumours per mouse comparing with the controls (Levin et al., 1984).

1.3.2.2 Human studies

Epidemiological studies in Germany and Sweden confirmed that occupational exposure to PAHs, such as from gasoline and coal tars industries, increased incidence of lung cancer and bladder cancer (Boffetta, Jourenkova, & Gustavsson, 1997). For non-occupational population, epidemiological studies

showed that high intake of barbequed/grilled meat products containing PAHs, had association with risk of stomach and esophagus cancer (Ward et al., 1997) and pancreatic cancer (Norell et al., 1986). However, the relationship between dietary intake of PAHs and risk of cancer is still not conclusive, since there are other carcinogens such as HCAs and N-nitroso compounds presented in meat products, which could also contribute to the posing risks.

1.3.2.3 Regulations

Europe (Belgium and Spain), Asia (China and Malaysia), Canada and USA have regulated occupational exposure limit is 0.2-mg/m^3 time-weighted average for the benzene-soluble fraction, including anthracene, BaP, Ch, phenanthrene and pyrene (IARC, 2010).

Cereal/bread and cooked meat are main contributors of dietary PAH intake (IARC, 2010). European Commission (EC) regulated that the maximum of BaP in smoked meat and fish products is 2 ng/g and total PAH4 should be less than 12 ng/g (Regulation No. 1881/2006, from 01/09/2014). It is also regulated that BaP in heat treated meat and commercial meat products, such as grilled/barbequed meat should be less than 6 ng/g, and PAH4 should be lower than 35 ng/g (No. 835/2011) (Purcaro et al., 2013).

1.4 HCAs/PAHs associated meat products in different countries

A number of epidemiological studies have reported dietary intake of HCAs and PAHs from cooked meat worldwide, including USA, Europe and Asia. The amount of individual HCAs and estimated daily intake of HCAs in different countries were summarized in Table 1-3. The amount of HCAs and estimated dietary intake in the US (407-1820 ng/person/day) were much higher compared with other countries, which could be explained by high consumption of grilled or fried meat in the US (Gibis, 2016). The HCAs intake in Spain was 663 ng/ person/ day and 554 ng/person/day in Malaysia. The difference in intake between the two countries could be due to cooking practice. Grilling and pan-frying are the popular cooking methods in Spain responsible of high amount of HCAs, compared with steaming and boiling, which are favorable in Malaysia. High frequency of meat consumption in these countries could also

contribute to the increase in HCAs intake (Busquets et al., 2004; Jahurul et al., 2010). Relatively lower intake of total HCAs was found in Singapore (50 ng/person/day) and Japan (64.5 ng/person/day). PhIP was the major contributor to dietary intake of HCAs, followed by MeQIx (Table 1-3).

Table 1-4 showed the amount of BaP, other PAHs and total PAHs intake in different countries. Since BaP is the well-known PAH with high carcinogenic potential, the amount of BaP in meat products has been determined in all studies. BaP content in cooked meat and fish products was high in Estonia (<0.3-31.2 ng/g) and Denmark (0-24 ng/g), followed by in Malaysia (0-12.5 ng/g) and China (0-10.5 ng/g). However, determination of other/ total PAHs was not consistent among studies. For example, some studies measured PAH4, while PAH8 or PAH16 were also determined in other studies. Aaslyng et al. (2013) determined PAH8 in barbequed beef, chicken and pork was 19.7-229 ng/g in Denmark, while Alomirah et al. (2011) reported 0.7-42.9 ng/g PAH8 in grilled and smoked meat products in Kuwait. The amount of PAH12 in meat and fish products was 0.59-11.34 ng/g in the UK (Dennis et al., 1983) and 5.7-20 ng/g in Estonia (Reinik et al., 2007). PAH16 (87.5-195.3ng/g) was estimated in commercial beef, chicken, pork and fish products in China (Xia et al., 2010). Due to difference in study design, it is difficult to compare dietary intake of PAHs accurately among countries. Since different meat products are consumed among countries, guideline about dietary intake of carcinogens from meat products should adapt factors including country and eating habit, in order to give more practical advice on intake of meat products for general public.

Table 1-3: The amount of HCAs in cooked meat and fish products and estimated dietary intake of HCAs (ng per person per day) reported in different countries

Country	MeIQx		4, 8-DiMeIQx		PhIP		Other HCAs		Total HCAs		References
	Content (ng/g)	Intake[1]	Content (ng/g)	Intake	Content (ng/g)	Intake	Content (ng/g)	Intake	Content (ng/g)	Intake	
USA	0.6-11.0	182.7	0.1-5.4	56.7	1.5-69.0	1164.8	IQ: 0.04-2.1	IQ: 19.6	2.24-87.5	1820	Layton et al. (1995)
USA	0-25.46	67.2	0-3.2	10.5	0.08-164.6	317.8	IQ: 0-33.1	IQ: 11.9	0.1-202.0	407.4	Keating and Bogen (2001)
Switzerland	0-0.7	91	0-1.5	42	0-4.3	168	IQ: 0, MeIQ: 0-0.7	IQ:42; MeIQ: 42	0-6.5	427	Zimmerli, Rhyn, Zoller, and Schlatter (2001)
Spain	0-2.9	11	0-1.8	5.2	0.6-46.9	307			1.3-51.6	663	Busquets et al. (2004)
Malaysia	0-24.3	312	0-0.7	16	0-30.6	195	MeIQ: 0-3.8		0-38.7	554	Jahurul et al. (2010)
Singapore	0-2.3	29	0.03-0.54	13	0-5.3	5.8			0-5.7	50	Wong et al. (2005)
Japan	0.01-0.2	11	0-0.1	6.3	0.3-0.6	47			0.4-0.9	64.5	Kobayashi et al. (2002)

[1]Intake Unit: ng per person per day

Table 1-4: The amount of PAHs in cooked meat & fish products and estimated dietary daily intake of PAHs (ng per person per day) reported in different countries

	BaP		Other PAHs		Total PAHs		References
Country	Content (ng/g)	Intake[1]	Content (ng/g)	Intake	Content (ng/g)	Intake	
UK	0-0.13	0-5.1	BaA: 0.03-0.09	BaA: 0-8.8	PAH12: 0.59-11.34	PAH12: 0-370	Dennis et al. (1983)
Estonia	<0.3-31.2	15-130[3]	BaA: <0.7-5.6		PAH12: 5.7-20	PAH12: 320-1600	Reinik et al. (2007)
Latvia	0-6.0	23.1	BaA: 0.05-14.2		PAH4: 0.2-34.7	PAH4: 203.7	Rozentāle et al. (2015)
Kuwait	0-5.8	0-37.1	Sum of BaP eq: 0.04-6.0	Sum of BaP eq: 0.7-89.9	PAH8: 0.7-42.9	PAH8: 2.6-641	Alomirah et al. (2011)
Denmark	0-24	2.0-4.1	PAH4: 1.1-17.3		PAH8: 19.7-229	PAH25: 528-1362	Aaslyng et al. (2013); Duedahl-Olesen, White, and Binderup (2006)
China[4]	0-10.5		Sum of BaP eq: 0.6-5.7		PAH16: 87.5-195.3	PAH16: 671	Xia et al. (2010)
Malaysia[5]	0-12.5		BbF: 0-9.67; Fl: 3.5-106		Sum of BbF, BaP and Fl: 3.5-51.1	1541.8	Farhadian et al. (2010)

Fl: Fluoranthene; PAH4: Sum of BaP, Ch, BaA and BbF; PAH8: Sum of BaP, Ch, BaA, BbF, BkF, BgP, DhA and IP
PAH12: Sum of PAH8, Dibenzo[a,e]pyrene, Dibenzo[a,l]pyrene, 5-Methylchrysene, Benzo[g,h,i]perylene
PAH16: Sum of PAH8, Naphthalene, Acenaphthylene, Acenaphthene, Fluorene, Phenanthrene, Antracene, Benzo[g,h,i]perylene, Benzo[g,h,i]perylene
PAH25: Sum of PAH12, Acenaphthylene, Acenaphthene, Fluorene, Phenanthrene, Antracene, Fluoranthene, Pyrene, Cyclopenta[c,d]pyrene, 5-methylchrysene, Anthanthrene, Coronene, 5-methylchrysene, Dibenzo[a,i]pyrene.
[1] Intake Unit: ng per person per day
[2] Intake of PAHs from Adult
[3] Through daily consumption of 100g
[4] Contribution of pork (10.45–12.49%) and fish (6.90–8.03%) to total PAHs (3543–8140 ng/d)
[5] Per capita consumption of poultry, beef and fish is respectively reported at 33.8, 5.8 & 60.5 kg

1.5 Factors affecting the formation of HCAs in cooked meat

Factors affect the formation of HCAs are cooking process, meat composition and other ingredients in meat products (Jinap et al., 2013; Rahman et al., 2014). A higher cooking temperature with prolonged time under direct heat source could lead to produce more carcinogens. However, adding ingredients with antioxidant capacity such as spices and vitamins, could inhibit the formation of HCAs (Gibis, 2016).

1.5.1 Cooking methods

The common thermal cooking methods of meat processing are grilling/barbecuing, frying, roasting/baking, smoking and boiling. They are all dry heat methods except from boiling (Fellows, 2002). Quantitation of HCAs has been reported in boiled meat (Skog & Solyakov, 2002a), microwaved meat (Oz, Kaban, & Kaya, 2010), pan-fried meat (Gibis, Kruwinnus, & Weiss, 2015; Janoszka et al., 2009; Quelhas et al., 2010; Viegas et al., 2012), deep-fried meat (Jinap et al., 2016), roasted meat (Zeng et al., 2014; Zeng et al., 2016; Zeng et al., 2017) and grilled meat products (Gibis & Weiss, 2015; Hasnol, Jinap, & Sanny, 2014; Jinap, Iqbal, & Selvam, 2015). Cooking method has a great impact on the formation of HCAs. Previous research showed grilled/ barbequed meat products contained a higher level of HCAs compared with boiled meat (Gibis, 2016; Liao et al., 2010; Liao, Xu, & Zhou, 2009; Skog & Solyakov, 2002b).

1.5.1.1 Grilling/barbequing

Grilling or barbequing is a thermal process with applying dry heat/flame directly over food surface (Fellows, 2002). This method could generate abundant amount of HCAs, since flame with high temperature was directly applied over the food surface and led to high moisture loss (Skog & Solyakov, 2002b; Skog, Johansson, & Jaègerstad, 1998). Much higher total HCAs was found in barbequed chicken (50.82 ng/g), compared with pan-fried chicken (3.78 ng/g) with the same doneness level (Iwasaki et al., 2010). Similar result was also reported in beef. Grilled beef (140.68 ng/g) contained 10 times more total HCAs than in pan-fried samples (13.53 ng/g) (Jinap et al., 2013; Puangsombat, Jirapakkul, & Smith, 2011). Oz and Kotan (2016) showed

barbequed fish contained significantly higher amount of total HCAs (3.09 ng/g) than dry heated (1.20 ng/g), microwaved (1.12 ng/g) and oven roasted fish (0.79 ng/g) ($p<0.05$). Jinap et al. (2013) and Manar (2014) detected 14.37-73.96 ng/g IQ in beef grilled at 270-320 ^{o}C, compared with none in boiled beef and 8.81 ng/g in fried beef.

1.5.1.2 Frying

Frying offers food a crust outer layer with a moist interior when food immersed in heated fat at high temperature (Fellows, 2002). During frying, internal moisture transports outwards when surface water evaporated, which brings water-soluble precursors (amino acids, creatine and sugars) of carcinogens to the surface of meat to generate medium level of HCAs (Balogh et al., 2000). The amount of total HCAs (IQ, MeIQx, 4,8-DiMeIQx and PhIP) was 2.16 ng/g in deep-fried chicken and 1.47 ng/g in duck, which were significantly lower than chargrilled ones, i.e. 31.06 ng/g in chicken and 11.80 ng/g in duck ($p<0.05$) (Liao et al., 2010). Similarly, Janoszka et al. (2009) found that pork chop, collar and mince chop fried at 170 ^{o}C had 7-15 ng/g total HCAs including MeIQ, MeIQx, DiMeIQx and PhIP. Ground beef fried at 175-225 ^{o}C patties contained up to 16.4 ng/g IQ, 12.3 ng/g MeIQ and 53.7 ng/g PhIP (Balogh et al., 2000). However, much higher total HCAs (4.7-17.8 ng/g) was detected in pan-residues instead of in beef (1.9-3.2 ng/g) fried with different frying fats, including margarine, butter, sunflower oil and rapeseed oil (Johansson et al., 1995). Inconsistent results of HCAs content might be attributed to different frying condition, fatty acids profile, depth of frying and water evaporation rate in the studies (Johansson et al., 1995).

1.5.1.3 Roasting

Roasting usually generates medium amount of carcinogens, as meat products were cooked under heated air (110-300 ^{o}C), which resulted in the crispy surface of cooked meat but with high internal moisture (Fellows, 2002; Skog & Solyakov, 2002b). There were less than 0.1 ng/g total HCAs in roasted chicken breast cooked at 175-240 ^{o}C for 25-40 min (Skog & Solyakov, 2002b). Liao et al. (2010) suggested that roasting produced the lowest amount of DiMeIQx in chicken, compared with other cooking methods including pan-

frying and grilling when similar cooking temperature and time were used. IQ was not detected in chicken (Liao et al., 2010) and pork loin (Busquets et al., 2004) roasted at 175-200 °C. 0.2-0.8ng/g of PhIP and 0.1-3.1 ng/g MeIQx were found in ham baked 225 °C for 10-20 minutes (Gibis & Weiss, 2012).

1.5.1.4 Boiling/steaming

Since boiling/steaming only involves moist heat, it results in high moisture in cooked meat (Fellows, 2002). Skog and Solyakov (2002a) reported that no HCAs (MeIQx, DiMeIQx and PhIP) detected in boiled chicken for 23-240 min. Lu, Kuhnle, and Cheng (2017a) reported 0.57-4.49 ng/g total HCAs (IQ, MeIQ, MeIQx, 4, 8-DiMeIQx and PhIP) in ready-to-eat (RTE) ham products. Commercial ham and smoked ham are usually cooked by placing the reformed ham in a hot water bath (80- 90 °C) or steam till the interior temperature reaches to 72 °C, which result in very high moisture content and low amount of HCAs (Turesky, 2010). Chen, Pearson, and Gray (1990) reported that high moisture content in the products might dilute the concentration of HCAs and led to low level of HCAs.

1.5.2 Cooking temperature and time

Heating temperature and time have major impact on the formation of HCAs. Cooking with higher temperature and longer time resulted in higher cooking loss, which might correspond to a high level of HCAs in meat products (Gibis, 2016; Knize et al., 1994; Olsson & Pickova, 2005; Skog et al., 1998).

Formation of HCAs initiates at 125 °C and significant amount of IQ, MeIQ, MeIQx, DiMeIQx and PhIP could be detected at temperature over 160 °C (Gibis, 2016). Jinap et al. (2013) reported that IQ increased by approximately 3 times in beef and chicken when grilling temperature increased from 270-300°C to 300-350 °C. The amount of MeIQx was also increased from 2-3 ng/g to15 ng/g in grilled chicken and nd to 7.3ng/g in fried beef patties when temperature increased from 190 °C to 320 °C and 150 °C to 230 °C respectively (Jinap et al., 2013; Knize et al., 1994). However, some HCAs such as DiMeIQx and PhIP, decreased at higher temperature (> 250 °C) (Jinap et al., 2013). Felton et al. (1994) reported that frying temperature increased from 200 °C to 250 °C could reduce the formation of DiMeIQx in

beef patties. This result was agreed with the finding obtained from a heated chemical model system, which noticed that 4, 8-DiMeIQx started degradation at temperature above 225 ^{o}C (Arvidsson et al., 1997).

The prolonged cooking time could promote the accumulation of total HCAs. Total HCAs (IQ, MeIQ, MeIQx, DiMeIQx and PhIP) increased significantly in fried beef patties with cooking time extended from 12 min to 20 min ($p<0.05$). PhIP increased from 27ng/g to 480 ng/g in grilled chicken and 1.33ng/g to 7.36ng/g in boiled pork with longer cooking time (Lan, Kao, & Che, 2004; Sinha et al., 1995).

1.5.3 Composition of meat

Amino acids, creatine and reducing sugars are the three main precursors involved in the formation of polar HCAs (IQ, MeIQ, MeIQx, DiMeIQx and PhIP). The amount and type of major precursors have significant effect on the level of HCAs (Felton et al., 1994; Hasnol et al., 2014; Puangsombat et al., 2011). Chemical model system has been established to mimic the formation of HCAs and to investigate the effect of various compounds on the formation of HCAs (Arvidsson et al., 1997; Johansson & Jägerstad, 1996; Moon & Shin, 2013; Shin, Strasburg, & Gray, 2002c; Zhu et al., 2016).

1.5.3.1 Reducing sugar, creatine and free amino acids content

Creatine is necessary for the formation of imidazole ring in the structure of imidazoquinoline and imidazoquinoxaline (Gibis, 2016). During heating, creatine is hydrolysed into creatinine, which can further react with glucose and amino acids into 2-methyl-pyridine or 2, 5-dimethyl-pyrazine. The 2-methyl-pyridine could react with other amino acids in meat, such as glycine or alanine to form IQ and MeIQ, respectively (Puangsombat et al., 2012). PhIP usually could be generated from the mixture of creatinine, phenylalanine and glucose under heating, the amount may increase in glucose-abundant meat products (Felton et al., 1994). Khan et al. (2013) stated that there was a positive correlation (correlation coefficient $r= 0.71$, $p<0.05$) between the amount of PhIP and the content of glucose and amino acids in fried fish products. Puangsombat et al. (2011) further confirmed that greater level of PhIP was

found in salmon with higher glucose and creatine content, compared with that in cod under the same cooking condition.

Amino acids other than creatine are also vital for the development of HCAs during cooking. In order to understand amino acids profile on the formation of different HCAs, heated chemical model system has been established including mixture of amino acids, reducing sugar such as glucose/ fructose and creatine (Bordas et al., 2004). It has been reported that amino acids such as glycine, alanine, lysine, threonine and phenylalanine are responsible for the formation of imidazoquinoline and imidazoquinoxaline (Bordas et al., 2004; Gibis, 2016). Particularly, glycine, threonine, alanine and lysine contributed to the formation of MeQIx, while threonine, alanine and lysine participated in the formation of 4, 8-DiMeIQx (Gibis, 2016), while phenylalanine is critical for generating PhIP (Zöchling & Murkovic, 2002).

1.5.3.2 Fat content and lipid oxidation

The source of fat in meat products could be either endogenous (fat content of raw meat) or exogenous (added fat or lipids during preparation/cooking process). High fat content could reduce HCAs owing to the dilution effect of substrates (Hwang & Ngadi, 2002; Knize et al., 1994), while fat could also accelerate heat penetration to meat system and lead to high level of HCAs formation (Knize et al., 1994). Johansson and Jagerstad (1994) stated that the formation of IQ had a positive correlation with fat content ($r= 0.774$, $p<0.01$) in well-done bacon. Jinap et al. (2013) found more MeIQ in cooked meat marinated with extra palm oil (10mL/kg) than samples without marinades. In addition, free radicals, aldehydes and ketones generated from lipid oxidation could interact with Maillard reaction to promote the formation of HCAs (Johansson & Jagerstad, 1994; Zamora & Hidalgo, 2007).

The pathway of nonenzymatic lipid oxidation through free radical reaction includes 3 steps, initiation, propagation and termination (see below). In the step of initiation, a free radical (R·) is produced from triglycerides or free fatty acids with the presence of oxygen/ metal ions/ metal-protein. Propagation starts when R· reacts with O_2 to form peroxide radical ROO·, which can react with fatty acids to generate another free radical R· and hydroperoxides

(ROOH). These unstable hydroperoxides are readily decomposed into aldehydes, ketones and hydrocarbons that could further react with amine groups of protein. The termination occurs when 2 radicals react or a stable substance formed (Guillen-Sans & Guzman-Chozas, 1998; Shahidi & Ambigaipalan, 2015).

Initiation $RH \rightarrow R\cdot + H$

Propagation $R\cdot + O_2 \rightarrow ROO\cdot$

$ROO\cdot + RH \rightarrow R\cdot + ROOH$

Termination $R\cdot + R\cdot$

$R\cdot + ROO\cdot$

$ROO\cdot + ROO\cdot$

Lipids and lipid oxidation interacted with Maillard reaction through enhancing the formation of Maillard reaction intermediates. Johansson and Jagerstad (1994) reported that fat could improve the production of pyridine-containing intermediates, which resulted in accumulating HCAs. Furthermore, decomposed compounds from cleavage of hydroperoxides, such as aldehydes and ketones in lipid oxidation could react with amino acids, which might promote Maillard reaction (Zamora & Hidalgo, 2007). Zamora et al. (2012) reported that the presence of lipid oxidation products, such as 4-oxo-2-nonenal significantly increased the amount of PhIP in chemical model system ($p<0.05$). However, antioxidants, i.e. polyphenols and tocopherols presented in lipids showed inhibitory effect on the formation of HCAs (Bordas et al., 2004; Johansson & Jagerstad, 1994; Lu, Kuhnle, & Cheng, 2017b). Thus, the effect of fat/lipids needs to be justified with the consideration of fatty acids profile and presence of antioxidants.

1.5.4 Addition of antioxidants

Since free radical mechanism has been proposed in the formation of HCAs, antioxidants with scavenging capacity have been applied into meat products and/or chemical model system to explore the effect on HCAs formation.

Previous research studied the effect of antioxidants on HCAs in meat products through 3 approaches, including adding single or mixture of chemical compound(s) (Cheng, Chen, & Wang, 2007a; Wong, Cheng, & Wang, 2012; Zeng et al., 2017), food extract (Cheng et al., 2007b; Gibis & Weiss, 2012) and whole food (Britt et al., 1998; Oz & Kaya, 2011a; Oz & Kaya, 2011b). Synthetic antioxidants, such as butylated hydroxyanisole (BHA), propyl gallate (PG) and tert-butylhydroquinon (TBHQ) have showed good inhibitory efficiency on the formation of HCAs (Vitaglione & Fogliano, 2004). However, natural antioxidants including phenolic/polyphenols and vitamins become more favourable to consumers because of the potential toxic hazard of synthetic antioxidants.

1.5.4.1 Phenolic antioxidants

Phenolic antioxidants, including phenolic acids and flavonoids, have showed antioxidant capacity owing to the reactivity of phenol moiety in the structure (Rice-Evans, Miller, & Paganga, 1997; Shahidi & Ambigaipalan, 2015). They could also scavenge pyridine and pyrazine radicals, so that prohibit further reactions with creatinine to prevent the formation of HCAs (Vitaglione & Fogliano, 2004). The mechanism of radical scavenging by phenolic compounds has been summarized by Shahidi and Ambigaipalan (2015). Phenolic antioxidants (AH) can donate hydrogen atom(s) to free radicals, such as alkoxys and hydroperoxides, in a result of forming stable compounds and antioxidant radicals, which can further react with other free radicals.

$$R\cdot/RO\cdot/ROO\cdot + AH \rightarrow A\cdot + RH/ROH/ROOH$$

$$RO\cdot/ROO\cdot + A\cdot \rightarrow ROA/ROOA$$

$$ROO\cdot + RH \rightarrow ROOH + R\cdot$$

Pure polyphenol compounds, such as quercetin and rutin (structure in Figure 1-4) could significantly reduce total HCAs (MeIQx, 4, 8-DiMeIQx and PhIP) in fried beef patties (Cheng et al., 2007a; Zhu et al., 2016). Flavonoids such as naringenin also inhibited MeIQx, 4, 8-DiMeIQx and PhIP effectively in a heated chemical model system (Zeng et al., 2016). The efficiency of scavenging radical in antioxidants is dependent on structure of the

compounds and its thermal behaviour (Shahidi & Ambigaipalan, 2015; Zeng et al., 2016). Generally speaking, radical scavenging capacity (antioxidant capacity) increases with the number of hydroxyl groups and decreases with glycosylation. For example, rutin showed less antioxidant activity and thermal stability than quercetin due to the presence of rhamnoglucoside moiety in the structure (da Costa et al., 2002; Shahidi & Ambigaipalan, 2015). The basic structure of flavonoids is illustrated in Figure1-4, which consists of the fused A and C rings with the phenyl B ring. Polyphenols with strong antioxidant activity usually have the structure including (1) ortho 3',4'- dihydroxyl groups on the B ring, (2) 3- hydroxyl on the C ring and (3) meta 5,7-dihydroxyl groups on the A ring and 2,3-double bond with the carbonyl group on the C ring (Rice-Evans et al., 1997).

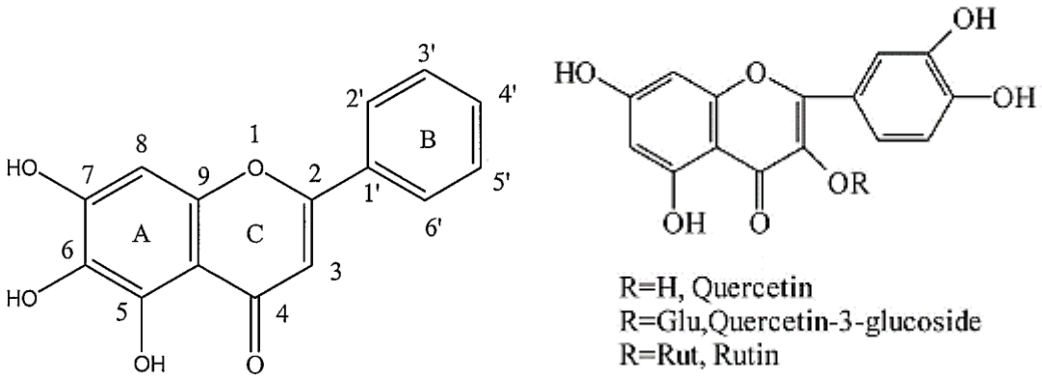


Figure 1-4: The basic structure of flavonoids, quercetin and rutin[1]

[1]Source from Cheng et al. (2007a) and Rice-Evans et al. (1997).

Adding plant/food extracts has showed reduction on HCAs in meat products. Grape seed extract (Cheng et al., 2007b; Gibis & Weiss, 2012), and dried apple peel extract (Sabally et al., 2016) showed 54-70% inhibition on total HCAs (MeIQx, 4, 8-DiMeIQx and PhIP) in cooked beef patties, which could be attributed to strong antioxidant capacity of epicatechin, proanthocycanidin and chlorogenic acid presented in these extracts.

Effect of whole food on the formation of HCAs has also been widely studied. Britt et al. (1998) reported that flaked cherry tissue containing isoflavones and anthocyanins could reduce 87% total HCAs (IQ, MeIQ, MeIQx and DiMeIQx) in fried beef patties. Persson et al. (2003) stated that virgin olive oil containing

tocopherol and polyphenols could inhibit the formation of DiMeIQx, MeIQx and PhIP in fried beef burgers. Black pepper (1%, w/w) could reduce total HCAs by 11-33% in meatballs fried at 175 °C and 200 °C, especially by 100% in samples fried at 225 °C due to the presence of quercetin and piperine (Oz & Kaya, 2011a). Green tea infusion marinade (1g/125ml hot water) (Quelhas et al., 2010) and red pepper (Wong et al., 2012) could reduce up to 75% PhIP in pan-fried beef patties. They proposed that phenolic compounds could directly trap phenyl acetaldehyde, which is a major precursor of PhIP to inhibit PhIP formation.

1.5.4.2 Vitamins

Vitamins with antioxidant potency could inhibit the formation of HCAs. Addition of water-soluble ascorbic acid powder could reduce PhIP in fried beef compared with non-treated samples (Wong et al., 2012). Cheng et al. (2007) also reported that marinating with lemon juice could reduce about 30% MeIQx in grilled beef. Oz and Kaya (2011b) reported that red pepper containing abundant of ascorbic acid and provitamin A carotenoids could reduce 75%-100% total HCAs (IQ, MeIQ, 4, 8-DiMeIQx and PhIP) in fried pork chop. Ascorbic acid could act as inhibitors to prevent HCAs formation, through radical quenching and free radical scavenging activity (Wong et al., 2012).

Fat-soluble vitamin E also has antioxidant capacity due to the presence of tocopherols (Figure 1-5), which not only act as radical scavenger, but also as singlet oxygen quencher (Shahidi & Ambigaipalan, 2015).

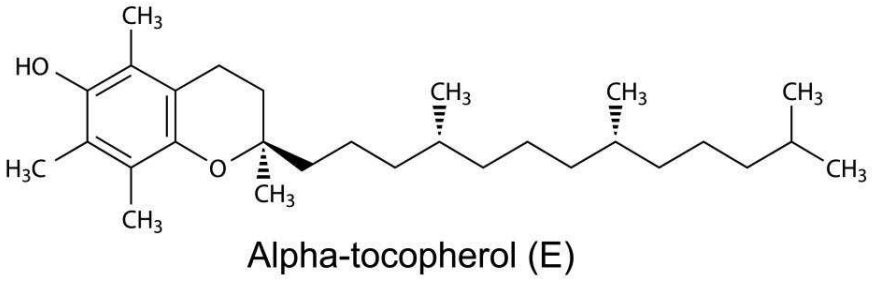


Figure 1-5: Chemical structure of α-tocopherol[1]

[1]Source from Shahidi & Ambigaipalan (2015).

Tocopherols (T-OH) perform as antioxidant by donating hydrogen to peroxyl (ROO·) or alkoxy (RO·) radicals to form stable tocopherol radical (TO·), which could obstruct the propagation of oxidation. The efficiency of hydrogen-donating is α > β > γ > δ (Frankel, 1998; Shahidi & Ambigaipalan, 2015).

T-OH + ROO· → TO· + ROOH

T-OH + RO· → TO· + ROH

Liao et al. (2009) and Sugimura et al. (2004) proposed that vitamin E could prohibit the formation of HCAs through removing free radicals in Maillard reactions. Rounds et al. (2012) and Balogh et al. (2000) found that 1% vitamin E spray on the surface of beef patties could reduce the concentrations of IQ, MeIQ, MeIQx, DiMeIQx and PhIP significantly by 45% to 75%. Similar result was also reported by Lan et al. (2004), who found that 70% of total HCAs (IQ, MeIQ, MeIQx, 4, 8-DiMeIQx and PhIP) was prohibited when 0.2% α-tocopherol was added into ground pork 1h before cooking.

1.5.4.3 Organosulfur compounds

Organosulfur compounds, including cysteine, diallyl disulfide (DAD), diallyl sulphide (DAS), dipropyl disulfide (DPD), diallyl trisulfide, have also been reported with inhibitory effect on the formation of HCAs. Pure compounds, diallyl disulfide and dipropyl disulfide could prohibit 70-78% total HCAs (MeIQx, DiMeIQx and PhIP) in both fried beef patties (Shin et al., 2002b) and chemical model system (Shin et al., 2002c). 20-68% total HCAs (MeIQx, DiMeIQx, and PhIP) could also be inhibited by adding fresh garlic (Shin et al., 2002a) and onion (Janoszka, 2010) in fried pork and beef patties. Reduction of HCAs might be via trapping intermediates in Maillard reactions (Puangsombat et al., 2011). DAD and DPD could also react with glucose in Maillard reaction, which resulted in less substrate available for the formation of HCAs (Shin et al., 2002c).

1.6 Factors affecting the formation of PAHs in cooked meat

1.6.1 Cooking process

Determination of PAHs has been conducted in grilled/ barbequed meat (Alomirah et al., 2011; Chung et al., 2011; Farhadian et al., 2010; Park et al.,

2017; Viegas et al., 2014; Wongmaneepratip & Vangnai, 2017), roasted meat (El-Badry, 2010; Lu et al., 2017b), fried meat (El-Badry, 2010; Janoszka, 2011; Rose et al., 2015) and smoked meat products (Alomirah et al., 2011; Djinovic, Popovic, & Jira, 2008; Lorenzo et al., 2011; Santos, Gomes, & Roseiro, 2011; Wretling et al., 2010). Most studies about PAHs have been focusing on grilled and barbequed meat products that are usually cooked above 250 ^{o}C, since PAHs with more rings are usually generated at 300-500 ^{o}C.

1.6.1.1 Grilling

High level of PAHs has been detected in grilled or barbequed meat products heated directly over flame with smoke generated from fat dripping (Kazerouni et al., 2001). The amount of PAHs varied from 0-130 ng/g and BaP ranged 0-50 ng/g in grilled meat and fish (Chung et al., 2011; Farhadian et al., 2010; Viegas et al., 2012). Different types of grilling and source of heat also had effect on the formation of PAHs, such as gas grilling, charcoal grilling or oven grilling. Farhadian et al. (2010) found that charcoal grilled meat contained higher amount of PAHs (BaP, BbF and Fln) than those grilled by gas. Coconut charcoal is characterized as smokeless and flameless charcoal. Research showed grilling with this charcoal could significantly reduce PAHs (BaP, BaA, Ch, BbF, $p<0.05$) in salmon even with high fat content, compared with wood charcoal (Viegas et al., 2012). Other factors, such as distance to heat source and doneness could also affect the level of PAHs in grilled meat. Rose et al. (2015) found that beef burgers grilled at distance 7cm over charcoal had 3 times less BaP and PAH4 than those grilled with distance 4cm.

1.6.1.2 Smoking

Smoking is a process used to extend shelf life, enhance colour and flavour of meat products. Traditionally, meat was hang and exposed to smoke produced from wood or charcoal with less O_2 (Roseiro, Gomes, & Santos, 2011). Traditional smoking can be divided into two groups according to temperature of smoke: cold smoking and hot smoking (Ledesma, Rendueles, & Díaz, 2016). Common meat products, such as salami and chorizo are produced by cold smoking (15-30^{o}C), while commercial ham and sausages are produced by hot smoking (50-85 ^{o}C) (Toldrá, 2009). Meat products are contaminated by

PAHs during smoking via (1) incomplete combustion of fuel, (2) fat dripping on fuels and contacting with fire. Average 1.88-13.5 ng/g BaA and 0.6- 36.9 ng/g BaP were determined from a wide range of smoked products including smoked pork, chicken, beef and sausage products (Ledesma et al., 2016; Rozentāle et al., 2015; Wretling et al., 2010).

Increasing smoking time could result in accumulating high level of BaP in smoked beef, ham and bacon (James & Dorcas, 1984). In addition, more PAHs were detected in meat products smoked with less heating distance (Roseiro et al., 2011). However, modern techniques of smoking, such as liquid smoke has been developed with short processing time and being more eco-friendly. The concentration of smoke, humidity and temperature are controlled precisely during liquid smoking, and carcinogens such as PAHs can be filtered before exposed to meat products (Ledesma et al., 2016; Škaljac et al., 2014).

1.6.1.3 Frying

Common frying temperature applied in meat products is 160-240^{o}C (Katragadda et al., 2010). PAHs were found in both heated meat and oils. El-Badry (2010) reported that 3.8 ng/g BaP and 21 ng/g total carcinogenic PAHs were detected in fried chicken. Perelló et al. (2009) reported fried fish contained 0.38-1.17 ng/g BaA and 0.04 ng/g BaP, and had the highest total PAHs16 (13.3- 35.42 ng/g) compared with grilled and roasted fish. BaA with 0.1-97 ng/g and BaP with 0.3-67 ng/g were determined in selected commercial oils, including coconut oil, palm oil and olive oil (Barranco et al., 2003). In addition, fatty acids profile of frying medium could also affect the formation of PAHs. Min, Patra, and Shin (2017) reported that the formation of PAH increased with the degree of unsaturation of lipids, and the highest content of total PAHs was found in the model system with methyl linolenate (34.30 ng/g), followed by methyl linoleate (32.46 ng/g) and methyl stearate (29.64 ng/g). Unsaturated fatty acids in edible fats/oils are readily oxidized at high temperature and generate hydroperoxides, which could further initiate cyclic compounds through intramolecular reaction and accelerate the

formation of PAHs (Chen & Chen, 2001; Olatunji et al., 2014; Singh et al., 2016).

1.6.2 Fat content

Fat content in meat also plays an important role in the formation of PAHs. Total amount of PAHs was positively related to fat content in meat and fish (Essumang, Dodoo, & Adjei, 2012). Dost and İdeli (2012) indicated that no PAHs were detected in barbecued low fat trout or bass, while 1.66 ng/g BaP was determined in grilled salmon due to high fat content in salmon. Similarly, BaP increased from 0.28 to 1.37 ng/g in Frankfurter-type sausages when fat content increased from 10% to 39% (Pöhlmann et al., 2013). Fat/lipids might be involved with PAHs formation at high temperature cooking through lipid pyrolysis, which occurred in fat dripping on heating source and adhering on surface of meat products (El-Badry, 2010). In addition, free radicals generated during combustion of fuel could induce polymerization between hydrocarbons to form heavy PAHs (Singh et al., 2016). Accumulation of PAHs in the fat/lipid rich products might be owing to their lipophilic characters (El-Badry, 2010; Ledesma et al., 2016).

1.6.3 Antioxidants

The addition of antioxidants, such as spices and beer marinades is believed having inhibitory effect on the formation of PAHs (El-Badry, 2010; Farhadian et al., 2011; Viegas et al., 2014). Since free radicals promote the recombination of fragmented hydrocarbons to form stable heavy PAHs, marinating meat with ingredients containing antioxidants might reduce the formation of PAHs due to free radical scavenging property (Viegas et al., 2014). Farhadian et al. (2012) studied the effect of different types of marinades on the formation of PAHs (BaP, BbF and Fl) in chargrilled beef, and they found out that basic marinade (sugar, water, onion, turmeric, lemon grass, salt, garlic, coriander, cinnamon), basic-lemon juice and basic-oil-lemon juice significantly reduced total PAHs by 32%, 56% and 27%, respectively. Janoszka (2011) confirmed that chopped garlic (30g/100g pork) and onion (15g/100g pork) could reduce BaP by approximately 50%, compared with those without any marinades. Similar result was also reported

by El-Badry (2010), who found that spices (cumin, coriander, black pepper, and rosemary), marinades (tomato juice, garlic paste, onion, cumin, coriander, and black pepper) and garlic paste could reduce BaP from 5.30 ng/g to 0.02 ng/g in chicken cooked by microwave oven, pan frying, direct and indirect grilling.

1.7 Determination of HCAs and PAHs

1.7.1 Determination of HCAs

Multiple steps of extraction /separation are necessary for HCAs with low level (ppb/ppm) presented in complex meat matrices (Jonaszka et al., 2009). Liquid-liquid extraction (LLE) was used for isolating HCAs from meat matrix according to interaction between HCAs and solvents with different solubility (Puangsombat et al., 2011). However, large amount of solvent and longer time is required for LLE, thus, researchers have developed the method that combining alkali hydrolysis with solid phase extraction (SPE) by using propyl sulfonic acid (PRS) and C-18 cartridges to improve efficiency of extraction and purity of target compounds (Jonaszka et al., 2009; Gibis and Weiss, 2002; Damašius et al., 2011).

Table 1-5 shows that common techniques for determining HCAs are high performance liquid chromatography (HPLC) with UV detector and liquid chromatography-mass spectrometry (LC-MS). Jinap et al. (2013), Haskaraca et al. (2017), Viegas et al. (2015), Unal et al. (2017) and Oz and Kotan (2016) determined HCAs by using HPLC with diode array detection /UV. HCAs have been identified through retention time along with comfirmation of UV spectrum, and quantified from internal or external calibration curve (Gibis, 2016). Chen et al. (2017) and Zeng et al. (2017) used UPLC -MS to analyse HCAs in cooked meat products. Limit of detection and recovery rate were comparable with HCAs determined by using HPLC-DAD /UV.

Table 1-5: Prepration method, detection method, recovery rate, limit of detection, limit of quantification of polar HCAs in different meat products

Meat products	Sample preperartion	Detection method	Recovery rate (%)	Limit of detection (LOD) (ng/g)	Limit of quantification (LOQ) (ng/g)	References
Roast beef patties	Acid hydrolysis and SPE	UPLC-MS[1]/MS	57.6-92.4	0.01-0.05	0.03-0.16	Chen et al. (2017)
Fried chicken breast	SPE	HPLC-UV/FLD[2]	46.4-83.1	0.02-14.3		Gibis (2009)
Fried beef patties	SPE	HPLC-DAD	40-70		0.02	Gibis and Weiss (2012); Gibis (2007)
Chicken burgers nuggets	SPE	HPLC-UV	36.2-82.2	0.01-0.02	0.03-0.08	Haskaraca et al. (2017)
Chicken and beef satay	SPE	HPLC-DAD	43-72	0.48-1.55	1.24-5.42	Jinap et al. (2013)
Roast beef patties	SPE	UPLC-MS	52.7-80.6	0.01-0.11	0.02-0.20	Zeng et al. (2017)
Charcoal-grilled pork	SPE	HPLC-DAD		0.02-0.06	0.2-2.6 ng/g	Viegas et al. (2015)
Barbecued sucuk	SPE	HPLC-DAD	45.1-82.1	0.008-0.024	0.025-0.085	Unal et al. (2017)
Roast beef patties	SPE	HPLC-DAD	61-72	0.15	0.5	Puangsombat et al. (2011)
Cooked fish	SPE	HPLC-DAD	55.6-87.1	0.008-0.024	0.025-0.081	Oz and Kotan (2016)
Pan-fried beef patties	SPE	HPLC-DAD	47-61	0.017-0.03		Natale et al. (2014)

[1] Mass selective detector

[2] Fluorescence detecto

1.7.2 Determination of PAHs

Similar to HCAs, the occourrence of PAHs in meat products is at low level (ppb/ppm), interference such as fat content could reduce the effiency of extraction, approporiate extraction should be selected (Purcaro et al., 2013).

In the clean-up step, saponification with potassium hydroxide (Alomirah et al., 2011) followed by toluene extraction (Dost and Ideli, 2012) was applied, to eliminate bias from fatty acids (Santo et al., 2011). SPE (Fardadian et al., 2010; Janoszka, 2011), Soxhlet (Mohammadi and Valizadeh-kakhki, 2016) and solid-phase microextraction (Purcaro et al., 2007) have been used widely in previous research. Inconsistent results of recovery could be obtained due to the complexity of meat system.

HPLC (Babaoglu et al., 2017; Farhadian et al., 2012; Oz and Yuzer, 2016; Viegas et al., 2012) and GC (Mohammadi and Valizadeh-kakhki, 2016; Olatunji et al., 2014; Park et al., 2017) are technologies frequenctly used in determing PAHs. Fluorescence detector can be used for detecting fluorescent PAHs (Babaoglu et al., 2017; Farhadian et al., 2012). In addition, PAHs can also be identified and quantified by mass spectrometer (MS) according to mass-to-charge ratio and molecular weight (Mohammadi and Valizadeh-kakhki, 2016; Park et al., 2017). Jonaszka et al. (2009) suggested that LC–MS was selective and efficient in quantifing PAHs.

Table 1-6: Prepration method, detection method, recovery rate, limit of detection, limit of quantification of PAHs in different meat products

Meat products	Sample preperartion	Detection method	Recovery rate (%)	Limit of detection (LOD) (ng/g)	Limit of quantification (LOQ) (ng/g)	References
beef and chicken kebab	Soxhlet with column chromatography	GC-MS	78-88	0.059-0.25	0.4-0.82	Mohammadi and Valizadeh-kakhki (2016)
Beef and lamb kokorec	SPE	HPLC-FLD[1]	89.2-92.6	0.027-0.069	0.09-0.23	Babaoglu et al. (2017)
Grilled beef	SPE	HPLC-FLD	75-89	0.01-0.03	0.04-0.10	Farhadian et al. (2012)
Pan-fried pork	SPE	HPLC-FLD	51.9-60.9	0.0005 ng/column		Jonoszka (2011)
Smoked, grilled & boiled meats	SPE	GC-FID[2]	83.7-92.4	0.1-0.3	0.3-0.9	Olatunji et al. (2014)
Barbecued beef steak	SPE	HPLC-DAD	89.2-92.3	0.027-0.069	0.09-0.23	Oz and Yuzer (2016)
Grilled pork belly	Alkali hydrolysis and SPE	GC-MS	70.1-94.9	0.05	0.18	Park et al. (2017)
Chargrilled pork	SPE	HPLC-DAD	15.4-145	0.07	0.22	Viegas et al. (2012)

[1] Fluorescence detector
[2] Flame ionization detector

1.8 Research gaps

Strong evidence from epidemiological studies provides that increasing red and processed meat intake could elevate the risk of cancer. The presence of HCAs, PAHs and Nitro-some compounds has been considered as one of the effective contributors. In general, food frequency questionnaires (FFQs) with interview has been one of the key approaches for assessing dietary HCAs and PAHs exposure by estimating the level of HCAs and PAHs from food diary. Since the level of HCAs and PAHs can be significantly affected by meat type, cooking methods, degree of doneness etc, it is useful to provide accurate measurement of HCAs and PAHs in more diverse range of meat products for accurate estimation of dietary exposure. Diet in the general public has dramatically changed in the last decades. Convenience food, such as RTE products/canned meat and recipe improved meat products are more favoured to comsumers because of increasing awareness of health and change of lifestyle (Singh et al., 2016). However, risk exposure to carcinogenic HCAs and PAHs in these commercial meat products has been received limited attention.

It is also important to develop strategies to control the formation of carcinogenic HCAs and PAHs in meat products, in order to modify and minimize dietary exposure to HCAs and PAHs, which would be beneficial for public health. Intensive researches have been conducted on altering cooking process to reduce the formation of HCAs and PAHs, but the effects of meat composition and ingredients are still not consistent, which need further investigation. With the high demand of healthy meat products from consumers, developing low fat/sugar and high fibre meat products has been well addressed by food industry. Researchers have been focusing on manipulating original recipes with more sustainable and healthier ingredients while maintaining their acceptability. Replacing fat with oils (Rodr´ıguez-Carpena, Morcuende, & Est´evez, 2011), water/oil emulsion (Youssef & Barbut, 2011) and oil/fibre emulsion (Choi et al., 2010; Lorenzo et al., 2016) have been well established in meat products. However, the effect of fat replacement on the formation of HCAs and PAHs in cooked meat products has yet been validated.

Although using wide range of antioxidants to reduce HCAs and PAHs has been conducted in both meat products and chemical model system, inconclusive results are found because of the complexity of whole food vehicle and synergistic or supressing effects between functional compounds. With the intention of better understanding effect of antioxidants on the formation of carcinogenic HCAs and PAHs, standardized antioxidants model system should be used to understand their inhibitory efficiency and pathway. The results would provide useful information about food safety and quality for industry and dietary guideline for public health.

Therefore, the objectives of this project are the following:

- To determine the concentration of HCAs and PAHs in selected popular RTE meat products on UK's market, to assess the dietary intake of carcinogens that RTE meat products contributed and provide useful guideline about dietary meat intake for general public.
- To explore the effect of partially replacing pork back fat with vegetable oils at different cooking temperatures on the formation of HCAs and PAHs in pork patties.
- To investigate the effect of common spices on the formation of HCAs and PAHs in two meat systems, beef and chicken in consideration of the heme iron level.
- To establish regression model for predicting the efficiency of different pure antioxidant compounds from spices on the formation of HCAs and PAHs in meat products.

1.9 References

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Chapter 2. Heterocyclic amines and polycyclic aromatic hydrocarbons in commercial ready-to-eat meat products on UK market

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2.1 Abstract

HCAs and PAHs, which are developed during meat processing, may play key roles in the imposing health risk. The consumption of RTE meat products has increased dramatically in recent years due to their convenience. Therefore, it is essential to evaluate its health risk and provide dietary intake guidance to the general public. 11 RTE meat products were selected from UK market including chicken, pork and fish to investigate their health risks in concern of HCAs and PAHs levels. HCAs and PAHs were extracted by solid-phase extraction and analysed by HPLC- Diode array UV/ FLD. Chargrilled chicken contained the highest amount of HCAs (37.45±4.89ng/g) and PAHs (3.11±0.49ng/g), followed by roasted bacon (HCAs 15.24±1.31ng/g, PAHs 1.75±0.17ng/g) and honey roast salmon (HCAs 17.12±5.86ng/g, PAHs 0.38±0.09ng/g). The high dietary intake of HCAs was from chargrilled chicken and ham, which could contribute to the increase risk in breast cancer and colorectal adenoma. While cancer risk associated with PAHs intake from RTE meat products was relatively low according to the Lifelong Average Daily Intake of UK consumers.

Key words: Chargrilled chicken; Roasted bacon; Ham; Fish; Dietary intake; Health risk.

2.2 Introduction

The average consumption of total red meat and processed meat was 70g per day for all adults in UK (NDNS, 2011). In processed meat products, the presence and hazard of HCAs and PAHs become a major concern for both consumers and researchers. HCAs represent a class of carcinogenic compounds that were identified from protein-rich food in the 1970s (Rahman et al., 2014). Five of them, including 2-amino-3-methylimidazo [4,5-f]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4,8-DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) are reasonably regarded as human carcinogens (IARC, 1993). On the other hand, PAHs are hydrocarbons that contain two or more benzene rings, which could be produced in processed meat products through incomplete combustion or pyrolysis of carbon and hydrogen. They can be accumulated in barbequed, grilled, fried and smoked food (PHE, 2008). PAH4, including benz[a]anthracene (BaA), benzo[a]pyrene (BaP), benzo[b]fluoranthene (BbF) and chrysene has recently been reported as indicator of carcinogenic potency of PAHs in food (Janoszka, 2011). In PAH4, both BaA and BaP are considered as probable carcinogens in human (Group 2A) comparing with other PAHs (less carcinogenic) according to the updated IARC (2010) report. Thus, it is necessary to evaluate the dietary intake of BaA and BaP from processed meat products. Particularly, BaA largely exists in smoked meat and is widely examined by researchers, BaP is one of PAHs with the highest Toxicity Equivalency Factor (TEF_{BaP}=1, TEF_{BaA}=0.1, TEF_{BbF}=0.1 and TEF_{BkF}=0.1) (Janoszka, 2011; Rozentāle et al., 2015; Saito et al., 2014; Santos, Gomes, & Roseiro, 2011).

Epidemiological studies indicate that high meat intake could increase the risk of cancer, since a high level of carcinogenic compounds could be produced during high-temperature constantly cooking, such as HCAs (Egeberg et al., 2013; Janoszka, 2010; Liao et al., 2010; Oz and Kaya, 2011). González et al.

(2006) carried out a cohort study and found out that there might be a close association between red and processed meat intake and gastric non cardiac cancer. Stefani et al. (1997) suggested that red meat intake increased the risk of breast cancer in the cohort study. Well-done meat such as beef steak and bacon contained more HCAs, which might be a factor that caused breast cancer (Zheng et al., 1998). Exposure to PAHs has been probably associated with causing lung and skin cancer (PHE, 2008). However, these cohort studies did not provide solid evidence that increased cancer risk was caused by the amount of carcinogens in red and processed meat, in particular because of the complexity of processing conditions, meat type and composition of processed meat products. Although IARC (1993) has already classified processed meat as carcinogenic to human, the level of carcinogens in meat products varies from not detectable to 500 ng/g due to different manufacturing process and food materials (Rahman et al., 2014). With the aim of understanding the relationship of red/processed meat and health risk, it is useful to study the impact of meat processing and ingredients on the formation of carcinogens. RTE meat consumption increased nearly two-fold (115g to 190g consumed per person per week) from 1975-2010 because of its convenience, they can be found either in packed sandwiches or meal dishes (Chalabi, 2013). Therefore, the main focus of this study was to determine the concentration of HCAs and PAHs in selected RTE meat products that are popular on UK's market, in order to assess the dietary intake of carcinogens that RTE meat products contributed and provide useful guideline about dietary meat intake for general public.

2.3 Material and methods

2.3.1 Meat samples

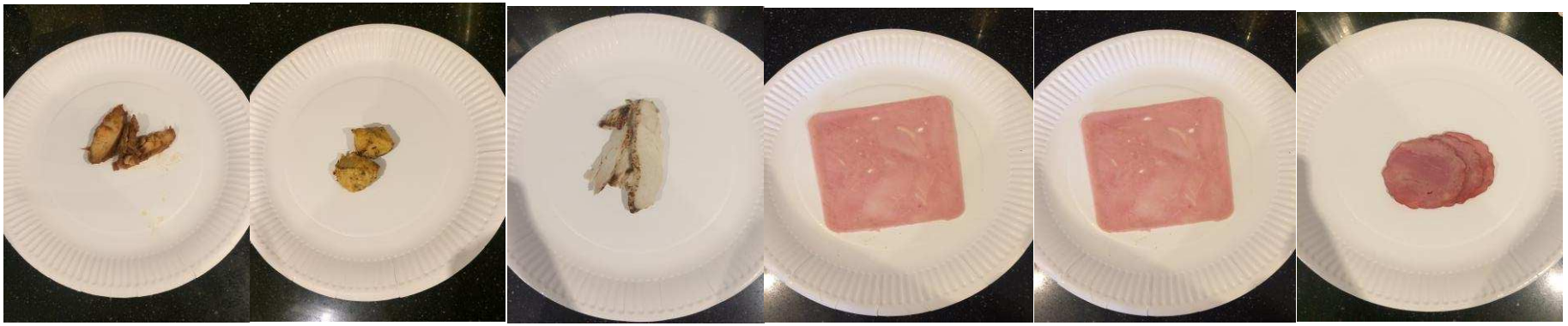
11 RTE meat products were purchased from a local supermarket (Reading, UK) including BBQ British chicken breast slices, tikka British chicken breast slices, Chargrilled British chicken breast slices, British smoked ham slices, British ham slices, classic roasted bacon, crispy smoked streaky bacon, sliced pork sausage, Swedish meatballs, honey roast salmon flakes and sweet chilli salmon flakes (Figure 2-1). These 11 RTE meat products have been selected based on the relatively higher amount of average daily consumption (g/day)

from NDNS (2015) (raw data, unpublished) with the consideration of variety of meat products, including chicken, pork and fish. All chicken products were produced in UK by using British chicken. The supplier information and ingredients information were listed in Table 2-1. All the samples were stored at 4^{o}C, and analysis were carried out within 10 days. All samples were purchased at 3 different occasions to take into account the batch effect.

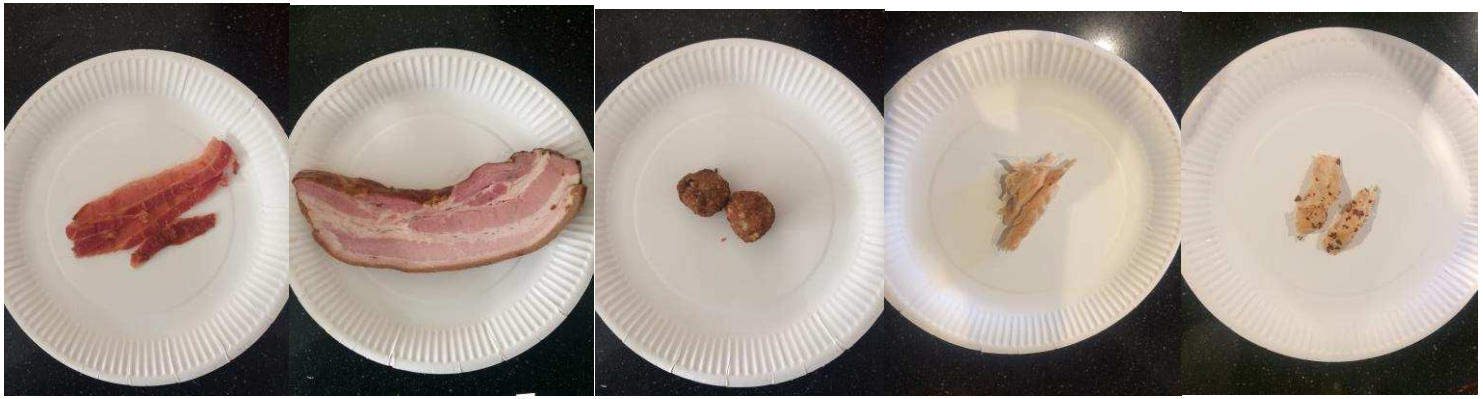
Table 2-1: Ingredients in Selected RTE meat samples

Sample	Ingredients	Supplier information	Packaging information
BBQ British chicken breast slices	Marinated Chicken Breast (Chicken Breast, Stabiliser: Sodium Triphosphate, Brown Sugar, Dextrose, Salt), Barbecue Style Marinades (Sugar, Spirit Vinegar, Smoked Paprika, Rice Flour, Tomato Puree, Maltodextrin, Cornflour, Salt, Molasses, Potato Starch, Barley Malt Extract, Allspice, Sunflower Oil, Cumin, Rapeseed Oil, Paprika Extract, Clove). Made using 104g of chicken per 100g of marinated, cooked chicken.	Produced in UK by using British chicken	Plastic - PP tray Laminate film Lid Paper laminate label
Tikka British chicken breast slices	British Chicken Breast Fillet, Tikka Style Marinades (26%) (Yogurt (Cow's milk), Onion, Cornflour, Rice Flour, Sunflower oil, Ginger Puree, Chilli Puree, Garlic Puree, Coriander, Potato Starch, Salt, Lemon Juice, Cumin, Tumeric, Black Pepper, Cinnamon, Cardamom, Paprika Extract, Fenugreek, Rapeseed Oil, Dill, Ginger, Clove), Stabiliser: Sodium Triphosphate; Brown Sugar, Dextrose, Salt. Made using 104g of chicken per 100g of marinated, cooked chicken.	Produced in UK by using British chicken	Plastic - PP tray Laminate film Lid Paper laminate label
Chargrilled British chicken breast slices	British Chicken Breast Fillet, Rice Flour, Cornflour, Potato starch, Salt, Stabiliser: Sodium Triphosphate; Rapeseed Oil.	Produced in UK by using British chicken	Plastic - PP tray Laminate film Lid Paper laminate label
British smoked ham	Cured, cooked and smoked, reformed ham with less than 15% added water.	Produced in UK by using British pork	Laminate film Plastic - APET base Virgin Paper - FSC label
British ham slices	Cured, cooked and reformed ham with less than 15% added water.		

Sliced pork sausage	Pork meat (53%), Water, Mechanically separated pork meat (6.5%), Salt, Soy protein, Potato starch, Pork collagen protein, Stabiliser: E451, E407a, E415, E331; Glucose, Sucrose. Maltodextrin, Antioxidant: E316; Flavour enhancer: E621; Flavourings (contains Wheat and gluten), Preservative: E250; Colour: E120.	Country of origin: Poland Made in Poland	Tray & Heat Sealed
Classic roasted bacon	Pork belly (98%), Water, Salt, Stabiliser: Potassium and Sodium Tri and Di phosphates, Carrageenan, Sodium citrate, Xanthan gum, Locust bean gum, Soya protein, Pork protein (Collagen), Modified starch, Antioxidant: Sodium erythorbate; Flavour enhancer: Monosodium glutamate; Flavourings, Spice extracts, Preservatives: Sodium nitrite.	Produced in UK by using British pork	Laminate film bag Paper label
Crispy smoked streaky bacon	Pork belly, Salt, Rosemary extract, Antioxidant: Sodium ascorbate, Preservatives: Sodium nitrite. Prepared with 405g raw pork per 100 of finished product.	Produced in UK by using British pork	Laminate film Plastic - APET base Virgin Paper - FSC label
Swedish meatballs	Meat 70% (Pork 55%, beef 15%), Water, Chopped whole potato, Potato starch, Onion, Iodized salt, Potato fibre, Spices, Dextrose, Sugar, Spice extract, Flavourings.	Country of origin: Sweden Made in Sweden	Tray & Heat Sealed
Honey roast salmon flakes	Salmon (84%), Honey, Demerara Sugar, Salt.	Produced in UK by using Scottish salmon	Plastic - RPET tray Plastic - APET film
Sweet chilli salmon flakes	Salmon (91%), Sugar, Water, Sweet Chilli Spice Mix (Sugar, Salt, Garlic Powder, Onion Powder, Cornflour, Coriander Leaf, Lemon Grass, Sunflower Oil, Paprika Extract); Salt, Red Wine, Vinegar, Red Chilli Puree, Red Pepper, Ginger Puree, Cornflour, Cayenne Pepper.	Produced in UK by using Scottish salmon	Plastic - RPET tray Plastic - APET film



(1) (2) (3) (4) (5) (6)



(7) (8) (9) (10) (11)

Figure 2-1: Pictures of 11 RTE meat products[1].

[1](1): BBQ chicken; (2): Tikka chicken; (3): Chargrilled chicken; (4): Smoked ham; (5): Ham; (6): Pork sausage; (7): Crispy bacon; (8): Roasted bacon; (9): Swedish meatballs; (10): Honey roasted salmon; (11): Sweet chilli salmon.

2.3.2 Chemicals

The HCA standards IQ (2-amino-3-methyl-imidazo [4,5-f]quinoline), MeIQ (2-amino-3,4-dimethyl-imidazo [4,5-f]quinoline), MeIQx (2-amino-3,8-dimthylimidazo [4,5-f]quinoxaline), 4,8-DiMeIQx (2-amino-3,4,8-trimethyl-imidazo [4,5-f]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine, BaA (Benz[a]anthracene) and BaP (benzo[a]pyrene) were purchased from Toronto Research Chemicals (Toronto, Canada). Ammonium acetate, triethylamine, acetonitrile (HPLC grade), methanol (HPLC grade), ethyl acetate (>98%), hydrochloric acid, water (HPLC grade) and sodium hydroxide were purchased from Fisher Scientific (Loughborough, UK). Phosphoric acid was obtained from Sigma Aldrich (Sigma Aldrich, UK). Extrelut NT 20 columns and diatomaceous earth refill material were purchased from Merck (Darmstadt, Germany). Bond Elut propyl-sulfonic acid (PRS) cartridges (100 mg, 10ml), C-18 cartridges (7 ml) were purchased from VWR Inc (Lutterworth, UK).

2.3.3 pH and composition analysis

pH was measured by homogenizing 5g sample and 45ml distilled water (Puangsombat et al., 2011). The moisture content was determined by drying 3g meat at 100℃ for 24 hours. Samples were dried firstly in an oven for 4 h and analysed in Soxhlet extraction system to determine the fat level. The protein content was determined by the Kjeldahl method (Horwitz and Latimer, 2005). The creatine content was measured based on the method used by Puangsombat et al. (2011). 0.25g well homogenized sample was stirred with 60ml trichloroacetic acid (30g/L in distilled water) for 5 minutes. The mixture was then filtered with a filter paper (No.1, Filter speed: medium fast & qualitative, 100 circles, 18.5cm, Whatman Ltd). 10ml diethyl ether was added to 20ml filtrate to dissolve fat. The mixture was shaken well and held for 10min to complete separating 2 phases. 4ml of defat layer was added with 2 ml of diacetyl (0.2 g/L in distilled water) and 2 ml of 1-naphthol (25 g/L in 20 g/L of sodium hydroxide solution). The blend was heated for 5 min at 40 ℃. The absorbance of solution was measured at 520nm against a reagent blank in an UV spectrophotometer. The creatine content was expressed as milligram per gram of the meat sample. Standard curve was made from 5 gradient

concentrations (0-20 mg/L) of creatine monohydrate (>98%, Sigma Aldrich, UK).

2.3.4 Determination of HCAs

HCAs extraction was based on the methods proposed by Puangsombat et al. (2011). To minimize the variation and bias due to the unevenly distribution of sauce on the surface of meat, all samples were blended well before analysis. 3g ground meat sample was blended well with 12ml 1M sodium hydroxide. The mixture was then transferred into an Extrelut 20 column with 17g diatomaceous earth. The HCAs were eluted by 60ml ethyl acetate in Extrelut column, and transferred into PRS cartridge which was pre-conditioned with 7ml ethyl acetate. A PRS cartridge was then washed with 6ml 0.1M HCl, 15ml methanol/0.1M HCl (45/55, v/v) and 2ml pure water to remove interferences from the PRS cartridge. The HCAs were then eluted by 20ml 0.5M ammonium acetate (pH 8.5) from the PRS cartridge and transferred into a C-18 cartridge that was conditioned with 5ml methanol and 5ml pure water. Finally, HCAs were eluted with 1ml methanol/ammonium hydroxide (9/1, v/v) from C-18 cartridge into a 2ml amber vial, followed by drying the mixture under nitrogen stream for 1.5h at room temperature. The contents of the vial were dissolved with 50µl methanol and submitted for HPLC analysis. 0.1ml of each 5 standard compounds mixtures (50 ng/ml) was spiked into samples before extraction for measuring the recovery rate. Three replicates were carried out for each sample.

IQ, MeIQ, MeIQx, 4, 8-DiMeIQx and PhIP were analysed using HPLC (HP1635 Series, Agilent ChemStation, Agilent Technologies, Kidlington, UK) connected with a diode array UV detector (RF 2000). The HCAs were separated gradually by a reversed-phase Luna 5u C18 column (250 × 4.60 mm, 5 µm, 100A, Product No: 00G-4252-E0, Phenomenex, UK). Mobile phase A was 0.01 M triethylamine (adjusted pH 3.6 with phosphoric acid) and phase B was acetonitrile (>99%, HPLC grade). The solvent contained 95% A and 5% B at beginning, then linearly changed to 75% A and 25% B within 30 min at flow rate 1.0 ml/min. The temperature of column was 40 ℃. The UV detector was set at 252 nm (Puangsombat et al., 2011).

$$Amount\ of\ Individual\ HCA\ (ng/g)$$
$$= \frac{Amount\ of\ individual\ HCA\ from\ HPLC\ (ng/ml) \times Final\ volumn\ redissolving\ in\ methanol(ml)}{Sample\ weight\ (g)}$$

2.3.5 Determination of PAHs

5g meat sample was homogenised with 15ml 1M NaOH for 1 h. The homogenized sample was then mixed with 17g diatomaceous earth and loaded in an Extrelut 20 column. Elution of PAHs started from Extrelut columns, and was followed by propyl sulphonic acid (PRS) cartridges with 60ml of CH_2Cl_2 containing 5% toluene. The dichloromethane solution was then vacuum evaporated to small amount (0.5-1ml) and the rest of the solvent was dried by a nitrogen stream. The residue was re-dissolved in 1ml n-hexane and transferred to the top of a glass column packed with silica-gel (10 g). PAHs were then eluted by 25ml of n-hexane and 60 ml of 60:40 (v/v) n-hexane–CH_2Cl_2 mixtures. After evaporation to dryness the residues obtained were dissolved in 1ml acetonitrile (spiked and unspiked samples) before the HPLC analysis (Janoszka, 2011). 50ng of 2 standard mixtures was spiked for measuring the recovery rate.

BaA and BaP were analysed using HPLC (HP1635 Series, Agilent ChemStation, Agilent Technologies, Kidlington, UK) connected with a fluorescence detector. Mixture of 84% acetonitrile (>99%, HPLC grade) and 16% water (HPLC grade) were used as a mobile phase under isocratic condition. The separation was performed at 40°C with flow rate 1.0 ml/min. The fluorescence detection was performed by applying the following excitation (Ex)/emission (Em) wavelength program: 280/410 nm from 0 to 8.50 min (BaA), 376/410 nm from 8.50 to 15 min (BaP) (Janoszka, 2011).

$$Amount\ of\ Individual\ PAH\ (ng/g)$$
$$= \frac{Amount\ of\ individual\ PAH\ from\ HPLC\ (ng/ml) \times Final\ volumn\ redissolving\ in\ acetonitrle\ (ml)}{Sample\ weight\ (g)}$$

2.3.6 Assessment of dietary exposure of Carcinogen from HCAs

The assessment of exposure of individual HCA from RTE meat products (ng/g) was calculated below:

Daily exposure of individual HCA (ng/day) = daily consumption of meat (g/day) × Concentration of individual HCA (ng/g) in meat (Norrish et al., 1999).

The daily meat consumption (g/day) for adults and elderlies (both genders) were based on data from NDNS (2015) (raw data, unpublished).

2.3.7 Assessment of dietary exposure of carcinogens from PAHs

Risk assessment of dietary exposure of PAHs was carried out by calculating Lifelong Average Daily Intake (LADD) ng/kg BW/day:

$$LADD = (TEQ_a \times IR \times ED) / (BW \times LT),$$

where TEQ_a (toxicity equivalent) = Concentration of BaA × RPV_{BaA}. + Concentration of BaP × RPV_{BaP}.

The relative potency value (RPV) of BaP is 1 and for BaA is 0.1. IR is average intake of processed meat in exposure duration (g/day) based on NDNS (2015) (raw data, unpublished). ED is expected duration of exposure. BW is the average body weight during exposure duration (83.6kg for male and 70.2kg for female); and LT is the average life expectancy for carcinogen (79.3 years old for male, 83 years old for female in UK), according to ONS (2015).

$$Ingestion\ cancer\ risk = LADD \times CSF,$$

where LADD= Lifetime average daily intake (ng/kg BW/day), CSF= Cancer slope factor $(mg/kg\ BW/day)^{-1}$ (EPA, 2012).

2.3.8 Statistical analysis

Overall, 3 batches of samples were purchased for each product. Measurements of HCAs, PAHs, pH and composition were determined with three replicates. All the results were analysed by analysis of variance (ANOVA) using SPSS Statistics 21, while Duncan's multiple test was used to investigate the difference between means. Pearson's correlation was used to investigate the relationship between HCAs/PAHs and moisture/fat level. The minimum acceptable probability for difference between samples was $p<0.05$.

2.4 Results and discussion

2.4.1 Proximate composition and pH of selected RTE samples

Composition and pH of meat samples, including moisture, fat, protein and creatine content are listed in Table 2-2. Ham and smoked ham had the highest moisture (75%), followed by Tikka chicken (67.81%) and pork

sausage (68.07%) ($p<0.05$). Crispy bacon and honey roasted salmon had the lowest water content, which was 47.60% and 48.55%. Roasted bacon and crispy bacon contained the highest fat (25.84% and 26.49%) among all samples, which were lower than the value (37.9%) reported by Puangsombat et al. (2011). All 3 types of chicken products had the lowest fat level with less than 2%. Sweet chilli salmon had almost twice much of fat (12.16%) than honey roast salmon (6.04% fat), which could be attributed to the sunflower oil in marinade. In general, results of proximate composition were comparable with the nutritional clarification on the packaging. pH of 11 RTE samples ranged from 5.94 to 6.26. BBQ chicken, roasted bacon, crispy bacon and Swedish meatballs had the significantly low pH compared with all other RTE meat products ($p<0.05$). Protein contents varied greatly among samples. Honey roasted salmon had the highest protein content (25.40%), compared with pork sausage, which had only 9.87% protein (the lowest). The greatest level of creatine (10.09 mg/g) occurred in honey roasted salmon, which was significantly higher than those in chicken and pork samples. The result of ham and smoked ham was in the range of 2.31-5.00 mg/g creatine in cooked pork (del Campo et al., 1998; Vangnai et al., 2014). The creatine concentration in sweet chilli salmon was consistent with results (4.5 mg/g creatine in salmon) published in Balsom, Söderlund, & Ekblom (1994). However, Johansson and Jagerstad (1994) reported that 16.64 mg/g creatine was found in cooked salmon (from the Pacific Ocean), compared with 10.09 mg/g creatine in honey roasted salmon (from Scottish). Variation might be due to raw materials from different area.

Table 2-2: Proximate composition and pH of 11 selected samples (n=9)

Sample	Water content (%)	Fat (%)	pH	Protein (%)	Creatine (mg/g)
BBQ chicken	62.42±0.79^{d}	1.68±0.20^{a}	5.95±0.06^{a}	24.89±0.10^{de}	5.12±0.27^{d}
Tikka chicken	67.81±0.42^{e}	1.58±0.24^{ab}	6.17±0.13^{b}	24.91±0.14^{e}	3.29±0.15^{c}
Chargrilled chicken	57.48±1.50^{c}	1.49±0.17^{a}	6.26±0.02^{b}	22.79±0.28^{cd}	3.15±0.07^{c}
Ham	75.45±0.13^{f}	2.80±0.07^{b}	6.26±0.02^{b}	18.01±0.23^{b}	4.37±0.43^{d}
Smoked ham	75.74±0.20^{f}	3.08±0.14^{b}	6.17±0.06^{b}	18.13±0.39^{b}	2.94±0.13^{bc}
Roasted bacon	58.08±0.12^{c}	25.84±0.27^{f}	5.98±0.02^{a}	20.57±0.49^{c}	1.08±0.09^{a}
Crispy bacon	47.60±3.06^{a}	26.49±0.18^{f}	5.98±0.03^{a}	24.21±0.34^{e}	2.24±0.43^{b}
Pork sausage	68.07±0.57^{e}	15.00±0.26^{e}	6.19±0.03^{b}	9.87±0.26^{a}	0.66±0.07^{a}
Swedish meatballs	61.67±0.67^{cd}	16.57±0.40^{e}	5.94±0.03^{a}	11.49±0.25^{a}	1.00±0.12^{a}
Honey roasted salmon	48.55±2.94^{a}	6.04±1.03^{c}	6.22±0.01^{b}	25.40±0.47^{e}	10.09±0.77^{e}
Sweet chilli salmon	52.04±0.99^{b}	12.16±0.34^{d}	6.19±0.03^{b}	24.52±0.28^{de}	4.99±0.27^{d}

Results with different letters in the same column are significantly different at the level $p<0.05$.
Each value is represented as mean ± standard deviation (SD).

2.4.2 Limit of detection (LOD) and limit of quantification (LOQ) of HCAs and PAHs

LOD and LOQ of 5 HCAs were estimated based on the peak-to-peak noise magnitude near analyte peaks with a known concentration and signal-to-noise ratios (R=S/N) of 2 and 10, respectively (Shrivastava & Gupta, 2011). The 5 standard HCA compounds were identified through the retention time of the peaks and specturms (details see Appendix 8). The quantity of each individual HCA was determined according to the external standard calibration curves, which were established by the following standard solutions of each HCA at 0.5-250ng/ml (Appendix 10). The average recoveries of these 5 HCAs according to triplicates were 58.52±5.46% for IQ, 61.89±7.19% for MeIQ, 65.98±8.51% for MeIQx, 62.21±10.06% for 4, 8-DiMeIQx and 62.78±8.58% for PhIP as showed in Table 2-3. Extraction solvent could affect the recovery rate significantly. Janoszka et al. (2009) reported that the recovery rates for MeIQ and PhIP were only 20% and 25% when dichloromethane containing 5% toluene was used as extraction solvent, whereas in this work was used ethyl acetate to extract MeIQ and PhIP. Khan et al. (2008) indicated that the efficiency of extracting polar HCAs such as IQ, MeIQx by using dichloromethane was less than 50%. However, results were comparable with several published data (Balogh et al., 2000; Gibis, Kruwinnus, & Weiss, 2015; Hasnol, Jinap, & Sanny, 2014). The recovery rate was also affected by the complexity of sample matrix and extraction procedures.

LOD and LOQ of PAHs were determined as same as method of HCAs (Table 2-4). Quantitative determination was calculated by using an external calibration curve, which was established by individual standard solution at 0.5-50ng/ml concentration. The regression coefficients of calibration curves for each individual standard were at least 0.996, which implied that the peak area on the chromatogram were markedly related to the concentration. The recovery rate for BaA and BaP were 55.86±6.37% and 57.91±8.42% respectively, which were in line with published range of 50% - 115% (Farhadian et al., 2010; Ishizaki et al., 2010; Janoszka, 2011).

Table 2-3: The retention time, LOD and LOQ calibration curve for standard solution, regression coefficient and recovery rate of 5 HCAs determined (n=3)

HCA	Retention time (min)	LOD (ng/g)	LOQ (ng/g)	Calibration curve equation	Regression coefficient	Recovery rate
IQ	10.501	0.02	0.10	y=3.7854x-0.2474	0.996	58.52±5.46%
MeIQ	12.873	0.01	0.05	y=0.7767x-0.0280	0.997	61.89±7.19%
MeIQx	13.552	0.02	0.10	y=1.9182x+0.0516	0.995	65.98±8.51%
4,8-DiMeIQx	17.134	0.05	0.25	y=1.0502x+0.0204	0.997	62.21±10.06%
PhIP	24.993	0.03	0.15	y=0.3687x+0.0324	0.994	62.78±8.58%

Table 2-4: The retention time, LOD, LOQ, calibration curve for standard soluition, regression coefficient and recovery rate of 2 PAHs determined (n=3)

PAHs	Retention time (min)	LOD (ng/g)	LOQ (ng/g)	Calibration curve equation	Regression coefficient	Recovery rate
BaA	7.336	0.08	0.16	y=0.3019x-0.0184	0.996	55.86±6.37%
BaP	9.487	0.06	0.12	y=0.1020x+0.0495	0.997	57.91±8.42%

2.4.3 Determination of HCAs

Table 2-5 showed HCAs levels in 11 types of RTE meat products. The total amount of HCAs were varied from 0.57 ng/g to 37.45 ng/g. Chargrilled chicken contained the most HCAs (37.45±4.89ng/g), followed by BBQ chicken (18.81±9.02 ng/g), Tikka chicken (18.07±2.56 ng/g) and honey roast salmon (17.12±5.86 ng/g). Ham (4.49±1.19 ng/g), smoked ham (0.57±0.29 ng/g), pork sausage (5.19±1.00 ng/g), crispy bacon (5.22±0.39 ng/g), Swedish meatballs (9.13±7.31 ng/g) and sweet chilli salmon (2.59±0.55 ng/g) had relatively low total HCAs content. Only BBQ chicken and honey roasted salmon had all 5 types of HCAs. The dominating compounds of HCAs in RTE meat products were IQ (nd-22.68 ng/g) and 4, 8-DiMeIQx (nd-12.61 ng/g), where Puangsombat et al. (2011) reported that PhIP and MeIQx were the major HCAs in RTE products that selected from the United States' market, and the total HCAs amount of selected RTE products (0.05-13.07 ng/g) were lower than the UK ones. Popularity of different RTE meat products in two countries could explain the difference. Rotisserie chicken with skin had the highest HCAs among all the RTE products in USA (Puangsombat et al., 2011), while the highest total HCAs (37.45ng/g) was detected in chargrilled chicken which had high consumption in UK RTE meat market (NDNS, 2015) (raw data, unpublished). Chargrilled chicken was the main contributor to IQ and 4, 8-DiMeIQx in consideration of total HCAs (Table 2-5), while Rotisserie chicken was key determinant for PhIP and MeIQx in concern of total HCAs in USA RTE meats. Commercial process for different RTE meat products varies in different countries, such as diverse cooking temperatures and the efficiency of various cooking equipment.

Table 2-5: The level of HCAs in selected meat products (n=9)

Sample	IQ (ng/g)	MeIQ(ng/g)	MeIQx(ng/g)	4,8-DiMeIQx(ng/g)	PhIP(ng/g)	Total (ng/g)	RSD[1] of total HCAs (%)
BBQ chicken	9.16±7.58^{b}	0.07±0.07^{a}	1.87±0.31^{bc}	5.46±3.50^{ab}	2.27±0.36^{a}	18.81±9.02^{c}	47.94
Tikka chicken	9.74±2.25^{b}	Nd	Nd	2.88±1.27^{a}	5.39±1.54^{a}	18.07±2.56^{c}	14.14
Chargrilled chicken	22.68±1.99^{a}	2.72±0.59^{b}	2.93±1.08^{c}	9.11±1.49^{ab}	Nd	37.45±4.89^{d}	13.04
Ham	Nd	2.59±1.29^{b}	1.90±0.18^{bc}	Nd	Nd	4.49±1.19^{ab}	26.59
Smoked ham	Nd	Nd	0.31±0.29^{a}	0.30±0.02^{a}	Nd	0.57±0.29^{a}	85.05
Roasted bacon	Nd	2.64±0.75^{b}	Nd	12.61±0.92^{b}	Nd	15.24±1.31^{bc}	8.60
Crispy bacon	Nd	3.39±0.37^{b}	Nd	1.83±0.08^{a}	Nd	5.22±0.39^{ab}	7.43
Pork sausage	Nd	2.87±0.19^{b}	Nd	2.32±1.13^{a}	Nd	5.19±1.00^{ab}	19.30
Swedish meatballs	Nd	2.11±0.42^{b}	Nd	7.02±7.69^{ab}	Nd	9.13±7.31^{ab}	80.11
Honey roasted salmon	5.56±3.75^{b}	0.14±0.90^{a}	0.83±0.25^{ab}	4.88±3.80^{ab}	5.71±4.15^{a}	17.12±5.86^{c}	34.21
Sweet chilli salmon	2.09±0.19^{b}	Nd	0.30±0.52^{a}	0.47±0.44^{a}	Nd	2.59±0.55^{a}	21.10

Nd: Not detected. Results with different letters in the same column are significantly different at the level $p<0.05$.
Each value is represented as mean ± SD.
[1]RSD: Relative standard deviation

Proximate composition of meat, such as moisture content had an effect on the level of total HCAs. As showed in Table 2-7, moisture content and total HCAs level were negatively correlated ($r=-0.708$, $p<0.05$), which implied that meat products with more moisture content seemed to contain less HCAs. Ham and smoked ham had the highest moisture content (about 75%) compared with the 3 chicken products (approximately 65%), which could partially explain a lower level of HCA in ham products than chicken products. Given the processing technology of ham, phosphate is one of the key ingredients used in ham products to improve the water holding capacity and to offer high moisture level in the final products. Consequently, high level of moisture in the product could dilute the concentration of HCAs generated during the processing (Chen, Pearson, & Gray, 1990). Vangnai et al. (2014) found that salt/phosphate injection reduced the total amount of HCAs in pork loin, which was cooked at 200℃ for 16 min. In addition, Fiener (2006) stated that water could prevent HCAs precursors moving to the food surface so that declined the concentration of carcinogens.

There was no significant correlation observed between fat content and total amount of HCAs in this study. However, positive relationship about fat and HCAs was reported by Johansson and Jagerstad (1994). The complexity of heating and formation of HCAs could shed some lights for the discrepancy. Although lipids and lipid oxidation could interact with the Maillard reaction to produce pyridine-intermediates in the formation of HCAs and promote the production of carcinogens (Barnes, Maher, & Weisburger, 1983), high level of fat could also dilute the reaction system and lead to a low level of carcinogens in final products. Nevertheless, Knize et al. (1994) reported increasing fat content up to 15% could accelerate the heat penetration and lead to more HCAs being produced, but a reduced level of carcinogens was observed when the fat content was over 15% owe to the dilution effect. In this study, low fat chicken samples had significantly higher total HCAs than high fat bacon and sausage samples, which might indicate that other factors such as cooking method might dominate the level of HCAs formation instead of fat content.

IQ level ranged from not detectable to 22.68±1.99 ng/g. Chargrilled chicken had the highest level of IQ, followed by Tikka chicken (9.74±2.25 ng/g), BBQ chicken (9.16±7.58 ng/g), honey roasted salmon (5.56±3.75 ng/g) and sweet chilli salmon (2.09±0.19 ng/g). Similar result was reported by Hasnol, Jinap, & Sanny (2014) that grilled chicken breast cooked at 300°C for 8 min contained 18.6±0.61 ng/g of IQ. In current study, IQ was only found in chicken and fish samples, but not in selected pork and bacon products. MeIQ was not detected in Tikka chicken, smoked ham and sweet chilli salmon, but crispy bacon (3.39±0.37 ng/g), pork sausage (2.87±0.19 ng/g), chargrilled chicken (2.72±0.59 ng/g), roasted bacon (2.64±0.75 ng/g), ham (2.59±1.29 ng/g) and Swedish meatballs (2.11±0.42 ng/g) had significantly higher level ($p<0.05$) of MeIQ, compared with BBQ chicken (0.07±0.07 ng/g) and honey roasted salmon (0.14±0.90 ng/g). Similar level of MeIQ (up to 1.7ng/g) was also reported in pork sausage and bacon by Busquets et al. (2004) and Johansson and Jagerstad (1994). MeIQ level in salmon samples was consistent with the work of Oz and Kotan (2016) that up to 0.42ng/g MeIQ in cooked salmon was determined with various cooking methods including microwave, dry-heating in pan, oven roasting, hot plat and barbecuing. MeIQx was detected up to 2.93±1.08 ng/g for selected RTE meat products, which fell in the ranges reported by Gibis (2016) and Oz and Kotan (2016), nd - 9 ng/g for cooked chicken breast, 0.1 – 27 ng/g for cooked pork and bacon, nd - 2.13 ng/g for cooked salmon. 4, 8-DiMeIQx was detected in all selected samples except ham (ng-9.11±1.49 ng/g). The significantly higher amount of 4, 8-DiMeIQx was observed in roasted bacon (12.61±0.92 ng/g, $p<0.05$), whereas extremely low level of 4, 8-DiMeIQx was found in ham and smoked ham. In the present study, 4, 8-DiMeIQx in chicken, pork and bacon products were higher than data reviewed by Gibis (2016), i.e. nd-3.6 ng/g in cooked chicken, nd-5.2 ng/g in cooked pork and bacon. Relatively high standard deviation occurred in BBQ chicken, Swedish meatballs and honey roasted salmon, which could be attributed to the batch effect when sampling from supermarket.

PhIP was only detected in BBQ chicken (2.27±0.36 ng/g), Tikka chicken (5.39±1.54 ng/g) and honey roasted salmon (5.71±4.15 ng/g). Skog and Solyakov (2002) reported similar level of PhIP (2.4-5.3 ng/g) in commercial

roasted chicken cooked at 175℃. There was no PhIP detected in chargrilled chicken, which was consistent with the result of Skog and Solyakov (2002) that no PhIP was found in commercial grilled chicken. However, Hasnol, Jinap, & Sanny (2014) detected 29.2ng/g PhIP in grilled chicken cooked at 300℃ for 8 min with internal temperature higher than 80℃. The disagreement might be attributed to the difference in the doneness of final products, as Knize et al. (2002) pointed out that the exposure of PhIP was especially high in well-done chicken. PhIP was significantly inhibited in sweet chilli salmon (not detectable), compared with that in honey roast salmon (5.71±4.15 ng/g) ($p<0.05$), which might be due to marinating ingredients in sweet chilli salmon, such as red pepper (Table 2-1). Wong, Cheng, & Wang (2012) indicated that polyphenol compounds (in red pepper) could directly trap phenyl acetaldehyde (a major precursor of PhIP) and inhibit the formation of PhIP.

In chicken products, chargrilled chicken breast contained the highest level of IQ, MeIQ and total HCAs, compared with BBQ chicken and Tikka chicken (commercially cooked in oven). Researchers discovered that grilling and barbecuing produced higher level of carcinogens than any other cooking methods such as boiling, since the high temperature of flame directly applied over the food surface and promoted greatly moisture loss (Liao et al., 2010; Skog, Johansson, & Jaègerstad, 1998). Roasting usually would not generate high amount of carcinogens, as meat products were cooked under heated air (110-300℃), which resulted in the crispy surface of cooked meat but high internal moisture (Skog and Solyakov, 2002). On the other hand, marinating ingredients could attribute to the low or non-detectable level of MeIQ and MeIQx in Tikka chicken and BBQ chicken (Table 2-5). Tikka chicken was processed with lemon juice which contained high level of Vitamin C, while BBQ chicken contained spices such as cumin, paprika and clove (Table 2-1). Both ascorbic acid and spices have been found to reduce HCAs formation due to their antioxidant capacity (Vitaglione and Fogliano, 2004). Cheng et al. (2007) stated that marinated beef with lemon juice could reduce about 30% MeIQ in grilled products.

In observed RTE meat products, pork and bacon products contained relatively low level of HCAs. It could be related to their processing methods. In pork and bacon products, commercial ham and smoked ham are usually cooked by placing the reformed ham in a hot water bath (80℃ - 90℃) or steam until the interior temperature reaches to 72℃, which lead to high moisture content and low amount of HCAs (Vangnai et al., 2014). On the other hand, to reach the desirable smoked flavour for crispy bacon and smoked ham, smoking can be delivered by cold smoking at 15-25℃, warm smoking at 25-50℃ and hot smoking at 50-85℃ by injecting smoking liquid with brine (Toldrá, 2009). Smoking at 15-85℃, a relatively low temperature range, only generated small amount of HCAs (Turesky, 2010), which could verify that smoked ham and crispy bacon contained less amount of HCAs.

In fish products, the occurrence of HCAs in sweet chilli salmon (2.59±0.55 ng/g) was nearly 8 times lower than those in honey roasted salmon (17.12±5.86 ng/g) (Table 2-5). It was supposed that spice powder (red pepper, garlic powder, onion powder, paprika extract) and sunflower oil in sweet chilli salmon played key roles in reducing the amount of HCAs (Table 2-1). Antioxidants in spices and oils such as polyphenols, organosulfides compounds, Vitamin C or Vitamin E have been proved to trap intermediates or remove free radicals to reduce the formation of HCAs (Janoszka, 2010; Lan and Chen, 2002; Salmon, Knize, & Felton, 1997). Diallyl disulfide and dipropyl disulfide (in garlic and onion powder) may contribute to trap intermediates in Maillard reaction so that prohibit further reactions, and also they may be regarded as scavengers of free radicals (Janoszka, 2010). In addition, sunflower oil (containing Vitamin E) added to sweet chilli salmon could also affect the formation of PhIP. Balogh et al. (2000) reported that spraying vitamin E on the surface of fried beef patties, resulted in the reduction of total HCAs by 71%. However, which type of compounds is more effective and the dosage effect need to be further investigated.

2.4.4 Determination of PAHs

The amount of individual PAH (BaA and BaP) and total PAHs in selected RTE meat products were listed in Table 2-6. The concentration of total PAHs

ranged from not detectable to 3.06 ng/g. Chargrilled chicken contained the highest amount of total PAHs, followed by Swedish meatballs (2.36±0.33ng/g), roasted bacon (1.75±0.17 ng/g) and crispy bacon (1.08±0.21 ng/g). There were no BaA and BaP detected in BBQ chicken, Tikka chicken, ham and sweet chilli salmon. While both PAHs were observed in roasted bacon, crispy bacon, sausage and Swedish meatballs. It was noticed that total PAHs level was negatively correlated to moisture content ($r=-0.734$, $p<0.01$) and positively correlated to fat content ($r= 0.414$, $p<0.05$) (Table 2-7). Similar trend that total PAHs increased with fat level in meat was reported by Chung et al. (2011), Fretheim (1983) and Janoszka (2011). Dost and İdeli (2012) indicated that there were no PAHs in barbecued trout or bass, because of low fat content in those types of fish. However, grilled salmon at 290^{o}C for 20 min contained high level of BaP (1.66 ng/g) due to high fat content. Pöhlmann et al. (2013) found that total PAH level was reduced in hot smoked Frankfurter-type sausages when back fat decreased from 30% to 20%. The positive relationship between fat level and PAHs could be attributed to the pyrolysis of heated fat which dropped on charcoal or other heating resources and deposited on the surface of meat (Fretheim, 1983).

Table 2-6: The level of PAHs in selected meat samples (n=9)

Sample	BaP (ng/g)	BaA (ng/g)	Total PAHs (ng/g)	RSD[1] of total PAHs (%)
BBQ chicken	Nq	Nq	Nq	N/a
Tikka chicken	Nq	Nd	Nq	N/a
Chargrilled chicken	Nq	3.06±0.50^{d}	3.06±0.50^{e}	16.34
Ham	Nd	Nq	Nq	N/a
Smoked ham	Nq	0.19±0.16^{ab}	0.19±0.16^{a}	84.21
Roasted bacon	1.09±0.11^{d}	0.66±0.07^{b}	1.75±0.17^{d}	9.71
Crispy bacon	0.71±0.12^{c}	0.37±0.09^{ab}	1.08±0.21^{c}	19.44
Pork sausage	0.21±0.03^{ab}	0.67±0.10^{b}	0.89±0.10^{bc}	11.24
Swedish meatballs	0.18±0.11^{ab}	2.18±0.22^{c}	2.36±0.33^{d}	13.98
Honey roasted salmon	0.35±0.06^{b}	Nq	0.35±0.06^{b}	17.14
Sweet chilli salmon	Nd	Nd	Nd	N/a

Nd: Not detected, Nq: Not quantified.
Results with different letters in the same column are significantly different at the level $p<0.05$.
Each value is represented as mean ± SD.
[1]RSD: Relative standard deviation
N/a: Not applicable

Table 2-7: Correlation coefficients between total carcinogens level and moisture/fat content in meat samples

Components	Correlation coefficient(r)	p-value
Total HCAs/Moisture	-0.708	0.001
Total HCAs/Fat	-0.213	0.317
Total PAHs/Moisture	-0.734	0.001
Total PAHs/Fat	0.414	0.046

The range of BaP in RTE meat products was from nd to 1.09±0.11 ng/g. The highest level of BaP was found in roasted bacon, followed by crispy bacon (0.71±0.12 ng/g), pork sausage (0.21±0.03 ng/g) and Swedish meatballs (0.18±0.11 ng/g). BaP was not detectable/quantifiable in chicken and ham products. Olatunji et al. (2014) revealed that similar level of BaP in smoked pork (1.28±1.61ng/g). Djinovic, Popovic, & Jira (2008) reported that BaP was detected 0.01-0.36 ng/g in smoked bacon and 0.08-0.33ng/g in smoked sausages, respectively. The amounts of BaP in all samples were all below 2ng/g, which is the maximum value that allows in smoked meat products that agreed by the Commission Regulation EC No 208/2005 and 1881/2006. BaP was proved that it was significantly correlated to the 6 IARC possible and probable carcinogens PAHs (BaA, BaP, BbF, benzo[k]fluoranthene, dibenzo[a,h]anthracene and indeno [1,2,3-cd]pyrene) in 4 Spanish traditional smoked sausage (Lorenzo et al. 2011; Lorenzo et al., 2010), hence it is necessary that reduce the amount of BaP in processed meat products. BaP was completely inhibited by marinating spice mix such as garlic, onion and pepper powder in sweet chilli salmon, compared with honey roasted salmon (0.35±0.06 ng/g). Farhadian et al. (2012) stated that marinating beef with onion, garlic, turmeric and lemon juice (70% w/w) was the most effective way to inhibit BaP in grilled beef. The mechanism of spice or vitamins affect the formation of PAHs has not fully understood yet, it could be possible due to the antioxidant capacity in marinating ingredients that interfere free radicals during cooking (Janoszka, 2011).

BaA was not detected in Tikka chicken and sweet chilli salmon, was not quantifiable in BBQ chicken, ham and honey roasted salmon. It could be found in pork and bacon products, but not chicken and fish except chargrilled chicken. Chargrilled chicken (3.06±0.50 ng/g) had significantly higher amount of BaA ($p<0.05$), compared with BBQ chicken and Tikka chicken. Janoszka (2011) reported that grilling could form more PAHs due to the incomplete combustion of fat and charcoal under direct heat source, which lead to be dripped into fuel, evaporated and attached on the surface of cooked meat. As it could be seen, 0.19±0.16 ng/g BaA was occurred in smoked ham, this was consistent with the previously published result (0.102-1.50 ng/g in industrially

smoked ham) (Djinovic, Popovic, & Jira, 2008). Smoking could also contribute to form more PAHs, as meat would be exposed by smoke produced from wood or charcoal with less O_2. Lawrence and Weber (1984) stated that more PAHs detected in smoked fish than in un-smoked fish. Olatunji et al. (2014) also concluded that smoked meat contained the most PAHs compared with boiled meat.

2.4.5 Dietary exposure of HCAs and PAHs from RTE meat products

Meat consumption data from NDNS 2015 (male and female, adults: 19-64 years old and elderly: over 65 years old) (Appendix 15) and levels of HCAs determined were used to estimate the daily exposure (ng/day) of IQ, MeIQ, MeIQx and PhIP from RTE meat products (ng/g) (Norrish et al., 1999). Table 2-8 showed that great daily intake of IQ (79.15-189.38 ng/day) from chargrilled chicken, MeIQ (9.49-19.92 ng/day) and MeIQx (7.14-14.61 ng/day) from chargrilled chicken and ham in both genders. It can be seen that daily intake of IQ from chargrilled chicken, MeIQ and MeIQx from chargrilled chicken and ham in female was relatively higher than those of male in both age groups. Sugimura et al. (2004) estimated that the median amount of HCAs daily intake for European people is 103ng/day, whereas the average intake of IQ from chargrilled chicken in female (both age groups) and male (adults group) were higher than 103ng/day (117.71- 189.38 ng/day). Recent case-control studies have been reported that the increasing intake of HCAs is related with colorectal adenoma and oxidative stress (Budhathoki et al., 2015; Carvalho et al., 2015). Carvalho et al. (2015) stated that high intake of HCAs including MeIQx, DiMeIQx and PhIP could aid in increasing oxidative stress regardless of lifestyle factors, in order to increase the risk of chronic diseases, such as cancer and heart disease. Budhathoki, et al. (2015) reported that a notably increased risk of colorectal adenoma (Odds Ratio: 2.10, 95% CI: 1.20–3.67, p=0.01) was related with the higher quartile of MeIQ (6.0 ng/day). The daily intake of MeIQ (male: 9.49-17.71 ng/day, female: 14.12-22.71 ng/day) from chargrilled chicken and ham were much higher than this threshold, it implies that increasing intake of these 2 RTE meat products has a higher risk of colorectal adenoma.

Table 2-8: Daily intake of IQ, MeIQ, MeIQx and PhIP from selected RTE meat samples

	Daily intake of HCAs			
	Male		Female	
	19-64 years old	>65 years old	19-64 years old	>65 years old
IQ (ng/day)				
BBQ chicken	0.27	0	2.47	0
Tikka chicken	15.39	0	10.51	14.66
Chargrilled chicken	147.65	79.15	189.38	117.71
MeIQ (ng/day)				
BBQ chicken	0.0021	0	0.018	0
Chargrilled chicken	17.71	9.49	22.71	14.12
Ham	10.77	9.74	15.31	19.92
Smoked ham	1.37	1.61	0.98	1.66
Roasted bacon	3.63	0.96	3.86	1.63
Crispy bacon	0.13	0.26	0.1	0.54
Pork sausage	0.78	0	1.19	0.49
Swedish meatballs	0.0021	0	0.018	0
MeIQx (ng/day)				
BBQ chicken	0.056	0	0.5	0
Chargrilled chicken	19.07	10.23	24.47	15.21
Ham	7.9	7.14	11.23	14.61
Smoked ham	0.13	0.02	0.05	0.12
PhIP (ng/day)				
BBQ chicken	0.07	0	0.61	0
Tikka chicken	8.52	0	5.98	8.62

The intake of HCAs might be linked with breast cancer. The consumption of IQ, MeIQx and PhIP from the lowest to highest quartile was associated with increasing breast cancer risk 2-3 fold, which was reported by Stefani et al. (1997). The amount of IQ in chargrilled chicken (22.68±1.99 ng/g) was greater than the highest quartile (1.02ng/g) (Odds Ratio: 3.34, 95%CI: 1.85-6.02, p=0.001), which indicate that high intake of chargrilled chicken could increase

at least 2-3 fold risk of breast cancer. They also revealed that exposure of PhIP was significantly linked with breast cancer and does-dependent, especially the cancer risk increased 3 times when the exposure of PhIP over 20.15ng/g (Odds Ratio: 3.31, 95%CI: 1.60-6.87, p<0.001). However, the amount of PhIP from chargrilled chicken was 5.39±1.54 ng/g, which were not enough to pose the risk. Overall, it should be pointed out that female consumers who have a high intake of chargrilled chicken and ham might increase the risk of breast cancer and colorectal adenoma. It is suggested that reducing the amount of HCAs in processed meat products, which would decrease the daily exposure of HCAs to UK processed meat consumers and lower the risk of breast cancer and colorectal adenoma.

The amounts of BaA and BaP in various selected RTE meat products ranged from not detectable-3.56 ng/g and not detectable-1.20 ng/g respectively. The concentration of BaP was ranged from 0.01-50 ng/g ng/g (Nisha et al., 2015).The LADDs of PAHs were estimated to assess the health risk imposing by selected RTE meat products (Table 2-9). The greatest LADDs were 0.0285 and 0.0177 ng/kg BW/day from chargrilled chicken in female, adults and elderly respectively. LADDs in the elderly group were lower than those in adults. LADDs were higher in chargrilled chicken, crispy bacon and roasted bacon than all other types of meat due to the higher average daily consumption. The cancer risk induced by PAHs was evaluated according to the carcinogenic potency factor (slope factor) of BaP, which was1.3-1.4 mg/kg BW/day^{-1} by oral exposure, which caused tumours in mice (Schneider et al., 2002). It was estimated that ingestion cancer risk associated with the dietary intake of in RTE meat products to be 2.5:10^{8} for male with average body weight of 83.6kg and 2.4:10^{8} for females with average body weight of 70.2kg (ONS, 2015). The acceptable risk level of developing cancer is a one in a million chance of additional human cancer over a 70 year lifetime (1/10^{6}), and a serious level is that one in ten thousand (1/10^{4}) (Alomirah et al., 2011; Chen et al., 2013). However, the acceptable risk level would be higher during lifetime exposure, as the lifetimes of both genders in UK are greater than 70 years. Even though the risk of PAHs in commercial RTE meat products is relatively low, it could be higher than the acceptable level of cancer risk if the

average daily intake (g/day) of chargrilled chicken is over 292 g/day in female adults.

Table 2-9: Lifelong Average Daily Intake (LADD) of PAHs from RTE meat products in the UK

	LADD (ng/kg BW/day)			
	Male		Female	
Meat	19-64 years old	> 65 years old	19-64 years old	> 65 years old
BBQ chicken	trace	trace[1]	trace	trace
Tikka chicken	trace	trace	trace	trace
Chargrilled chicken	0.0184	0.0099	0.0285	0.0177
Ham	trace	trace	trace	Trace
Smoked ham	0.0001	trace	trace	0.0001
Roasted bacon	0.0057	0.0066	0.0049	0.0083
Crispy bacon	0.0097	0.0026	0.0124	0.0053
Pork sausage	0.0001	0.0003	0.0001	0.0006
Swedish meatballs	0.0011	trace	0.0020	0.0008

[1] trace: <0.0001

2.5 Conclusions

The total HCAs level in selected RTE meat products ranged from 0.57±0.29 ng/g to 37.45±4.89 ng/g, nd-3.06±0.50 ng/g for PAHs. Dominating compounds were IQ and 4, 8-DiMeIQ in chicken, fish and pork products respectively, BaP in bacon products and honey roasted salmon. Chargrilled chicken contained

the highest level of both types of carcinogens. Tikka chicken, BBQ chicken and honey roast salmon contained a significantly higher level of total HCAs than those in ham, smoked ham and sweet chilli salmon, whereas chargrilled chicken contained the highest amount PAHs. This work offered the accurate level of carcinogens from both HCAs and PAHs in RTE meat products, which could be used to support the guideline of dietary intake of HCAs from RTE meat products. The dietary intake of HCAs, particularly IQ and MeIQ from chargrilled chicken and ham were relatively high, which might contribute to the increased risk of breast cancer and colorectal adenoma. The Lifelong Average Daily Intake of PAHs from chargrilled chicken, crispy bacon and roasted bacon were higher than all other types of meat products. Overall, intake of chargrilled chicken could increase breast cancer and colorectal adenoma risk, other types of meat had relatively lower health risk. However, if the average dietary intake of chargrilled chicken increased to 292 g/day, the health risk would be over the acceptable level. Since the consumption of meat is unlikely to change, it is recommended that processing methods should be optimized to reduce the amount of these carcinogens. Therefore, the health risk from intake of RTE meat products could be reduced for the general public.

2.6 References

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Chapter 3. The effects of vegetable oil and temperature on the formation of heterocyclic amines and polycyclic aromatic hydrocarbons in fat-replaced pork patties

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3.1 Abstract

Formation of HCAs and PAHs was examined to evaluate the impact of using vegetable oil as fat replacement on carcinogens formation in meat product. Pork patties were formulated with 40% fat replacement by olive oil, sunflower oil or grape seed oil and cooked at 180°C or 220°C, respectively. Control patties contained the highest amount of HCAs compared with all other patties at both temperatures. Olive oil and sunflower oil replacement completely inhibited formation of MeIQ, while grape seed oil completely inhibited MeIQx, 4, 8-DiMeIQx and PhIP in patties. Grape seed oil achieved the highest inhibition capacity compared with sunflower oil and olive oil. HCAs increased significantly with cooking temperature ($p<0.05$), but no difference was observed in total PAHs for patties cooked at different temperature ($p>0.05$). In conclusion, fat replacement with sunflower oil, olive oil or grape seed oil in

pork patties could reduce the formation of HCAs without compromising eating quality.

Key words: Antioxidants; Fat modification; Maillard reaction; Oxidation.

3.2 Introduction

Fat plays an important role in the human diet. It not only creates a unique sensation of food, but also helps maintain health. The consumption of pork in the world has dramatically increased from 18 to 110 million tons per year (1950-2010) (Brown, 2013). Research found that increased saturated fatty acid intake could elevate the risk of cardiovascular disease, but monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) could reduce the risk and maintain cardiovascular health (McAfee et al., 2010; Sadler, 2014). Therefore, changing fatty acids profile of meat products by replacing saturated fatty acids with unsaturated fatty acids has attracted lots of attention in both academic research and meat processors. Adding olive oil could dramatically increase the percentage of MUFAs in final products, whereas sunflower oil and grape seed oil could greatly raise the level of PUFAs in fat replaced meat products (Gunstone, 2002; Matthäus, 2008). Rodríguez-Carpena et al. (2012) successfully replaced 50% fat with avocado, sunflower and olive oil in cooked pork patties and reported that avocado and olive oil could even offer better aroma to the final products than control ones. Vural and Javidipour (2002) successfully substituted beef fat in Frankfurters with the mixture of interesterified palm, cottonseed and olive oil without changing physical parameters and total sensory scores. Choi et al. (2010) used pre-emulsified grape seed oil and 2% rice bran fibre to develop pork batters with 50% fat replacement and reported that the fat-reduced pork batters could achieve the comparable eating quality with control samples. Domínguez, et al. (2016) replaced 100% pork back fat with olive oil in pork pâté, which significantly increased the content of tocopherols and MUFAs in cooked products without altering physio-chemical properties. Domínguez et al. (2017) and Lorenzo et al. (2016) replaced 25%-75% backfat with olive oil, microencapsulated fish oil and the mixture of fish oil and olive oil, which significantly increased the percentage of PUFAs in frankfurter type sausage

and Spanish salchichón. These results indicate that vegetable oil could be used successfully to replace fat partially or completely to offer products comparable eating quality with healthier fatty acids profile, i.e. high level of MUFAs and PUFAs.

However, unsaturated fatty acids in vegetable oil may pose risk in domestic cooking due to their oxidation and decomposition at high temperature. For example, linoleic acid was found associated with the formation of potentially toxic compounds, such as free radicals, aldehydes and ketones (Guillén & Uriarte, 2012; Katragadda et al., 2010). These reactive oxygen species (ROS) initiated by unsaturated fatty acids peroxidation could induce the decomposition of Amadori compounds and generate 1- and 3- deoxysone that are intermediates for Strecker aldehydes, pyrazines and pyridines in Maillard reaction. Consequently, it might promote the formation of HCAs (Morello, Shahidi & Ho, 2002; Turesky, 2010; Zamora & Hidalgo, 2007). Some hydroperoxides generated from the decomposition of the unsaturated hydrocarbons during heating, such as linolenate can also undertake aromatization and de-hydrocyclization, further cleave into benzaldehydes and other benzene ring-containing compounds, which are precursors of PAHs (Chen & Chen, 2001; Lorenzo et al., 2010; Lorenzo et al., 2011; Singh, Varshney & Agarwal, 2016).

HCAs, PAHs and N-nitrous compounds are well-known carcinogens which were detected in processed meat products (Hasnol, Jinap & Sanny, 2014; Jinap et al., 2013; Liao et al., 2010; Oz & Kaya, 2010; Salmon, Knize & Felton, 1997). HCAs are mainly formed with the presence of free amino acids, carbohydrates and creatine under high cooking temperature (Rahman et al., 2014). IARC (1993) classified the following 5 aminoimidazoarenes (AIAs) compounds as human carcinogens, including 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,4-methylimidazo[4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4, 8-DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). PAHs are hydrocarbons that contain two or more benzene rings, such as pyrene, anthracene and naphthalene. They

can be formed through incomplete combustion or pyrolysis of organic components, including fat, protein and carbohydrates at the temperature over 200 ^{o}C. Grilling, roasting and smoking meat products likely contain high level of PAHs (Alomirah et al., 2011). Benz[a]anthracene (BaA) and benzo[a]pyrene (BaP) are the most potent carcinogenic PAHs in processed meat products (PHE, 2008). The metabolite of BaP, BaP-7,8-diol-9,10-epoxide, has been reported with the highest tumour-inducing activity due to causing DNA adducts (Purcaro, Moret & Conte, 2013).

Vegetable oils contain various antioxidants such as vitamin E, ß-carotenes and phenolic compounds (Ramírez-Anaya et al., 2015). These antioxidants have been characterized as free radical scavengers during cooking, which might inhibit the formation of carcinogens (Janoszka, 2011; Wong, Cheng & Wang, 2012). Cheng, Chen and Wang (2007) reported that marinating beef patties with phenolic compounds such as epicatechin gallate, rosmarinic acids and carnosic acid could significantly reduce HCAs by 24%-70% in final cooked products. Balogh et al. (2000) found that HCAs (IQ, MeIQ, MeIQx, DiMeIQx and PhIP) were inhibited by 45%-75% when sprayed 1% vitamin E (w/w) on the surface of beef patties before frying. Therefore, in the concern of the carcinogens level in processed meat products, replacing saturated fat with vegetable oils rich in unsaturated fatty acids needs to be justified. Thus, the objectives of this study were to (1) explore the effect of partially replacing pork back fat with sunflower oil, olive oil and grape seed oil on the formation of HCAs and PAHs; (2) examine the effect of different cooking temperatures on the formation of carcinogens in fat reduced pork patties.

3.3 Materials and methods

3.3.1 Materials

Three batches of lean pork leg and pork back fat were purchased from Jennings Caversham (Reading, UK) at different time point to consider the batch effect. In average, pork back fat consists of 40.3% SFA, 43.4% MUFA and 10.0% PUFA (McCance & Widdowson, 2002). Excess visible fat on pork legs was trimmed, then minced by a Kenwood Food processor (Chef Titanium KM010, 4.6, Kenwood Limited) and vacuum packed separately. Raw

materials were stored at -18ºC and defrosted 24h at 4 ºC before use. Commercial grape seed oil (Waitrose©, produced in Italy) with 12.4% SFA, 20.2% MUFA, 68.2% PUFA, 10-15mg tocopherols and 5.9-11.5mg/100g polyphenols (Bail, Stuebiger, Krist, Unterweger, & Buchbauer, 2008), sunflower oil (Morrisons©, produced in UK) with 14.3% SFA, 20.5% MUFA, 63.3% PUFA and 50mg/100g tocopherols (McCance & Widdowson, 2002) and refined olive oil (Filippo©, phenols were removed by industrial process, produced in Italy) with 14.3% SFA, 73.0% MUFA, 8.2% PUFA and 5-300mg tocopherols (McCance & Widdowson, 2002) were purchased from local supermarket (Reading, UK). Oils were kept in refrigerator (4ºC) before making patties and further analysis.

The 5 HCAs and 2 PAHs standards were purchased from Toronto Research Chemicals (Toronto, Canada). Ammonium acetate, triethylamine, acetonitrile (HPLC grade), bovine serum albumin (BSA), dinitrophenylhydrazine (DNPH), ethyl acetate 99.5% 0.9000g/ml, 6M guanidine HCl (pH 6.5), hydrochloric acid solution 0.1M, methanol (HPLC grade), HPLC grade water, sodium hydroxide 1M, perchloric acid (99.8%), sodium phosphate buffer (pH 6.5), thiobarbituric acid (TBA), and trichloroacetic acid (TCA) were purchased from Fisher Scientific (Loughborough, UK). 2,2-Azobis(2-methylpropionamidine) dihydrochloride granular 97% (ABAP), 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), phosphate buffer solution 0.1 M and phosphoric acid were purchased from Sigma-Aldrich (Gillingham, UK). The solid-phase extraction Extrelut NT 20 columns and diatomaceous earth refill material were purchased from Merck (Darmstadt, Germany). Bond Elut propyl-sulfonic acid (PRS) cartridges (100 mg, 10 ml), C-18 cartridges (7 ml) were purchased from VWR Inc (Lutterworth, UK).

3.3.2 Procedures for preparing and cooking pork patties

The formulation of control patties (as shown in Table 3-1) included 700g lean pork mince, 180g distill water, 100g pork back fat and 20g salt per kilogram. For the fat partially replaced patties, 40% of pork back fat was replaced with sunflower oil, olive oil or grape seed oil respectively. Overall, there were 4

types of pork patties prepared in this study, i.e. control (100% back fat, C patties), sunflower oil patties (S patties), olive oil patties (O patties) and grape seed oil patties (G patties). All ingredients were homogenized at 5000rpm for 5min in the Kenwood Food processor to reach a uniform emulsion. Each patty was weighed 100g, shaped in a foil cup (9.0cm diameter * 2.5cm height) for standardization.

Table 3-1: Formulation of control and fat replaced pork patties

	Ingredients in recipe represent as g/1kg				
Treatment	Lean pork leg	Oil	Pork back fat	Salt	Water
Control, pork back fat (C patties)	700	0	100	20	180
Fat replaced with Sunflower oil (S patties)	700	40	60	20	180
Fat replaced with Olive oil (O patties)	700	40	60	20	180
Fat replaced with Grape seed oil (G patties)	700	40	60	20	180

Patties were cooked in an air-forced oven at either 180 ^{o}C for 26 min or 220^{o}C for 22 min until the core temperature of patties reached to the range of 72.6-73.4 ^{o}C, which was monitored by applying a thermal probe (KM330 Industrial Thermometer, Comark Instruments, UK). After cooking, pork patties were covered by foil and chilled in cold room at 4 ^{o}C for 24 hours. Physical properties including texture and colour were measured on the following day of cooling. While part of samples was homogenized and stored in -18 ^{o}C for further chemical analysis. Cooking loss was determined according to the equation: Cooking loss (%) = $(W_r - W_c)/W_r$ *100, where Wr was the weight of raw pork patties, and Wc was weight of cooked pork patties. Three batches of control and fat replaced pork patties cooked at 180 ^{o}C or 220 ^{o}C (8 treatments in total) were manufactured. For each replicate, 8 patties were made for each treatment.

3.3.3 pH and composition analysis including moisture, fat and protein content

The procedure of analysis was the same with Chapter 2.3.3.

3.3.4 Lipid/protein oxidation and antioxidant capacity

3.3.4.1 Lipid oxidation--Thiobarbituric acid-reactive substances (TBARS) value

The degree of lipid oxidation in samples was expressed by TBARS values. It was determined by the method orinignally reported by Tarladgis et al. (1964) with slight modification, which is extraction method. Pork patties that for TBARS analysis were vacuum packed and kept frozen (-18ºC) up to 30 days. Three batches of vacuum-packed pork patties were deforsted at 4 ºC for 24h and homogenized to uniform bebfore analysis. 5 g homogenized pork patty was homogenized with 15 ml perchloric acid (3.86%) and 0.5ml BHT (4.2% in ethanol) in a beaker, which was immersed in an ice bath to minimize oxidative reactions in samples during extraction. The mixture was then filtered and centrifuged at 3000 rpm for 4 min, 2 ml supernatant was mixed with 2 ml thiobarbituric acid (0.02 M) in test tube. The test tubes were then placed in a boiling water bath (100 ºC) for 45 min. After cooling, the absorbance was measured at 532nm using a spectrophotometer (6315, Bibby Scientific Ltd, UK). The standard curve was prepared using 1,1,3,3-tetraethoxypropane (TEP) in 3.86% perchloric acid with the concentration of 0, 0.5, 1.0, 2.5, 5.0 and 10.0μM.

3.3.4.2 Total protein carbonyl value (Protein oxidation)

The degree of protein oxidation can be evaluated by calculating the total carbonyl value according to the method originally described by Oliver et al. (1987). Pork patties that for protein carbonyl analysis were vacuum packed and kept frozen (-18ºC) up to 30 days. Three batches of vacuum-packed pork patties were deforsted at 4ºC for 24h and homogenized to uniform bebfore analysis. 1g of homogenized pork patties 1:10 (w/v) was mixed in 20 mM sodium phosphate buffer containing 0.6 M NaCl (pH 6.5) for 30 s. Two equal aliquots of 0.2ml mixture were then dispensed in 2 ml eppendorf tubes, respectively. 1ml cold trichloroacetic acid (TCA) (10%, w/w) was added into

tubes and centrifuged for 5 min at 5000 rpm. One pellet was mixed with 1 ml 2M HCl in order to measure protein concentration, while the other pellet was mixed with 1 ml of 0.2% (w/v) dinitrophenylhydrazine (DNPH) in 2M HCl in order to measure carbonyl concentration. Both tubes were incubated for 1 h at room temperature. Subsequently, 1 ml 10% TCA was added into tubes and pellets were washed twice with 1 ml ethanol: ethyl acetate (1:1, v/v) to remove excess of DNPH. The pellets were then mixed with 1 ml of 20 mM sodium phosphate buffer containing 6M guanidine HCl (pH 6.5), stirred and centrifuged for 2 min at 5000 rpm to remove insoluble fragments. Protein concentration (mg/ml) was calculated from absorption at 280 nm using BSA as standard. The carbonyl concentration (nmol/ml) was calculated by using the Beer-Lambert Law: A = e*c*l, where A is the absorbance at 370nm, e is the absorption coefficient of DNPH for absorbance at 370 nm (21.0 nM^{-1} cm^{-1}), c is the carbonyl concentration of sample and the l is the length of cuvette (1cm). The protein carbonyl content in samples was expressed as nmol of carbonyl per mg of protein.

Protein carbonyl content (nmol/mg) = (Carbonyl nmol/ml) / (protein content mg/ml)

3.3.4.3 Trolox equivalent antioxidant capacity (TEAC) of vegetable oils/ back fat

TEAC was used to evaluate the total antioxidant capacity of vegetable oils and pork back fat. The measuring procedures were based on the method reported by van den Berg et al. (1999). An ABTS radical solution was prepared by mixing 2ml 2.5 mM ABAP with 20ml 20 mM $ABTS^{2-}$ stock solution in 25ml 100 mM phosphate buffer (pH 7.4), which contained 2ml 150 mM NaCl. The solution was covered with foil and heated at 60℃ for 12 min, then cooled down to room temperature. 1g sample was diluted with 10ml 80% n-hexane. 40 µl of the sample solution was mixed with 1960 µl of the freshly prepared ABTS solution. Difference of absorbance at 734 nm in 6 min was recorded. A calibration curve was made by measuring the difference of absorbance in 6min for Trolox at the concentration of 0, 0.5, 1.0, 2.5, 5.0, 7.5

and 10.0 μM. The TEAC of vegetable oils or back fat was presented on a molar basis to Trolox (μmol Trolox/100g).

3.3.5 Determination of HCAs

Approximate 2mm samples surface were trimmed and blended well before measuring. The procedure of analysis was the same with Chapter 2.4.3.

3.3.6 Determination of PAHs

The procedure of analysis was the same with Chapter 2.4.4.

3.3.7 Recovery rate of HCAs and PAHs

The 5 standard HCAs (IQ, MeIQ, MeIQx, 4, 8-DiMeIQx and PhIP) and 2 PAHs (BaA and BaP) compounds were identified through the retention time of the peaks and spectrum, and the quantity of each individual compound was determined according to the standard calibration curves, which was established by the standard solution at 0.5-250ng/ml. Limit of detection (LOD) for IQ, MeIQ, MeIQx, 4, 8-DiMeIQx, PhIP, BaA and BaP were for 0.02 ng/g, 0.01 ng/g, 0.02 ng/g, 0.05 ng/g, 0.03 ng/g, 0.07 ng/g and 0.06 ng/g. The average recoveries of these 5 HCAs based on triplicates were 60.01% for IQ, 61.76% for MeIQ, 53.64% for MeIQx, 60.57% for 4,8-DiMeIQx and 55.98% for PhIP. Results were comparable with several published data (Gibis, Kruwinnus & Weiss, 2015; Messner & Murkovic, 2004; Oz & Cakmak, 2016; Yao et al., 2013). The recovery rate for BaA and BaP was 54.37 and 49.54% respectively, which was comparable with published results of 50% - 115% (Farhadian et al., 2010; Ishizaki et al., 2010; Iwasaki et al., 2010; Janoszka, 2011). Recovery rate could be affected by sorbing materials, flow rate through cartridges, organic modifier quality and/or content, interfering effects of eluting solvents (Busetti et al., 2006). Stevens et al. (2006) reported that overlapping peaks on chromatogram indicating insufficient separation procedures could result in recovery rate over 110%, and AOAC (2016) recommends that 40-120% recovery rate is acceptable for compounds at 1ng/g concentration.

3.3.8 Inhibitory efficiency of HCAs and PAHs

Inhibitory efficiency was determined according to the equation:

Inhibitory efficiency (%) = $(A_c - A_t)/A_c \times 100$

where A_c was the average total amount of HCAs/PAHs in control samples (ng/g), and A_t was the average total amount of HCAs/PAHs in fat partially replaced patties (S/ O/ G patties) (ng/g).

3.3.9 Physical parameters

Texture and colour parameters were measured on pork patties to determine the overall eating quality of products that affected by reformulation.

3.3.9.1 Colour

Colour feature including L*, a* and b* was measured using Hunter Lab Colour instrument (Hunter Associates Laboratory, Virginia, USA, 2003). Each sample was measured at 3 different locations, while the average was recorded as the colour feature of the sample.

3.3.9.2 Texture

Texture profile analysis (TPA) was performed at room temperature with a Stable System Texture Analyzer (Middleboro, USA). Cyclinder samples (D=18mm, H=22mm) were prepared using a stainless cork borer. Before the analysis, samples were tempered at room temperature (20^{o}C) for 30min. The settings for texture analysis were: load cell 5 kg, head speed 1.6 mm/s, and compression depth 10.0 mm. The calculation of TPA values was based on the compression curve with force (y-axis) and time (x-axis). Values for hardness (N) was defined as the absolute peak force in the 1st compression cycle, cohesiveness as the area of work in the 2nd compression divided by the area of work in the 1st compression, chewiness as the product of hardness*cohesiveness*springiness, and springiness as the force that sample return to its initial pattern after compression (Sánchez del Pulgar, Gázquez & Ruiz-Carrascal, 2012). Each sample was measured at least 5 times, and the average was recorded as the value of the sample.

3.3.10 Statistical analysis

Statistical significance test was carried out by using SPSS Statistics 21. The significant difference in chemical composition, physical properties, levels of HCAs and PAHs for the 8 treatments were carried out by one-way analysis of variance (ANOVA) at the significant level 0.05, and *Duncan* test was selected for multiple comparisons if equal variances assumed, otherwise *Tamhane's*

T2 test was used. To analyse the effect of factors and the interaction between factors (cooking temperature and replacing oil type), two-way ANOVA was employed at the significant level 0.05. Multivariate linear regression model was employed to explore the effect of multi independent factors, including lipid oxidation, protein oxidation and antioxidant capacity of oils on the formation of HCAs and PAHs at the significant level 0.05. Pearson correlation was employed for the measurements as a prerequisite for the regression.

3.4 Results and discussion

3.4.1 Effect of replacing oil and cooking temperature on pH, proximate composition and physical parameters of reduced fat pork patties

3.4.1.1 Proximate composition and pH

Table 3-2 shows the effect of replacing oil and cooking temperature on proximate composition and pH values in patties. Type of replacing oil did not pose any effect on all proximate composition and pH, but cooking temperature had significant influence on moisture and cooking loss ($p<0.01$). Interaction between type of oil and cooking temperature was only observed in cooking loss ($p<0.01$).

Moisture content varies from 63.36±0.37% to 67.53±0.26% in fat partially replaced patties, which are consistent with the results reported by Rodríguez-Carpena et al. (2012). They found that pork patties with 50% fat replaced with sunflower oil, olive oil and avocado oil had moisture level at 61.48-63.39% when patties were cooking at 170 ^{o}C for 18 min in a forced-air oven. Type of oil did not affect moisture content in the patties (with or without fat replacement) ($p>0.05$), which indicated that replacing back fat with vegetable oils did not affect moisture level in the final products. However, cooking temperature significantly affected the moisture content in the final products ($p<0.05$). Higher cooking temperature at 220 ^{o}C led to low moisture level in cooked patties, compared with low cooking temperature at 180 ^{o}C ($p<0.01$), which were 63.58% vs 69.15% for control, 63.36% vs 65.91% for olive oil treatment, 63.49% vs 66.34% for sunflower oil treatment and 65.90% vs 67.53% for grape seed oil treatment. Low moisture level in the final products are

directly associated with high cooking loss, as cooking loss is mainly composed of water and water soluble nutrients such as proteins (Sánchez del Pulgar et al., 2012). Pork patties with or without fat replacement had cooking loss ranged from 20.30%- 24.75%, which was consistent with the results reported by Rodr´ıguez-Carpena, Morcuende and Est´evez (2011). They found that cooked pork patties at 170 ^{o}C for 18 min had cooking loss at 20.69%- 22.20%. Fat and protein content ranged from 9.49%-10.01% and 15.03%-15.34% respectively, as expected, fat and protein content in fat modified patties were comparable with those in C patties. pH ranged from 5.85 to 5.99. Cooking temperature and type of oil did not affect pH of patties, there was no interaction between temperature and type of oil as well ($p>0.05$).

Table 3-2: Proximate composition and pH values of 4 types of patties cooked at 180°C and 220°C [a,b]

Treatment	Cooking temperature(°C)	Moisture (%)	Cooking loss(%)	Fat (%)	Protein (%)	pH
Control	180	69.15±0.30[d]	20.51±1.59[a]	10.01±0.85[a]	15.19±1.56[a]	5.99±0.01[a]
	220	63.58±0.60[a]	24.75±1.24[d]	9.98±1.05[a]	15.03±1.69[a]	5.85±0.02[b]
Olive oil	180	65.91±0.16[b]	20.30±0.60[a]	9.62±1.21[a]	15.21±1.98[a]	5.85±0.02[b]
	220	63.36±0.37[a]	23.21±0.84[bcd]	9.49±1.25[a]	15.09±1.37[a]	5.86±0.01[b]
Sunflower oil	180	66.34±0.63[b]	22.54±0.29[bc]	9.68±1.17[a]	15.28±1.59[a]	5.88±0.01[b]
	220	63.49±0.15[a]	24.23±0.76[cd]	9.70±1.05[a]	15.17±1.94[a]	5.87±0.02[b]
Grapeseed oil	180	67.53±0.26[c]	21.88±0.31[ab]	9.76±1.14[a]	15.34±1.32[a]	5.87±0.02[b]
	220	65.90±0.09[b]	24.72±1.09[d]	9.68±1.32[a]	15.17±1.46[a]	5.87±0.01[b]
p-value (Type of oil)		0.21	0.43	0.74	0.54	0.065
p-value (Temperature)		<0.01	<0.01	0.33	0.28	0.16
p-value (Interaction between oil* temperature)		0.18	<0.01	0.49	0.15	0.16

[a] Results with different letters in the same column are significantly different at the level $p<0.05$.
[b] Values represented as the Mean ± SD, n=3.

3.4.1.2 Physical parameters

3.4.1.2.2 Texture

Texture property of cooked pork patties was examined including hardness, cohesiveness and chewiness through a typical texture profile analysis, with the purpose of assessing the comparable eating quality of fat replaced patties. Results were listed in Table 3-3. Cooking temperature affected hardness, cohesiveness and chewiness ($p \leq 0.05$). Fat replacement with vegetable oils did not affect any of the texture attributes ($p > 0.05$). There was no interaction observed between oil replacement and temperature for hardness and chewiness except cohesiveness ($p > 0.05$).

High cooking temperature led to high hardness. Control patties cooked at 220℃ had significantly higher hardness (26.65±3.15N) than that cooked at 180℃ (20.14±2.81N) ($p < 0.05$). Roldán et al. (2013) observed that the elevating cooking temperature resulted in higher hardness, while the increased hardness in pork patties might be associated with high cooking loss. There was no difference observed in hardness for fat modified patties regardless of temperature ($p > 0.05$), which agreed with the results reported by Rodr´ıguez-Carpena et al. (2011). They stated that patties partially (50%) replaced with sunflower oil, olive oil and avocado oil had the same hardness with control patties. However, Hur, Jin and Kim (2008) reported that olive oil replacement in pork patties resulted in lower hardness compared with control patties. The cooking temperature could help to explain the disagreement. In their study, patties were cooked at 100 ºC in water bath, while samples were cooked in convection oven at 180ºC or 220ºC. The hardening effect of high cooking temperature could be neutralized by the softening effect of the replacing vegetable oil. As a result, there was no difference observed in hardness for fat modified patties and control samples ($p > 0.05$).

Temperature affected cohesiveness greatly ($p = 0.001$), because the texture parameters are mainly determined by denaturation of the structural protein system, i.e. actomyosin complex and collagen (Palka & Daun, 1999). Type of oil did not have any effect on the cohesiveness of pork patties ($p > 0.05$). Rodríguez-Carpena et al. (2012) also reported that there was no difference in

cohesiveness between control and patties with 50% fat substitution using avocado, sunflower or olive oil. The interaction between temperature and type of oil on cohesiveness was observed ($p<0.05$). For control, olive and sunflower oil treatment, patties cooked at 220°C had higher cohesiveness than these cooked at 180°C ($p<0.05$), but there was no difference in cohesiveness for grape seed oil samples cooked at different temperatures ($p>0.05$). This interaction between cooking temprature and type of oil could be explained by the emulsion stability of oil/fat emulsion. Youssef and Barbut (2009) and Rodr´ıguez-Carpena et al. (2011) reported that vegetable oil with high PUFAs had small fat globules in meat emulsion, which could offer a stronger fat-protein interaction. Grape seed oil contained high level of PUFAs compared with back fat and olive oil, which led to a stable emulsion in G patties. In addition, polyphenol compounds in grape seed oil emulsion could help maintain the protein functionality through inhibition of protein oxidation during processing (Ganhão, Morcuende & Estévez, 2010). Thus, grape seed oil/meat emulsion was less sensitive to temperature changes in relation to cohesiveness of the final products.

For chewiness, temperature significantly affected it ($p<0.05$). High cooking temperature resulted in high chewiness. Chewiness remained similar for all patties cooked at 180 °C (from 3.94 to 4.11N.s), but increased to 5.92±0.77N.s in C patties, and 5.35±0.41 N.s in O patties cooked at 220 °C. Greater hardness, cohesiveness and chewiness at higher cooking temprature could be due to the more severe denaturation of myosin (40-60 °C) and actin (66-73 °C) (Sánchez del Pulgar et al., 2012). In addtion, chewiness is also associated with the water retention in meat products (Roldán et al., 2014). Patties cooked at 180°C had significantly higher mositure content than thoes cooked at 220°C (as shown in Table 3-2). Therefore, high chewiness would be expected in samples cooked at 220°C due to high moisture loss.

Table 3-3: Texture parameters (hardness, cohesiveness and chewiness) and colour parameters (lightness L*, redness a* and yellowness b*) in 4 types of patties cooked at 180 °C and 220 °C [a,b]

Treatment	Cooking temperature (°C)	Hardness (N)	Cohesiveness	Chewiness (N.s)	L*	a*	b*
Control	180	20.14±2.81[a]	0.34±0.02[ab]	4.06±0.77[ab]	70.71±0.08[bc]	2.30±0.11[c]	16.98±0.80[abc]
	220	26.65±3.15[b]	0.37±0.01[d]	5.92±0.77[c]	65.80±1.82[a]	1.46±0.22[ab]	18.73±1.15[c]
Olive oil	180	18.02±3.58[a]	0.34±0.01[a]	3.94±0.41[a]	69.80±1.76[bc]	2.82±0.08[d]	16.23±0.71[ab]
	220	21.75±0.37[a]	0.38±0.01[d]	5.35±0.41[bc]	65.33±3.69[a]	1.18±0.02[a]	17.44±1.90[bc]
Sunflower oil	180	17.98±2.45[a]	0.35±0.01[abc]	3.96±0.35[a]	70.10±0.57[bc]	2.86±0.13[d]	15.41±0.73[a]
	220	19.40±2.81[a]	0.38±0.02[d]	4.34±0.99[ab]	65.97±2.05[a]	1.43±0.22[ab]	17.59±0.45[bc]
Grape seed oil	180	17.86±1.12[a]	0.37±0.01[cd]	4.11±0.30[ab]	71.41±1.74[c]	3.24±0.37[d]	17.03±0.38[abc]
	220	21.55±2.37[a]	0.36±0.01[bc]	5.22±1.02[abc]	68.15±0.47[ab]	1.66±0.43[b]	18.47±0.15[c]
p-value (Type of oil)		0.10	0.87	0.50	0.062	0.54	0.65
p-value (Temperature)		0.05	0.001	0.001	0.05	0.005	0.05
p-value (Interaction between oil*temperature)		0.41	0.02	0.33	0.083	0.84	0.04

[a] Results with different letters in the same column are significantly different at the level $p<0.05$.
[b] Values represented as the Mean ± SD, n=3.

3.4.1.2.3 Colour

Effects of vegetable oil and cooking temperature on colour characteristics of cooked pork patties including lightness (L*), redness (a*) and yellowness (b*) were summarized in Table 3-3. Temperature significantly affected all three parameters ($p \leq 0.05$), especially a* with $p=0.005$. Different vegetable oils did not have any impact on the colour parameters, while the interaction between oil and temperature was observed in yellowness (b*). Patties cooked at 220°C had lower L* than those cooked at 180 °C ($p<0.01$), which agreed with the results of Sánchez del Pulgar et al. (2012). The decrease in surface lightness could be attributed to the brown pigments formed from caramelization of sugars and Maillard reaction when samples were cooked at temperature over 90 °C (Girard, 1992). In addition, the lightness was also associated with the moisture content in meat products. Qiao et al. (2001) reported that there was positive correlation between lightness and moisture content in broiler breast fillet. Presence of heme pigments, containing 90-95% myoglobin in muscles gives meat red colour (a*). At 180 °C, a higher a* value was found in all pork patties with oil replacement than control patties ($p<0.05$). The antioxidants in these vegetable oils, such as vitamin E could prohibit the oxidation of oxymyoglobin and lead to a high redness in the final products (Hui, 2001; Sánchez del Pulgar et al., 2012). Cooking temperature could significantly affect a* as well. All patties cooked at 220 °C had significantly lower a* than those cooked at 180 °C. The reduction of a* caused by increased temperature could be associated with the denaturation of myoglobin (Nollet, 2012). Liao, Xu and Zhou (2009) found that a* of stir fried pork floss decreased significantly by 30% when cooking temperature increased from 125°C to 150°C.

Yellowness b* ranged from 15.41-18.73 in all cooked patties. Both type of oil and cooking temperature had no effect on b* values, but the interaction between type of oil and cooking temperature was observed. Jamali et al. (2016) also found that b* value in beef patties was not affected by cooking temperature (160 °C and 220 °C). The results of b* in control samples (16.98-18.73) were comparable with Vittadini et al. (2005).

3.4.2 Effects of vegetable oils and cooking temperature on the formation of HCAs

Concentration of HCAs (IQ, MeIQ, MeIQx, 4, 8-DiMeIQx and PhIP) in control patties and fat modified patties cooked at 180°C and 220°C were listed in Table 3-4. Type of oil affected all individual HCAs compound except IQ, cooking temperature significantly affected the total amount of HCAs and all individual HCAs compounds except MeIQx. Interaction between oil and cooking temperature was observed in total HCAs, IQ, MeIQ, 4, 8-DiMeIQx and PhIP, but not MeIQx. At both temperatures, all fat modified patties had significantly lower amount of MeIQ, 4, 8-DiMeIQx and total HCAs than control patties ($p<0.05$). MeIQx, 4, 8- DiMeIQx and PhIP were not detectable in G patties. Tocopherols (average 50mg/100g in sunflower oil, 5-300mg/100g in refined olive oil and 10-15mg/100g in grape seed oil) and polyphenols (common profile in grape seed oil: catechin, epicatechin and epicatechin gallate) in these oils could play roles in reducing the final HCAs in patties (Bail et al., 2008; McCance & Widdowson, 2002; Rombaut et al., 2014). Tocopherols have been found to block dialkyl-pyrazine radicals for further reaction with creatine to form HCAs, or react with precursors of 4, 8-DiMeIQx to inhibit the formation of HCAs (Pearson et al., 1992; Vitaglione & Fogliano, 2004). Polyphenols could also prevent the formation of imidazoquinoxaline-type HCAs through trapping pyrazine cation radicals and some other carbon-centred radicals generated either from pyrazine cation radicals or different pathway during Maillard reaction (Kato et al., 1996). In addition, polyphenols compounds have the ability to directly trap phenylacetaldehyde, which is a major intermediate during the formation of PhIP (Cheng et al., 2007).

Total HCAs ranged from not detected (Nd) to 140.57±22.03 ng/g. Control patties cooked at both cooking temperatures contained significantly higher amount of total HCAs (67.56±17.29 ng/g and 140.57±22.03 ng/g), followed by S patties (5.98±1.10 ng/g and 23.88±2.44 ng/g) and O patties (4.11±0.87ng/g and 20.03±2.25 ng/g), while G patties achieved the lowest total HCAs in both temperatures (Nd and 1.90±0.04 ng/g). Control samples cooked at 220°C contained all types of HCAs, whereas none of HCA compounds were detected in G patties cooked at 180°C. The dominating compounds of HCAs

were MeIQ (59.70±0.98ng/g) and 4, 8-DiMeIQx (43.37±15.67ng/g) in C patties, while PhIP in O (14.78±1.49ng/g) and S patties (22.70±1.95ng/g). The total HCAs in C patties were higher than some published results. Vangnai et al. (2014) reported MeIQx (7.59±0.43ng/g), PhIP (13.12±0.72 ng/g) and total HCAs (22.35±1.17 ng/g) in fried pork loins cooked at 204 ºC for 16 minutes. The total level of HCAs in pan-fried well-done pork was 49.7ng/g with cooking ended at 80 ºC core temperature (Iwasaki et al., 2010). The sampling procedure for measuring HCAs could help explain the difference. HCAs were extracted from the 2mm outer layer surface of samples in this study, while lots of researchers extracted HCAs from entirely ground samples. The precursors of HCAs, such as creatine, glucose and free amino acids would migrate to the surface of meat and enhance Maillard reaction during cooking (Gibis & Weiss, 2015). As a result, surface could accumulate much higher level of HCAs compared with internal part of the sample. Therefore, a higher concentration of HCAs would be expected than that exacted from entirely ground samples.

Table 3-4: HCAs in cooked pork patties with partial replacement of fat by vegetable oils at 180 °C and 220 °C [a,b,c]

Treatment	Cooking temperature (°C)	IQ (ng/g)	MeIQ (ng/g)	MeIQx (ng/g)	4,8-DiMeIQx (ng/g)	PhIP (ng/g)	Total (ng/g)	Inhibitory efficiency
Control	180	Nd	18.26±14.46[a]	8.34±1.78[ab]	25.66±1.51[b]	11.43±6.33[a]	67.56±17.29[c]	N/a
	220	3.88±3.50[a]	59.70±0.98[b]	13.45±7.43[b]	43.37±15.67[c]	24.07±1.99[b]	140.57±22.03[d]	N/a
Olive oil	180	0.58±0.01[b]	Nd	3.50±0.68[a]	Nd	Nd	4.11±0.87[a]	93.90%
	220	1.30±0.42[b]	Nd	2.52±0.36[a]	1.31±0.22[a]	14.78±1.49[a]	20.03±2.25[b]	85.75%
Sunflower oil	180	Nd	Nd	4.32±0.50[a]	1.02±0.50[a]	Nd	5.98±1.10[a]	91.15%
	220	0.64±0.16[b]	Nd	4.31±0.55[a]	5.12±0.35[a]	22.70±1.95[b]	23.88±2.44[b]	83.01%
Grape seed oil	180	Nd	Nd	Nd	Nd	Nd	Nd	100%
	220	0.59±0.04[b]	1.31±0.06[c]	Nd	Nd	Nd	1.90±0.04[a]	98.64%
p-value (Type of oil)		0.12	<0.01	<0.01	<0.01	<0.01	<0.01	
p-value (Temperature)		0.037	<0.01	0.37	0.039	<0.01	<0.01	
p-value (Interaction between oil*temperature)		0.040	<0.01	0.24	0.035	<0.01	<0.01	

[a] Results with different letters in the same column are significantly different at the level $p<0.05$.
[b] Values represented as the Mean ± SD, n=3.
[c] Nd: Not Detected.

IQ was detected up to 3.88 ng/g in cooked patties, which was in the range of 0.7-5.3 ng/g in fried ground beef patties (Balogh et al., 2000). At 180 °C, IQ was not detected apart from O patties, but cooking at 220°C generated high level of IQ in all patties ($p<0.05$). Different vegetable oils did not affect the formation of IQ ($p>0.05$), but interaction between vegetable oil and temperature was observed in formation of IQ. IQ is generally formed through reactions between creatinine, pyridine radicals and formaldehydes (Vitaglione & Fogliano, 2004). Vegetable oils could decompose into hydroperoxides, and then aldehydes and ketones at high cooking temperature, which further react with amino acids in Maillard reaction (Johansson, Skog, & Jagerstad, 1993; Zamora & Hidalgo, 2007). Olive oil with high level of oleic acid has been reported decomposed into aldehydes (-CHO) much faster (3-15 times) than sunflower oil and grape seed oil containing high amount of linoleic and linolenic acid at 190°C (Guillén & Uriarte, 2012b). This might explain IQ was only detected in olive oil patties at 180°C, while the detailed pathway between fatty acids profile and formation of IQ need further investigation. The highest MeIQ was found in control patties cooked at 220 °C (59.70±0.98 ng/g), followed by in control patties cooked at 180 °C (18.26±14.46 ng/g). Janoszka (2010) reported that 6.28 ng/g MeIQ was detected in pan-fried pork patties cooked at 170°C for 12 minutes, which was similar with this study. Formation of MeIQ was completely inhibited by olive oil and sunflower oil at both cooking temperatures. Grape seed oil could only inhibit formation of MeIQ at low cooking temperature, while 1.31ng/g was detected in G patties cooked at 220°C. The inhibitory effect on MeIQ could be attributed to the antioxidants such as vitamin E and polyphenols in vegetable oils. Rounds et al. (2012) and Liao et al. (2009) also reported that vitamin E and polyphenols could reduce the formation of MeIQ. Balogh et al. (2000) found that vitamin E had stronger inhibitory effect on MeIQ with reduction rate 64.3% than phenolic compound in Oleoresin rosemary extract with reduction rate 47.9% in fried beef patties. Since olive oil and sunflower oil contained higher level of vitamin E than grape seed oil, therefore, stronger inhibition of MeIQ would be expected in O patties and S patties. Cooking temperature did not affect MeIQx level in patties ($p>0.05$), but formation of MeIQx was significantly affected by different

vegetable oils. S patties and O patties had similar MeIQx with control samples. For G patties cooked at both temperatures, there was no MeIQx detected. However, sunflower oil and olive oil did not affect MeIQx in patties, although both oils were rich of vitamin E. No MeIQx was detected at all which was in agreement with Rounds et al. (2012), who also reported grape seed extract could completely inhibit the formation of MeIQx in cooked beef patties. Temperature significantly increased formation of 4, 8-DiMeIQx as evidenced in C patties cooked at 180 ^{o}C and 220 ^{o}C (25.66±1.51 ng/g and 43.37±15.67 ng/g) ($p<0.05$). All vegetable oils effectively reduced 4, 8-DiMeIQx in patties. Grape seed oil was the most effective one among the three vegetable oils as 4, 8-DiMeIQx was not detected in G patties cooked at both temperatures.

PhIP ranged from Nd to 24.07±1.99 ng/g. A similar level of PhIP (18.4±11.5 ng/g) in fried pork patties was reported by Zhang et al. (2013), when patties were cooked at 180^{o}C for 5 min. At 180 ^{o}C, PhIP (11.43±6.33 ng/g) was totally inhibited by all 3 vegetable oils, but only grape seed oil could completely inhibit the formation of PhIP at both cooking temperatures. The stronger inhibitory effect on PhIP in G patties could be attributed to the polyphenol compounds in grape seed oil. Gibis and Weiss (2012), Jamali et al. (2016) and Oguri et al. (1998) found that catechin, epicatechin and epicatechin-3-O-gallate in grape seed extract may be responsible for 50%-90% reduction of PhIP in both oven cooked beef patties and chemical model system. Zamora and Hidalg (2015) suggested phenolic compounds could effectively scavenge the carbonyl compounds in the Strecker degradation of phenylalanine to produce phenylacetaldehyde (major intermediate in the development of PhIP). Temperature significantly affected PhIP level in pork patties ($p<0.05$). PhIP increased significantly in C patties from 11.43±6.33 ng/g to 24.07±1.99 ng/g, O patties from Nd to 14.78±1.49 ng/g and S patties from Nd to 22.70±1.95 ng/g when cooking temperature increased from 180 ^{o}C to 220 ^{o}C ($p<0.05$). The results agreed with Gibis and Weiss (2012) and Wong et al. (2012) that PhIP level was directly related to the cooking temperature. At 175 ^{o}C -200 ^{o}C, only very low level of PhIP (0-6.91 ng/g) could be formed even at varied cooking time, but it could increase

dramatically to 31.80ng/g with prolonged cooking time if temperature went above 200 ºC.

Type of vegetable oil significantly affected the level of total HCAs in cooked patties ($p<0.05$). Interaction between type of vegetable oil and cooking temperature was observed as well ($p<0.05$). Effect of fatty acids/oils on the formation of HCAs has been documented inconsistently. Johansson et al. (1995) reported that the most MeIQx and DiMeIQx found in burgers fried in rapeseed oil containing high level of oleic acid with high peroxides values, compared with butter, margarine and sunflower oil. However, Zamora et al. (2012) stated that both primary and secondary lipid oxidation products, hydroperoxides, such as methyl 13-hydroperoxyoctadeca-9,11-dienoate and alkenals could enhance PhIP, and low to medium oxidation degree of oil could accelerate the formation of PhIP comparing to highly oxidized oil in chemical model system. This inconsistency might be resulted from non-pyridine/pyrazine participated pathway of PhIP and more complexity of real meat system with the consideration of antioxidants in oils (Johansson, Skog, & Jagerstad, 1993). In the current study, reduction of total HCAs by 85.75%-93.90% was found in O patties, 83.01%-91.15% in S patties, while G patties achieved the highest reduction rate at 98.64%-100% (Table 3-4). Antioxidants in the vegetable oils could be responsible for the reduction of total HCAs as a strong negative correlation ($r=-0.618$, $p<0.01$) was disclosed between total HCAs level and antioxidant capacity (TEAC) of oils (Table 3-6). Grape seed oil had the highest TEAC value with 0.71±0.01 μmol Trolox/100g, followed by olive oil (0.52±0.05 μmol Trolox/100g) and sunflower oil (0.18±0.04 μmol Trolox/100g), while pork back fat had the lowest TEAC value (0.09±0.02 μmol Trolox/100g) (Figure 3-1). Therefore, reduction of HCAs in vegetable oils, especially grape seed oil was expected compared with control samples. Results were comparable with findings of Matthäus (2008) and Castelo-Branco and Torres (2012). Balogh et al. (2000) found that 1% vitamin E spray on the surface of beef patties could reduce the concentrations of IQ, MeIQ, MeIQx, DiMeIQx and PhIP significantly by 45% to 75%, because vitamin E could remove free radicals in Maillard reaction. Similar result was also reported by Lan, Kao and Che (2004). They found that 70% of total HCAs (IQ,

MeIQ, MeIQx, 4, 8-DiMeIQx and PhIP) were prohibited when 0.2% α-tocopherol was added into ground pork 1h before cooking. Polyphenol compounds, such as catechin, epicatechin-3-O-gallate, oligomer procyanidins and tocopherols in grape seed oil contributed to its antioxidant capacity (Agostini et al., 2012; Crews et al., 2006; Matthäus, 2008). Vitaglione and Fogliano (2004) suggested that mixture of antioxidant compounds could perform better than single antioxidant as they could inhibit various pathways in different steps of reactions. Therefore, polyphenols compounds might work synergistically with tocopherols to enhance each other to inhibit the formation of HCAs. However, the synergistic effect between different antioxidants needs to be further examined.

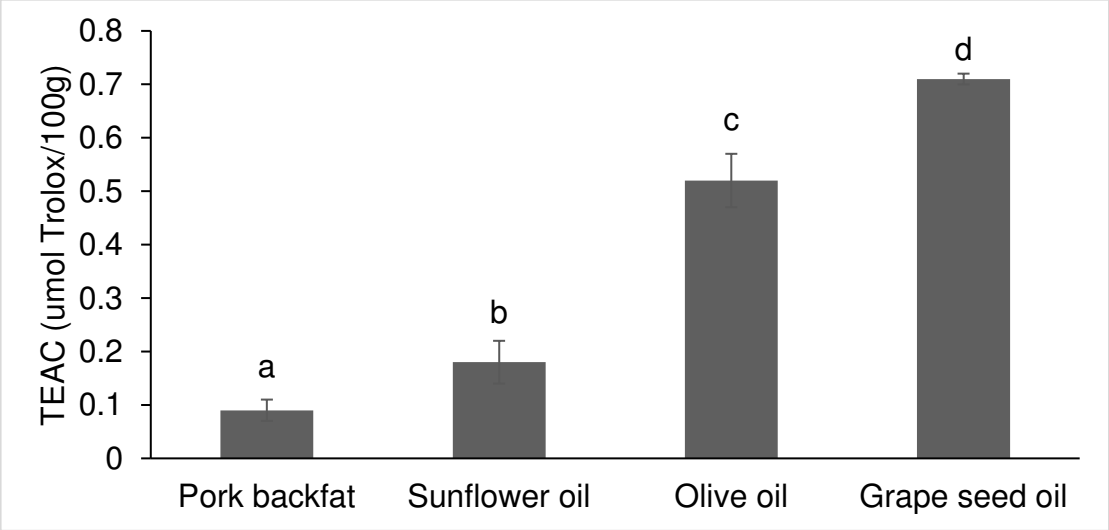

Figure 3-1: Trolox Equivalent Antioxidant capacity (umol Trolox/100g) of pork backfat and 3 vegetable oils[1].
[1]Results with different letters are significantly different at the level $p<0.05$.

Cooking temperature significantly affected total HCAs in pork patties ($p<0.01$) (Table 3-4). Patties cooked at 220 °C had significantly higher level of total HCAs than these at 180 °C ($p<0.01$). Effect of temperature on the formation of HCAs was well examined in previous research (Knize et al., 1994; Liao et al., 2009; Oz & Kaya, 2011). Thermal processing has vital influence on the formation of polar HCAs (IQ, MeIQ, MeIQx, DiMeIQx and PhIP), which are formed in meat products when samples are cooked at 160-250 °C, typical domestically cooking temperature. High cooking temperature generated more diverse types of HCAs, but also stimulate the accumulation of the amount of

HCAs on the surface of meat products (Olsson & Pickova, 2005; Skog, Johansson & Jaègerstad, 1998).

3.4.3 Effects of vegetable oils and cooking temperature on the formation of PAHs

Concentration of PAHs (BaA and BaP) in cooked pork patties with different cooking temperature was listed in Table 3-5. The range of total PAHs was from 1.59±0.26 ng/g to 3.84±0.21 ng/g. BaA ranged from 0.14-0.31 ng/g in cooked patties, while BaP ranged from 1.44 to 3.53ng/g. Temperature did not affect the formation of both BaA and BaP, but type of vegetable oil had significant effect on the formation ($p<0.05$). Interaction between type of oil and cooking temperature was also observed in both compounds (Table 3-5). BaP level in this study (1.44-3.53 ng/g) are consistent with results reported by Nisha et al. (2015) and Janoszka (2011), i.e. 1.52 ng/g of BaP in the oven broiled pork and 1.61 ng/g BaP in oven grilled pork chop (17min at 170°C). Olive oil and grape seed oil showed inhibitory effect on BaP when patties cooked at 220 °C, but no effect or even promoting effect was observed at 180°C. On the contrast, sunflower oil offered inhibition on BaP at 180 °C, but promotion at 220 °C. As BaP is one of the highest toxic potency compounds during meat cooking, EU Commission has regulated that the updated limit of BaP occurring in processed meat and seafood products is 2 ng/g (Purcaro et al., 2013; Wretling et al., 2010). Among all the patties, only O patties cooked at 220 °C and S patties cooked at 180 °C met the safety regulation of BaP. Therefore, it is necessary to develop any procedures or alternative methods that reduce the amount of BaP to safety limit.

Table 3-5: PAHs in in cooked pork patties with partial replacement of fat by vegetable oils at 180 °C and 220 °C [a,b]

Treatment	Cooking temperature (°C)	BaA (ng/g)	BaP(ng/g)	Total PAHs (ng/g)	Inhibitory efficiency
Control	180	0.15±0.01[a]	2.44±0.37[c]	2.58±0.36[c]	N/a
	220	0.21±0.03[b]	3.08±0.06[d]	3.28±0.07[d]	N/a
Olive oil	180	0.15±0.02[a]	2.24±0.40[bc]	2.38±0.40[bc]	7.75%
	220	0.15±0.01[a]	1.44±0.27[a]	1.59±0.26[a]	51.52%
Sunflower oil	180	0.14±0.01[a]	1.88±0.17[ab]	2.02±0.16[ab]	21.71%
	220	0.31±0.02[c]	3.53±0.20[e]	3.84±0.21[e]	-17.07%
Grape seed oil	180	0.18±0.01[ab]	3.29±0.15[d]	3.46±0.16[d]	-34.11%
	220	0.18±0.05[ab]	2.51±0.07[c]	2.71±0.07[c]	17.38%
p-value (Type of oil)		<0.01	<0.01	0.031	
p-value (Temperature)		0.1	0.076	0.43	
p-value (Interaction between oil* temperature)		<0.01	<0.01	<0.01	

[a] Results with different letters in the same column are significantly different at the level p<0.05.
[b] Values represented as the Mean ± SD, n=3.

The effect of oil and interaction between type of oil and cooking temperature on the formation of PAHs were significant ($p<0.05$). Cooking temperature did not affect the formation of PAHs ($p>0.05$). S patties cooked at 220 °C had the highest total PAHs (3.84±0.21 ng/g), followed by G patties (3.46±0.16 ng/g) cooked at 180 °C and C patties (3.28±0.07 ng/g) cooked at 220 °C. O patties cooked at 220°C obtained the lowest PAHs. PAHs were mainly associated with the pyrolysis of fat undertaken at high temperature (Viegas et al., 2012). Therefore, smoking point of vegetable oils may help explain the difference in PAHs. Sunflower oil and grape seed oil contain high content of PUFAs, especially linoleic acid and linolenic acid have lower smoke points (grape seed oil 216 °C, sunflower oil 227 °C), which make them easy to decompose, compared with olive oil (smoke point 242 °C). The decomposition of oil could generate more reactive free radicals to accelerate the production of PAHs (Chen & Lin, 1997; Elmore et al., 2002). They also concluded that hydroperoxides from lipid oxidation, could subsequently generate cyclic compounds through intramolecular reaction, and PUFAs could undergo further polymerization. In addition, vegetable oils themselves contained BaP, which might increase the total amount of PAHs in cooked meat. Fromberg, Højgard and Duedahl-olesen (2007) reported that olive oil, sunflower oil and grape seed oil approximately contained 0.12 ng/g, 0.4 ng/g and 1.0 ng/g BaP respectively. As a result, high level of PAHs was expected in sunflower oil and grape seed oil samples. Although vegetable oils contain antioxidants, the inhibitory effect on PAHs formation was not observed consistently. In O patties, the inhibitory efficiency at both temperatures were 7.75% and 51.52%, but sunflower oil and grape seed oil increased the formation of PAHs by 17.07% and 34.11%, respectively. It shows that antioxidants in vegetable oils were not involved in the formation of PAHs to a great extent, which is further confirmed by the correlation analysis. As indicated in Table 3-6, there is no correlation relationship observed between antioxidant capacity of oil (TEAC) and total PAHs. However, Wongmaneepratip and Vangnai (2017) reported that radical scavenging activity of commercial palm oil and sunflower oil could be correlated to the inhibitory effect on PAHs formation in grilled chicken. The discrepancy could be attributed to the difference of preparation process.

Longer marinating time of commercial oils with meat might provide enough time for antioxidants in oils to perform scavenging ability (Wongmaneepratip and Vangnai, 2017). The impact of tocopherols and polyphenol compounds on the formation of PAHs in processed meat was not well documented. *In vitro* study, Zhu et al. (2014) found that vitamin E intake could significantly prohibit free radicals induced by BaP and protect cellular damage in human lung, but the effect of antioxidants on formation of PAHs in food products has been scarce.

Table 3-6: Pearson correlation coefficient (r) between the level of total HCAs/PAHs (ng/g) and TBARS, protein carbonyl and TEAC

	TEAC	HCAs	PAHs
TBARS	-0.764**	0.826**	-0.154
Protein carbonyl	-0.606**	0.778**	0.019
TEAC	-	-0.618**	0.301

** Significant level 0.01

3.4.4 Correlation between lipid oxidation, protein oxidation and the formation of HCAs and PAHs

It is noted that the results from current extraction method were slightly different with those from distillation method described by Tarladgis et al. (1964), which could be attributed to the interference of haemoglobin. Jurdi-Haldeman et al. (1987) reported that TBARS values of cooked ground lamb was higher in distillation method with higher sensitivity, compared with in extraction method. However, extraction method was used in the consideration of popularity in recent publications and practical availability. In Figure 3-2, S patties, O patties and G patties had significantly lower TBARS values than control samples ($p<0.05$), while G patties had the lowest TBARS value compared with O and S patties ($p=0.001$). This inhibitory effect against lipid oxidation could be attributed to the antioxidants (tocopherols and polyphenol compounds) within the oils, since a significantly negative relationship was found between TBARS values and antioxidant capacity of oils ($r= -0.764$,

p<0.01) (Table 3-6). Wong et al. (2015) reported that 0.1-0.4 mmol vitamin E could inhibit 45% of lipid oxidation in beef patties, by obstructing the formation of some key aldehydes and ketones during lipid oxidation. Similar results were achieved by Ahn, Grün and Fernando (2002) as well. Frankel (1998) proposed that α-tocopherol could prevent the chain propagating and remove free radicals through reacting with either singlet oxygen or peroxyl radicals. Consequently lipid oxidation was reduced. Meanwhile, polyphenols, such as epicatechin (EC) and oligomer procyanidins were also sufficient to inhibit lipid oxidation by reducing free radicals and preventing chain propagation in cooked pork and beef (Rojas & brewer, 2007). They could also chelate metals (iron and cooper in meat) or react with ROS, and then turn into non-radical species. As a result, reactions were terminated (Roman et al., 2013). Moreover, Ahn et al. (2002) and Tang et al. (2001) reported that polyphenols such as catechin, epicatechin were more efficient in inhibiting lipid oxidation than α-tocopherol at the same concentration in cooked meat. This could explain why G patties had the lowest TBARS values than S and O patties. Although Gunstone (2002) stated that a higher degree of unsaturation of fatty acids could be easier to trigger the lipid oxidation and interacted with Maillard reaction, the presence of antioxidants should be also considered.

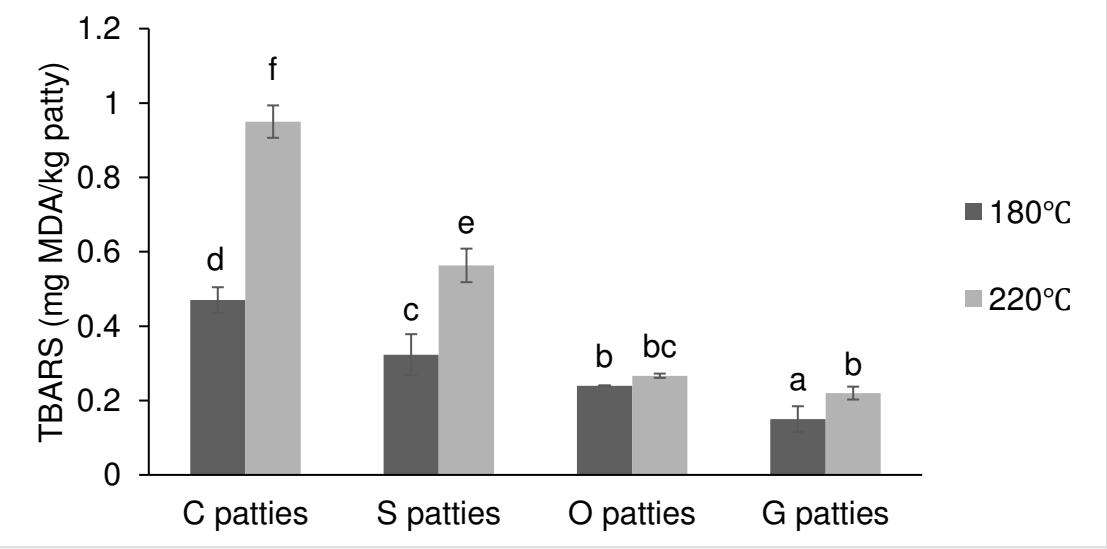

Figure 3-2: TBARS values in pork patties cooked at 180 ºC and 220 ºC[1]

[1] Results with different letters are significantly different at the level p<0.05.

Protein carbonyls are produced from protein oxidative degradation in meat products, which were used to analyse degree of protein oxidation (Figure 3-3). Significant effect of vegetable oils on the protein oxidation was observed ($p=0.001$). C patties had a significantly higher level of protein carbonyls (12.11 nmol/mg) than other 3 fat modified patties ($p<0.05$). The protein oxidation could be inhibited by the presence of antioxidants in oil, as negative correlation between antioxidant capacity of oils (TEAC) and protein carbonyl level was found with $r=-0.606$, $p<0.01$, as indicated in Table 3-6. Botsoglou et al. (2014) found that protein carbonyl value could be reduced significantly in cooked pork patties when 50mg/kg α-tocopherol was added ($p<0.05$). Vuorela et al. (2005) reported that phenolic compounds, including vinylsyringol and sinapic acid in rapeseed oil had good antioxidant capacity against protein oxidation in cooked pork patties. Ganhão et al. (2010) also found that arbutus-berries extract containing catechins significantly reduced protein oxidation by chelating heme iron in cooked patties. However, there was no difference in the protein carbonyl level among O, S and G patties ($p>0.05$).

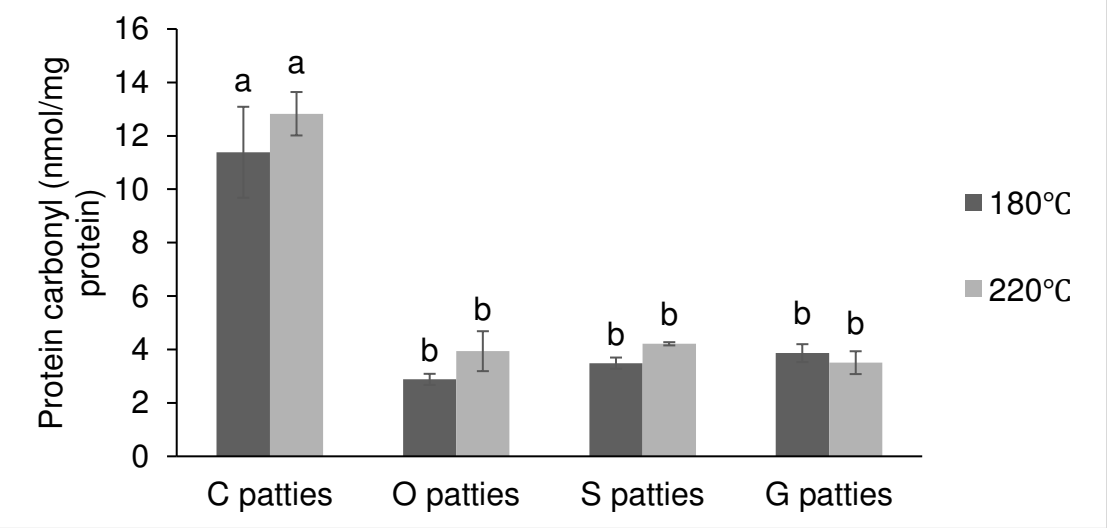

Figure 3-3: Protein carbonyl values in pork patties cooked at 180 °C and 220°C[1]

[1]Results with different letters are significantly different at the level $p<0.05$

In the development of HCAs under high temperature cooking, both lipid oxidation and protein oxidation are involved with Maillard reaction in the meat system (Johansson, Skog & Jagerstad, 1993; Zamora & Hidalgo, 2007). Lot of researchers believed that formation of HCAs could be primarily related to

interactions between free radicals generated from lipid oxidation and free radicals produced in Maillard reaction (Hwang & Ngadi, 2002; Skog et al., 1998). Therefore, it is useful to explore the relationship between lipid/protein oxidation and the formation of HCAs. In this study, correlation analysis was conducted between TBARS/protein carbonyl values and concentration of total HCAs in fat modified cooked patties (Table 3-6). Significant positive correlation was disclosed between total HCAs and TBARS ($r=0.826$, $p<0.01$) and between HCAs and protein carbonyl ($r=0.778$, $p<0.01$), which further confirmed that both lipid oxidation and protein oxidation participated the formation of HCAs during cooking process. In order to further examine the relationship between lipid oxidation/protein oxidation/antioxidant capacity of lipids and total HCAs in cooked patties, multivariate linear regression model was displayed below,

Total HCAs= -42.37+ 108.26 * TBARS + 5.647 * Protein Carbonyl.

It can be seen from the equation that TBARS (lipid oxidation) played a predominant role on the formation of HCAs, compared with protein carbonyl (protein oxidation). The factor 'TEAC (antioxidant capacity of lipids)' has been removed from the model, because the strong correlation between TEAC and TBARS/ protein carbonyl indicates that variance accounted for TEAC could be well accounted by TBARS/ protein carbonyl. In cross-validation, adding/ removing 'TEAC' caused little change in adjusted R square of the predicted models, which indicated variance caused by TEAC could be well explained by other independent factors in the model (Field, 2009).

Free radicals, aldehydes and ketones generated from lipids oxidation could interact with Maillard reaction by reacting with the polar head of an amino group to produce more HCAs (Jägerstad et al., 1998; Zamora & Hidalgo, 2007). On the other hand, active protein carbonyl residues, such as alkyl, peroxyl radicals that formed by muscle protein oxidation can be initialized by lipid oxidation, metal ions and other peroxided compounds (Cai et al., 2002). Subsequently, these carbonyls could interact with Maillard reaction via Schiff base and then generate Strecker aldehydes, which are intermediates of imidazoquinolines and imidazoquinoxalines (Estévez, 2011; Soladoye et al.,

2015). Researchers also suggested that lipid oxidation could trigger protein oxidation by reacting with heme iron that released from myoglobin (Ganhão et al., 2010; Vuorela et al., 2005). In this work, there was no correlation observed between TBARS /protein carbonyl and PAHs ($p>0.05$), which indicated that the involvement of lipid and protein oxidation in the formation of PAHs was only at null level. Thus, no linear regression model was fitted. The antioxidants in vegetable oils could not inhibit the formation of PAHs, which was evidenced by null correlation between TEAC and PAHs ($p>0.05$).

3.5 Conclusion

Control patties contained the highest amount of HCAs and relatively higher PAHs at 180 °C and 220 °C. All 3 fat modified patties had significantly lower HCAs, which could be attributed to antioxidants, such as tocopherols and polyphenol compounds existing in the oils. The negative correlation ($r= -0.618$, $p<0.01$) between the antioxidant capacity of lipids and the total amount of HCAs could be useful evidence to support this claim. Both lipid and protein oxidation contributed to the formation of HCAs, which were supported by the positive relationship between TBARS/ protein carbonyl values and total HCAs with $r= 0.826$ and 0.788 ($p<0.01$), respectively. Olive oil and sunflower oil completely prohibited MeIQ, whereas grape seed oil could inhibit MeIQx, 4, 8-DiMeIQx and PhIP. Grape seed oil could achieve the highest inhibitory effect on the formation of HCAs. However, effect of vegetable oils on the formation of PAHs was not consistent, which could be attributed to complexity of oil decomposition and antioxidants in the oils. The involvement of lipid oxidation and protein oxidation in formation of PAHs was limited or at a minimum level. Antioxidants in oils could not reduce the total amount of PAHs effectively. Therefore, it is necessary to explore other methods to reduce PAHs in processed meat. Overall, replacing pork back fat with vegetable oils in processed meat products could offer healthier meat products with reduced HCAs without compromising eating quality.

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Chapter 4. The effect of common spices and meat type on the formation of heterocyclic amines and polycyclic aromatic hydrocarbons in deep-fried meatballs

This chapter is ready for submiting to the journal 'Food Control'.

4.1 Abstract

Spices are commonly used as flavour enhancer and natural antioxidants in processed meat products. However, effect of spices on the formation of carcinogens especially heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) in different meat system has yet been investigated. In this study, 0.5% garlic, onion, red chilli, paprika, ginger and black pepper powder was added into beef and chicken meatballs fried at 180oC. Formation of HCAs and PAHs was examined to evaluate the inhibitory efficiency of spices in beef and chicken meatballs. Control meatballs (without adding spice) contained the highest amount of HCAs compared with all spice added meatballs of both beef and chicken. All the spices powder reduced the formation of total HCAs, while ginger powder achieved the highest inhibition efficiency compared with all other spices. The correlation coefficient (r) between antioxidant capacity of spices and total HCAs was - 0.853 ($p<0.01$) for TEAC and -0.712 ($p<0.05$) for ORAC. Chicken meatballs contained less HCAs than beef, but no difference was observed in total PAHs between beef and chicken meatballs ($p>0.05$). Both electron transfer and hydrogen donation were involved with the inhibitory effect of spices for developing HCAs, but only electron transfer mainly in the formation of PAHs. In conclusion, antioxidant capacity of spices determined their efficiency in prohibiting formation of HCAs and PAHs, and meat type affected the formation of HCAs, but not PAHs.

Key words: Antioxidant capacity; Free radicals; Phenolics; Thermal stability.

4.2 Introduction

In processed meat products, the presence and hazard of HCAs and PAHs become a major concern for both consumers and researchers. HCAs

represent a class of carcinogenic compounds that were identified in meat products cooked at high temperature (Rahman, Sahar, Khan, & Nadeem, 2014). Five of them, including 2-amino-3-methylimidazo [4,5-f]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4,8-DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) are reasonably regarded as human carcinogens (IARC, 1993). Meanwhile, PAHs are hydrocarbons that contain two or more benzene rings, which could be generated through incomplete combustion or pyrolysis of carbon and hydrogen. They can be accumulated in barbequed, grilled, fried and smoked food (PHE, 2008). PAH4, including benz[a]anthracene (BaA), benzo[a]pyrene (BaP), benzo[b]fluoranthene (BbF) and chrysene has recently been reported as indicator of carcinogenic potency of PAHs in food (Janoszka, 2011). In PAH4, both BaA and BaP are considered as probable carcinogens in humans (Group 2A) comparing with other PAHs (less carcinogenic) according to the updated IARC (2010) report.

In order to reduce HCAs and PAHs in the cooked meat products, research work about understanding their formation pathways has been extensively carried out. Readily oxidized and decomposed species, such as free radicals, aldehydes and ketones could interact with Maillard reaction during meat cooking to accelerate the formation of HCAs (Guillén & Uriarte, 2012; Katragadda et al., 2010). PAHs are formed mainly due to incomplete combustion or pyrolysis of organic components, including fat, protein and carbohydrates at the temperature over 200 ^{o}C (Alomirah et al., 2011). The whole process of forming HCAs and PAHs contained a series of radical reactions (D'Anna & Violi, 1998; Wang, Raj, & Chung, 2013). Antioxidants have been proved to interfere with these radical reactions to affect the formation of HCAs and PAHs. Vitaglione and Fogliano (2004) and Janoszka (2011) stated that antioxidants could trap free radicals, such as intermediates of HCAs and PAHs, to prevent the formation of HCAs and PAHs. Thus, incorporating antioxidants in meat products has been considered as an effective way to reduce the level of HCAs and PAHs in cooked meat products. Synthetic antioxidants, including butylated hydroxyanisole (BHA), butylated

hydroxytoluene (BHT), propyl gallate (PG) have been proved effective reduction on the formation of MeIQx and 4, 8-DiMeIQx in chemical model systems (Vitaglione & Fogliano, 2004). However, these synesthetic antioxidants have been banned in Europe and some other countries due to their carcinogenic potential to human (Oz & Kaya, 2011b). In addition, using natural ingredients in food processing have been highly demanded by consumers.

Natural source of antioxidants such as herbs and spices has been explored as ingredients in processed meats. Garlic and onion are both from Allium family, and they contain a high level of antioxidants, mainly organosulfides including diallyl sulfide (DAS) and diallyl disulfide (DAD). In addition, quercetin in onion has well documented for its antioxidant capacity and antibacterial properties (Mellado-García et al., 2015). Paprika and red chilli are members of the Capsicum family and have been reported to contain carotenoids and capsaicin both of which have high scavenging radical activity (Materska & Perucka, 2005). Quercetin and piperine in black pepper and gingerol in ginger were well known compounds with antioxidant capacity (Shobana & Naidu, 2000). The inhibitory effect of some spices on the formation of HCAs and PAHs in meat products has been documented. Janoszka (2010, 2011) reported that adding garlic and onion in pork could reduce 26%-36% of total HCAs and 50-60% of total PAH during frying. Black pepper could reduce 12%-100% of total HCAs in beef meatballs fried at 175°C, 200 °C and 225 °C (Oz & Kaya, 2011a). They also found sprinkling 1% (w/w) flaked red pepper on pork steak could reduce 75-100% of total HCAs during pan-frying at 175 °C, 200 °C and 225 °C (Oz & Kaya, 2011b). Researchers proposed that antioxidants could scavenge free radicals such as alkylpyridine and dialkylpyrazine for HCAs formation (Jägerstad et al., 1998), propargyl (C_3H_3·) for PAHs formation (El-Badry, 2010; Janoszka, 2011). For example, rosemary extract could reduce MeIQx by up to 92% and PhIP up to 85% in beef patties. Carnosol, carnosic acid and rosmanol within the extract could be attributed to the inhibitory effect as these compounds have reactive ortho-diphenol groups which could scavenge cation radicals (Puangsombat, Jirapakkul, & Smith, 2011). Meat marinades containing beer (Viegas et al., 2014) and spices

including garlic and onion (El-Badry, 2010) were reported with reduction of PAHs level in final products as antioxidants including lipophilic polyphenols, vitamin E and vitamin C presented, these marinades would scavenge free radicals both in fragmentation of hydrocarbons and cyclization of aromatic compounds during the formation of PAHs (Pulido, Hernandez-Garcia, & Saura-Calixto, 2003; Viegas et al., 2014). The scavenging capacity between different antioxidants may depend on antioxidant's molecular structure and particularly the potent reactivity of phenol or polyphenol moieties with free radicals (Meurillon & Engel, 2016).

High intake of meat with presence of metals has been associated with increasing cancer risk. Tasevska (2009) found that high intake of heme iron in meat products might trigger lung cancer, and it could induce endogenous carcinogens N-Nitroso compounds formation. In addition, iron can act as a pro oxidant that promotes lipid oxidation and the formation of heat-induced carcinogens at high temperature. Skog (2000) reported that adding iron into chemical model system could increase the amount of MeIQx. Free radicals generated from lipid oxidation induced by iron could interact with the formation of HCAs (Jägerstad, 1998). Rice-Evans, Miller, and Paganga (1997) discovered that polyphenols could particularly chelate iron and prevent the formation of transition metal-catalysed free radicals. As iron content varies between red meat and white meat, and it could significantly affect the formation of carcinogens during meat processing (Jinap et al., 2016). Spices containing different level and type of antioxidants may perform differently in different meat system. So far, most studies have been focused on the dosage effect or temperature effect on the formation of HCAs and PAHs, while limited attention was given to understand the efficiency of different spices or performance in different meat system. Therefore, 6 commonly used spices with equal dosage level were selected to investigate their performance on the formation of HCAs and PAHs in two meat systems, beef and chicken in consideration of the heme iron level.

4.3 Materials and methods

4.3.1 Materials

Lean beef steak, skinless chicken breast, tallow and chicken skin were purchased from Jennings Caversham (Reading, UK). Excess visible fat on beef and chicken was trimmed before cooking. Raw materials were stored at -18 ^{o}C and defrosted 24h at 4^{o}C before making meatballs. Commercial spices, ground black pepper, garlic granules, ginger powder, onion powder, paprika powder and red chilli powder from Schwartz®, were purchased from ASDA supermarket (Reading, UK). Sunflower oil (ASDA, UK) for deep frying and spices were kept in refrigerator (4^{o}C) before use.

4.3.2 Procedures of making meatballs and cooking process

The formulation of beef and chicken meatballs per kilogram was listed in Table 4-1 and Table 4-2, respectively. Fat level of beef meatballs were adjusted to 16% and chicken to 10%. Spices including garlic, onion, paprika, ginger, red chilli and black pepper were added to the meat batter according to the popularity of useage both in commercial recipes and previous research. Concentration of the spices (0.5%, w/w) was chosen based on the sensory result from Dwivedi et al. (2006), they reported that although adding spices powder could increase spice flavour and aroma in products, moderate spice flavour intensity (0.5%, w/w) in cooked beef products would be acceptable for public.

All ingredients were homogenized at 5000rpm for 5min in a Kenwood Food processor (Chef Titanium KM010, 4.6, Kenwood Limited) to reach a uniform batter. Each meatball was weighed 15±0.1g, and shaped to ball with 9.0±0.2 diameters by hand. Meatballs were deep fried in a rotary deep fryer (Delonghi, Type No: F283118) with the setting temperatures 180^{o}C for 3min until core temperature 71.8-73.1^{o}C reached, measured by a thermal probe (KM330 Industrial Thermometer, Comark Instruments, UK). After cooking, meatballs were placed on paper towel for 10min to remove excess oil on surface then final weight was recorded to calculate the cooking loss. Cooking loss was determined by the equation: Cooking loss (%) = $(W_r - W_c)/W_r \times 100$, where W_r was the weight of raw meatballs, and Wc was weight of cooked meatballs.

Cooked meatballs were chilled in cold room at 4 °C overnight, followed by stored at -18 °C for further analysis. Meatballs used for lipid oxidation and protein oxidation were vacuum packed and stored at -18 °C.

Table 4-1: Formulation of raw beef meatballs

	Minced lean beef (g)	Tallow (g)	Salt (g)	Powder (g)	Water (g)	Bread crumb (g)
Control	640	160	10	0	90	100
Garlic	640	160	10	5	85	100
Onion	640	160	10	5	85	100
Red chilli	640	160	10	5	85	100
Paprika	640	160	10	5	85	100
Ginger	640	160	10	5	85	100
Black pepper	640	160	10	5	85	100

Table 4-2: Formulation of raw chicken meatballs

	Minced lean chicken (g)	Chicken skin (g)	Salt (g)	Powder (g)	Water (g)	Bread crumb (g)
Control	700	100	10	0	90	100
Garlic	700	100	10	5	85	100
Onion	700	100	10	5	85	100
Red chilli	700	100	10	5	85	100
Paprika	700	100	10	5	85	100
Ginger	700	100	10	5	85	100
Black pepper	700	100	10	5	85	100

4.3.3 Proximate composition and physical analysis

4.3.3.1 pH, moisture, fat and protein content

The procedure of analysis was the same with Chapter 2.3.3.

4.3.3.2 Colour

The procedure of analysis was the same with Chapter 3.3.9.1.

4.3.4 Determination of HCAs

Approximate 2mm samples surface were trimmed and blended well before measuring. The procedure of analysis was the same with Chapter 2.4.3.

4.3.5 Determination of PAHs

The procedure of analysis was the same with Chapter 2.3.5.

4.3.6 LOD, LOQ and recovery rate of HCAs and PAHs

LOD and LOQ of 5 HCAs were estimated based on the peak-to-peak noise magnitude near analyte peaks with a known concentration and signal-to-noise ratios (R=S/N) of 2 and 10, respectively. The average recoveries of these 5 HCAs according to triplicates were 58.35% for IQ, 61.10% for MeIQ, 53.97% for MeIQx, 57.24% for 4,8-DiMeIQx and 55.99% for PhIP. Results were comparable with published data (Gibis, 2007; Oz, 2011). Similarly, recovery rate of BaA and BaP were 54.03% and 50.87% respectively, which was comparable with published results of 50% - 115% (Farhadian et al., 2010; Janoszka, 2011).

4.3.7 Inhibitory rate of HCAs and PAHs

Inhibitory rate was determined according to the equation:

$$\text{Inhibitory rate (\%)} = (A_c - A_t)/A_c \times 100$$

where A_c was the total amount of HCAs or PAHs in control samples (ng/g), and A_t was the total amount of HCAs or PAHs in spice added meatballs (ng/g).

4.3.8 Lipid oxidation and protein oxidation

4.3.8.1 TBARS value (Lipid oxidation)

TBARS analysis was completed by measuring from the meatballs that vacuum frozen (-18^{o}C) up to 30 days. The procedure of analysis was the same with Chapter 3.3.4.1.

4.3.8.2 Protein carbonyl value (Protein oxidation)

Protein carbonyl analysis was completed by measuring from the meatballs that vacuum frozen (-18^{o}C) up to 30 days. The procedure of analysis was the same with Chapter 3.3.4.2.

4.3.9 Total phenolic content (TPC)

Total phenolic content was determined using Folin–Ciocalteu agent according to the procedures published by Wojdyło, Oszmiański, and Czemerys (2007). 1g spice powder was added into a tube with 10ml mixture of methanol and water (8:2, v/v). The test tube was agitated firstly for 15min in a shaker (Multi Reax D-91126, Heidolph, Germany) and then covered with foil and left for 24 hours at room temperature for adequate extraction of polyphenols from spices. The extract was centrifuged for 10 min (1500rm) at room temperature and supernatant was collected for further analysis. 100 µl supernatant was mixed with 0.2 ml of Folin–Ciocalteu reagent and 2 ml of distilled H_2O for 3 min, and then incubated with 1 ml 20% sodium carbonate for 1 hour at room temperature. The absorbance of solution was measured at 734 nm with a UV spectrophotometer (6315, Bibby Scientific Ltd, UK). Standard curve was prepared using gallic acid solution at 0, 10, 25, 50, 100, 250 and 500 mg/L. The results were expressed as gallic acid equivalents (GAE) per gram dry weight (dry weight).

4.3.10 Antioxidant activity assay of 6 spice powder

4.3.10.1 TEAC

The procedure of analysis was the same with Chapter 3.3.4.3.

4.3.10.2 Oxygen radical antioxidant capacity (ORAC)

Determination of ORAC of spices was according to the method described by Ou et al. (2001). 1g of spice powder was mixed with 10ml acetone and water (1/1, v/v) in a test tube. The test tube was then agitated for 15min in a shaker (Multi Reax D-91126, Heidolph, Germany) with coverage of foil and left for 24 hours at room temperature. The extract was centrifuged for 10 min (1500rm) at room temperature. Phosphate buffer was made with 75 mM K_2HPO_4 solution and 75 mM $NaH_2PO_4.2H_2O$, 4.72/1 v/v, adjusted pH to 7.4. 100µM fluorescein solution was prepared by dissolving 3.76mg disodium fluorescein

into 100ml phosphate buffer. The blank was prepared by adding 25 µl of phosphate buffer to a well. The plate reader was switched on and the temperature was set to 37°C. Emission and excitation wavelengths were set at 535 and 485 nm respectively in the GENios TECAN plate reader (Serial No. 12900400464, TECAN, Austria Gesellschaft M.B.H.). 150µl fluorescein solution was kept at 37°C in an incubator for 30 min. 25 µl spice extraction followed by 150µl fluorescein were added to each well and the initial fluorescence was recorded in the plate reader. A volume of 75 µl of AAPH was then added to wells to start the kinetic measurement. Fluorescence measurements were taken every 1 min. The dynamics of fluorescence intensity with time was plotted. Area under the curve (AUC) is used to quantify the antioxidant capacity, expressed as the ORAC value. A calibration curve was prepared by linear concentrations of Trolox (1, 10, 25, 50, 75 and 100µM).

$$AUC = 1 + RFU_1/RFU_0 + RFU_2/RFU_0 + RFU_3/RFU_0 + \ldots.. + RFU_n/RFU_0$$

RFU_0 = relative fluorescence units at time point zero

RFU_n = relative fluorescence units at time points n

4.3.11 Statistical analysis

Statistical significance test was carried out by using SPSS Statistics 21. The significant difference in chemical composition, physical property, levels of HCAs and PAHs, TBARS and protein carbonyl values for the 14 samples were examined using one-way analysis of variance (ANOVA) at the significant level 0.05, and Duncan test was selected for multiple comparison if equal variances assumed, otherwise Tamhane's T2 test was used. The associations between total HCAs/PAHs, lipid oxidation, protein oxidation, and antioxidant capacity were examined by Pearson's correlation. To analyse the effect of factors and the interaction between factors (spice and meat type), two-way ANOVA was employed at the significant level 0.05.

4.4 Results and discussion

4.4.1 Antioxidant capacity and TPC of 6 spices

Single electron transfer and hydrogen atom transfer are two key mechanisms for understanding the antioxidant activity or radical scavenging property

(Shahidi & Ambigaipalan, 2015). TEAC assay is designed to measure single electron transfer during antioxidation, while ORAC is used to investigate the hydrogen atom transfer during antioxidation. TEAC values, ORAC values and TPC of 6 spices were reported in Table 4-3. In TEAC assay, black pepper with 13.57±1.63 µmol of Trolox/ 100 g of dry weight showed the highest antioxidant capacity in single electron transfer, followed by ginger with 11.19±1.15 µmol of Trolox/ 100 g of dry weight. Red chilli (8.45±1.35 µmol of Trolox/ 100 g of dry weight), paprika (7.86±1.29 µmol of Trolox/ 100 g of dry weight), garlic (7.5±0.94 µmol of Trolox/ 100 g of dry weight) and onion (6.55±0.90 µmol of Trolox/ 100 g of dry weight) had similar TEAC values, but their TEAC measurements were lower than those in black pepper and ginger ($p<0.05$). Mariutti et al. (2008) and Ho et al. (2010) also found that ginger and black pepper had higher TEAC value than onion and garlic. 6-gingerol and shogaol in ginger, kaempferol and quercetin in black pepper (Ho et al., 2010; Suhaj, 2006) have been considered as main phenolics with scavenging activity due to their structure of monophenolic moiety (Hinneburg, Dorman, & Hiltunen, 2006). The scavenging ability is dependent on the structure of active compounds. Great antioxidant properties of kaempferol and quercetin are resulted from the presence of catechol hydroxyl groups connected to phenyl and pyran fused rings compared with other phenolics (Shahidi & Ambigaipalan, 2015). Hossain et al. (2008) and Shahidi and Ambigaipalan (2015) found kaempferol and gingerol had higher antioxidant efficiency than capsaicin in TEAC assay. Therefore, high TEAC values in black pepper and ginger are expected as compared with that in red chilli and paprika.

For ORAC assay, red chilli had significantly higher antioxidant capacity (809.90±400.65 µM Trolox/g) than garlic (276.43±15.75 µM Trolox/g) ($p<0.05$), similar result was also found in the work of Ho et al. (2010). However, no significant difference in ORAC value was observed among onion, black pepper, paprika and ginger ($p>0.05$). Main antioxidants in red chilli are mixture of capsaicin, β-carotene, ascorbic acid and phenolic acids including sinapic acid and ferulic acid (Materska & Perucka, 2005). Carotenoids, such as β-carotene act as antioxidant through quenching the oxidation promoters, such as oxygen singlet (Shahidi & Zhong, 2010). Shahidi and Ambigaipalan

(2015) reported that hydroxyl group on the aromatic ring is responsible for the antioxidant capacity of sinapic acid and ferulic acid mainly through hydrogen atom donation. H-donation capacity of capsaicinoids in red chilli could be mainly attributed to the presence of a methoxy group in ortho position to -OH in the phenolic ring (Materska & Perucka, 2005). Phenolic acids were considered as one of the main types of antioxidants, while red chilli (3.47 mg GAE/g dry matter) contained the highest level of TPC. As a result, high ORAC was expected in red chilli. Black pepper showed moderate antioxidant capacity in ORAC owing to the presence of piperanine and piperine, which could scavenge free radicals through hydrogen-donation and chelate metals (Kapoor et al., 2009). Results of TPC in black pepper (2.97 mg GAE/g dry weight) and ginger (3.41 mg GAE/g dry weight) were consistent with data reported by Embuscado (2015) and Ninfali et al. (2005). Although garlic contains organosulfur compounds with antioxidant potential, low ORAC value of garlic (276.43±15.75 μM Trolox/g) might be attributed to the low phenolic content (0.90 mg GAE/g dry matter). In Table 4-7, TPC was correlated to both TEAC ($r=0.78$, $p<0.01$) and ORAC ($r=0.68$, $p<0.01$), which indicated that phenolic compounds in spices could scavenge free radicals through both electron transfer and hydrogen atom transfer. Similar mechanism was also proposed by Ho et al. (2010) and Shahidi and Zhong (2010).

Table 4-3: Antioxidant capacity and principle compounds of 6 common spices

Common name (Scientific name)	*Trolox* equivalent antioxidant capacity (TEAC) (µM of Trolox/100g of dry weight)	Oxygen radical antioxidant capacity (ORAC) (µM Trolox/g)	Total phenolic content (TPC) (mg GAE/g dry weight)	Principle compounds
Garlic (*Allium sativum*)	7.5±0.94[a]	276.43±15.75[a]	0.90±0.003[a]	Organosulfur compounds: disulfides, trisulfide (Iciek, Kwiecień, & Włodek, 2009)
Onion *(Allium cepa L.*)	6.55±0.90[a]	611.06±266.79[ab]	0.99±0.012[a]	Organosulfur compounds: Thiosulfinates Flavonols: Quercetin (Block, Naganathan, Putman, & Zhao, 1992; Nuutila, Puupponen-Pimiä, Aarni, & Oksman-Caldentey, 2003)
Red chilli (*Capsicum frutescens)*	8.45±1.35[a]	809.90±400.65[b]	3.47±0.036[d]	Capsaicin; phenolic acids; Carotenoids: β-carotene; Ascorbic-acid (Suhaj, 2006; Yanishlieva, Marinova, & Pokorný, 2006)
Paprika (*Capsicum annuum*)	7.86±1.29[a]	625.61±240.39[ab]	1.18±0.011[b]	Capsaicin; Carotenoids: lutein, β-carotene (Materska & Perucka, 2005; Vega-Gálvez et al., 2009)
Black pepper (*Piper nigrum L.*)	13.57±1.63[c]	703.81±125.01[ab]	2.97±0.019[c]	Piperine and piperine isomers Flavonols: Quercetin (Suhaj, 2006)
Ginger (*Zingiber officinale Rosc.*)	11.19±1.15[b]	696.54±234.05[ab]	3.41±0.028[d]	Phenolic: gingerol, shogaol and zingerol (Shan, Cai, Sun, & Corke, 2005; Yanishlieva et al., 2006)

Results with different letters in the same column are significantly different at the level $p<0.05$. Each value is represented as mean ± SD (n = 3).

4.4.2 Proximate composition and colour of deep fried beef and chicken meatballs

Table 4-4 shows the effect of spice and meat type on physical and chemical properties of meatballs. Type of spice did not pose any effect on cooking loss, pH, fat and protein, but moisture and colour ($p<0.05$), while meat type significantly affected all the physical and chemical properties of meatballs ($p<0.01$). There was no interaction observed between type of spice and meat type except colour (L*, a* and b*) ($p<0.01$).

Cooking loss in beef meatballs (25.59%-31.28%) was significantly higher than these in chicken (19.89%-23.18%) ($p<0.05$), while spices had no impact on cooking loss. Lan et al. (1995) found that cooking loss increased with lowering pH of muscle meat, as it might induce more protein denaturation and reduce the water holding capacity of meat products. Chicken had higher pH than beef, which could explain a low cooking loss in chicken compared with that in beef. Moisture content ranged from 39.43% to 46.67% in beef and 47.14% to 51.32% in chicken meatballs. Moisture content in chicken meatballs was comparable with Al-abdullah et al. (2011). They reported that chicken meatballs with similar ingredients that deep fried at 180^{o}C for 4min had moisture level of 46.9-57.20%. However, moisture content in lean beef meatballs deep fried at 160^{o}C for 3min was 58.8-65.1% (Galanakis, Tornberg, & Gekas, 2010), which was much higher than the results in this work. Higher level of water addition (15%) in their recipe and low cooking temperature might explain the difference. Low moisture level in the final products is usually corresponded to high cooking loss, as cooking loss is mainly composed of water and water soluble nutrients including proteins (Sánchez del Pulgar, Gázquez, & Ruiz-Carrascal, 2012).

Table 4-4: Cooking loss, proximate composition and colour attributes of deep fried beef and chicken meatballs

		Cooking loss (%)	Moisture (%)	pH	Fat (%)	Protein (%)	L*	a*	b*
Beef	Control	25.59±0.83^{d}	41.11±3.52^{a}	6.01±0.02^{a}	20.32±2.82^{c}	23.92±0.40^{c}	33.93±1.89^{a}	7.25±0.54^{a}	5.29±0.99^{c}
	Garlic	27.34±0.46^{d}	41.40±1.19^{a}	5.98±0.03^{a}	19.85±2.88^{c}	24.11±0.36^{c}	35.23±1.71^{a}	6.59±0.61^{a}	4.26±0.70^{c}
	Onion	27.09±0.47^{de}	44.75±1.56^{a}	6.00±0.03^{a}	21.05±0.77^{c}	24.09±0.24^{c}	34.35±1.19^{ab}	6.69±0.42^{a}	2.84±0.98^{ab}
	Red chilli	31.28±1.05^{e}	41.30±1.56^{a}	6.00±0.02^{a}	20.88±1.27^{c}	24.09±0.23^{c}	36.54±1.07^{bc}	7.12±0.45^{a}	3.41±0.57^{bc}
	Paprika	27.00±0.61^{de}	43.52±2.46^{a}	5.97±0.04^{a}	20.45±2.41^{c}	24.10±0.28^{c}	34.73±1.19^{ab}	6.61±0.10^{a}	2.41±0.91^{ab}
	Black pepper	27.05±0.90^{d}	39.43±1.99^{a}	5.98±0.03^{a}	20.90±1.36^{c}	24.17±0.37^{c}	34.49±1.01^{ab}	6.37±0.68^{a}	1.59±0.65^{a}
	Ginger	27.96±1.42^{d}	46.67±7.83^{ab}	5.97±0.04^{a}	19.62±1.43^{c}	24.15±3.16^{c}	35.03±0.81^{ab}	6.61±0.53^{a}	1.67±0.71^{a}
Chicken	Control	21.97±0.98^{bc}	51.32±4.21^{bc}	6.24±0.02^{bcd}	16.47±0.20^{b}	21.06±0.41^{b}	42.75±1.96^{e}	1.76±0.88^{b}	14.99±0.56^{f}
	Garlic	20.38±0.82^{ab}	47.14±5.19^{bc}	6.26±0.03^{cd}	15.18±1.32^{a}	20.28±0.41^{a}	43.30±2.08^{e}	1.64±0.99^{b}	14.37±0.94^{f}
	Onion	19.89±0.98^{a}	50.91±1.18^{c}	6.28±0.03^{d}	16.88±1.10^{ab}	20.51±0.92^{ab}	44.75±0.41^{e}	1.60±1.05^{b}	14.50±0.26^{f}
	Red chilli	23.18±0.50^{c}	50.07±2.82^{bc}	6.28±0.02^{d}	16.16±1.46^{b}	20.77±0.49^{ab}	36.47±0.34^{bc}	1.43±0.37^{b}	8.91±0.27^{d}
	Paprika	21.16±1.34^{ab}	49.27±2.22^{bc}	6.24±0.02^{bcd}	15.90±0.35^{a}	20.43±0.38^{ab}	39.13±1.17^{d}	1.74±0.14^{b}	9.83±0.71^{e}
	Black pepper	20.36±1.19^{ab}	50.85±1.30^{bc}	6.20±0.03^{b}	16.45±1.87^{b}	20.78±0.41^{b}	38.38±0.40^{cd}	1.45±0.81^{b}	8.35±0.55^{d}
	Ginger	20.72±0.67^{ab}	50.51±1.25^{bc}	6.22±0.02^{bc}	16.26±1.11^{b}	20.92±0.25^{ab}	42.97±0.74^{e}	1.53±0.45^{b}	10.75±0.16^{e}
p (Spice)		0.102	<0.01	0.318	0.792	0.621	<0.01	0.578	<0.01
p (Meat type)		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
p (Interaction between spice and meat type)		0.098	0.190	0.415	0.990	0.375	<0.01	<0.01	<0.01

Results with different letters in the same column are significantly different at the level p<0.05.
Each value is represented as mean ± SD (n = 3).

pH ranged 5.97-6.01 in beef and 6.20-6.28 in chicken meatballs, respectively. Similarly, Oz (2014) reported pH with 5.71±0.21 in fried beef meatballs. pH of chicken meatballs was consistent with the data published by Bhat, Kumar, and Kumar (2013), who observed that deep fried chicken meatballs with added 0-25% chicken skin had pH 6.2 on average. The pH of meatballs was not affected by spices, but meat type. Zeng et al. (2017) found adding 0.5% - 1.5% red chilli powder into beef patties did not change pH in final products. There was no significant difference in pH between control and beef patties added with 0.1%-2% paprika, red pepper and black pepper powder ($p>0.05$) (Martínez et al., 2006). pH of raw muscles varies originally, where beef with 5.53 and chicken with 6.19 (Lan et al., 1995), this might contribute to the variation of pH in the cooked products.

Beef had fat level at 19.62%-21.05% and chicken had fat level at 15.18%-16.88% (as indicated in Table 4-4), which were expected as 16% tallow and 10% chicken fat were mixed into meatballs exogenously. Protein content ranged from 23.92% to 24.17% in beef and 20.43% to 21.06% in chicken. The protein content in beef was slightly lower than the result reported by Ulu (2004), as they found 25.51% protein in shallow-fried beef meatballs formulated with 70% beef. Chicken had higher protein content than commercial chicken meatballs (9.93%-15.06%). The food starch and other food conditioner might dilute concentration of protein in final products, and lead to low protein level in commercial meatball products (Huda, Shen, & Huey, 2009).

Effects of spice and meat type on colour characteristics of deep fried meatballs including lightness (L*), redness (a*) and yellowness (b*) were summarized in Table 4-4. Spices affected L* and b*, while meat type affected all three parameters ($p<0.01$). The interaction between spices and meat type was observed in all three parameters ($p<0.01$).

L* (lightness) varied from 33.93 to 36.54 in beef meatballs and 36.47 to 44.75 in chicken meatballs. Rhee, Cho, and Pradahn (1999) also found lightness of cooked chicken was higher than that of beef, since chicken originally contained less heme pigments than beef. Adding red chilli, paprika and black

pepper significantly reduced L* in chicken meatballs ($p<0.05$). Similar results was also found in the work of Martínez et al. (2006). They reported adding 0.1%-2% red pepper, paprika and black pepper powder in pork sausage significantly reduce L* value of the final products compared with control samples ($p<0.05$). Although brown pigments generated during cooking process could reduce lightness, colour of spice itself added into meatballs should also be taken into account. The black pepper reduced the lightness of meatballs significantly, which could be attributed to the light grey colour of black pepper. In addition, Martínez et al. (2006) proposed that spices, such as red pepper were often added in dry form, which could absorb free water in meat products, and resulted in the reduction of L* consequently.

Redness a* ranged from 6.37 to 7.25 in beef and 1.43 to 1.76 in chicken, variation is directly associated with the myoglobin level in two muscles. Hazell (1982) reported that myoglobin content in beef (13.7-19.8 μg/g) was almost 40 times higher than that in chicken (0.3-0.5 μg/g), thus, a higher a* in beef was expected. Spice had no effect on a* in meatballs. Although pigments of *Capsicum* family, such as keto-carotenoids (red xanthophylls), capsanthin and capsorubin contribute to red colour, there was no significant difference observed between spices added samples and control samples. The low concentration of spice powder used in the products may be not high enough to initiate the significance (Martínez et al., 2006). Yellowness b* of control chicken meatballs was comparable with data reported by Huda et al. (2009). Higher L* and b* were observed in chicken meatballs compared with in beef, which might be attributed to less myoglobin content of chicken meat (Yılmaz, Şimşek, & Işıklı, 2002). Similar result was also found in Mitsumoto et al. (2005), they reported that cooked chicken patties had greater b* (8.3) than beef (6.1).

4.4.3 Effect of common spices on the formation of HCAs in deep fried beef and chicken meatballs

Concentration of HCAs including IQ, MeIQ, 4, 8-DiMeIQx and PhIP in control and spice added beef and chicken meatballs were listed in Table 4-5. Both spice and meat affected each individual HCAs compound and total HCAs

($p<0.01$). Interaction between spice and meat type was observed in IQ, MeIQ and PhIP, but not 4, 8-DiMeIQx and total HCAs. In control samples, the leading contributor to total HCAs was 4, 8-DiMeIQx in beef and MeIQ in chicken meatballs. Jinap et al. (2013) found similarly that MeIQ was major contributor to total HCAs in both cooked beef and chicken satay.

Table 4-5: HCAs in deep fried beef and chicken meatballs added with six common spices[1]

	Treatment	IQ (ng/g)	MeIQ (ng/g)	4,8-DiMeIQx (ng/g)	PhIP (ng/g)	Total (ng/g)	Inhibitory efficiency (%)
Beef	Control	11.29±1.88^{e}	15.38±0.75^{bc}	19.90±4.33^{d}	13.85±2.26^{c}	60.42±9.07^{e}	-
	Garlic	5.14±0.76^{bc}	10.59±6.33^{abc}	5.11±2.62^{b}	7.81±1.85^{b}	31.67±6.96^{c}	47.58
	Onion	4.02±0.80^{b}	18.09±1.04^{bc}	7.47±1.22^{c}	4.87±0.63^{b}	34.44±2.66^{c}	42.99
	Red chilli	4.17±0.22^{ab}	11.65±4.70^{abc}	7.43±0.75^{c}	4.67±0.57^{b}	27.92±3.77^{bc}	53.79
	Paprika	4.66±0.69^{b}	15.64±1.90^{bc}	8.16±0.82^{c}	4.05±0.72^{b}	32.46±1.28^{c}	46.28
	Black pepper	2.47±0.40^{a}	10.77±1.25^{bc}	5.31±0.16^{b}	2.81±0.87^{a}	21.36±0.12^{b}	64.65
	Ginger	2.46±0.22^{a}	3.03±1.59^{a}	4.72±1.50^{b}	2.79±0.45^{a}	12.99±1.52^{a}	78.50
Chicken	Control	8.96±0.93^{e}	12.61±1.17^{bc}	16.36±1.23^{d}	11.07±1.44^{c}	48.99±3.26^{d}	-
	Garlic	8.07±0.21^{de}	15.43±0.74^{bc}	1.61±0.13^{a}	1.28±1.59^{a}	26.39±2.04^{bc}	46.13
	Onion	8.07±0.57^{de}	11.56±6.99^{abc}	1.56±0.23^{a}	1.73±0.75^{a}	22.93±6.50^{bc}	53.19
	Red chilli	4.43±2.33^{b}	9.85±4.44^{ab}	1.51±0.55^{a}	1.46±1.35^{a}	17.26±8.05^{abc}	64.77
	Paprika	6.46±0.38^{cd}	11.82±2.79^{b}	1.83±0.67^{a}	2.09±0.79^{a}	22.20±1.27^{b}	54.68
	Black pepper	8.87±0.86^{e}	1.73±0.90^{a}	0.31±0.54^{a}	2.82±0.22^{a}	13.73±1.57^{a}	71.97
	Ginger	3.43±1.23^{ab}	0.70±0.39^{a}	Nd	2.36±0.93^{a}	6.49±0.63^{a}	86.75
p (Spice)		<0.01	<0.01	<0.01	<0.01	<0.01	
p (Meat type)		<0.01	<0.01	<0.01	<0.01	<0.01	
p (Interaction between spice and meat type)		<0.01	0.038	0.556	<0.01	0.613	

Results with different letters in the same column are significantly different at the level p<0.05. Nd: Not detected. Each value is represented as mean ± SD (n = 3); [1] MeIQx was not detected in all samples.

All 6 spices reduced IQ greatly in beef meatballs with maximum level of 5.14±0.76 ng/g in spices added samples vs 11.29±1.88 ng/g in control beef sample ($p<0.05$). However, only red chilli (4.43±2.33 ng/g), paprika (6.46±0.38 ng/g) and ginger (3.43±1.23 ng/g) were effective to inhibit IQ (8.96±0.93 ng/g in control chicken) in chicken meatballs ($p<0.05$). Similar result was found by Oz and Kaya (2011b) that average 88% of IQ was inhibited by 1% (w/w) flaked red pepper in fried beef chop. Viegas et al. (2012) also found that IQ was totally inhibited in pan-fried beef by marinating with beer, 2.9% (w/v) garlic, 0.4% (w/v) rosemary, 0.25% (w/v) thyme, 2.8% (w/v) ginger and 0.1% (w/v) red chilli pepper. Garlic and onion powder showed inhibition on IQ in beef but not in chicken. Tsai, Jenq, and Lee (1996) reported that DAD, which is an organosulfide compound that naturally existed in garlic and onion extract could inhibit the formation of IQ-mutagens in boiled pork juice. It has been proposed that organosulfide compounds could directly interact with glucose in Maillard reaction, in order to compete the substrate of forming HCAs (Shin, Strasburg, & Gray, 2002a). Knize and Felton (2005) determined that glucose content in chicken (0.47 mg/g meat wet weight) was much lower than in beef (7.03 mg/g meat wet weight), which might make organosulfur compounds less effective in chicken. MeIQ was only inhibited by ginger in beef (3.03±1.59 ng/g), ginger (0.70±0.39 ng/g) and black pepper (1.73±0.90 ng/g) in chicken ($p<0.05$), compared with control meatballs (15.38±0.75 ng/g in beef, 12.61±1.17 ng/g in chicken). Oz and Kaya (2011a) found that 1% black pepper reduced 25% MeIQ in beef meatballs fried at 200°C and 100% fried at 225°C respectively. MeIQ was reduced by 83% in medium grilled beef using marinade containing torch ginger (2.5-10%, w/w) (Jinap, Iqbal, & Selvam, 2015). The amount of MeIQ in control beef meatballs was consistent with the result that reported by Dong, Lee, and Shin (2011). However, they also stated that adding onion powder (12-16g/ 100g) could promote the formation of MeIQ in cooked beef patties, since reducing sugar within onion could promote Maillard reaction. 4, 8-DiMeIQx ranged from 4.72-19.90 ng/g in beef and nd-16.36 ng/g in chicken. Both meat type and spices significantly affected the level of 4, 8-DiMeIQx in the meatballs. Spices reduced the level of 4, 8-DiMeIQx significantly both in beef and chicken samples, but there was no

difference of inhibiting efficiency among 6 spices in chicken, while ginger completely inhibited the formation of 4, 8-DiMeIQx in cooked chicken meatballs. In beef samples, 4, 8-DiMeIQx in ginger, black pepper and garlic added samples were significantly lower than that in onion, red chilli and paprika, which indicated that ginger, black pepper and garlic had better inhibitive effect on the formation of 4, 8-DiMeIQx in beef samples. Jinap et al. (2016) reported that ginger (10%) showed the greatest inhibition (about 45%) on DiMeIQx, compared with turmeric, lemon grass and curry leaves in deep fried lamb with both medium and well-done degree of doneness. Strong inhibitory effect on 4, 8-DiMeIQx was also reported by Oz and Kaya (2011a) and Oz and Kaya (2011b) that black pepper could completely inhibit the formation of 4, 8-DiMeIQx in pan-fried beef chop (225^{o}C for 15min) and deep fried beef meatballs (225^{o}C for 15min) respectively.

The PhIP content was same in beef (13.85 ng/g) and chicken (11.07 ng/g) meatballs, but adding spices reduced the level of PhIP significantly in both beef and chicken meatballs ($p<0.05$). Black pepper (2.81±0.87 ng/g) and ginger (2.79±0.45 ng/g) had notably the lowest amount of PhIP in beef, followed by paprika (4.05±0.72 ng/g), red chilli (4.67±0.57 ng/g), onion (4.87±0.63 ng/g) and garlic (7.81±1.85 ng/g). PhIP in control beef and chicken samples were higher than the result of Keşkekoğlu and Üren (2014), who reported 0.69ng/g PhIP in beef and 0.30ng/g in chicken meatballs. Lower frying temperature (150^{o}C) in their study and different sampling procedures (homogenization of whole sample vs surface sampling) might explain the difference (Gibis, 2016; Lu, Kuhnle, & Cheng, 2017b). There was no difference in PhIP content for all spices added chicken meatballs, which indicated that all the spices had similar inhibitory effect on PhIP in chicken meatballs. Rounds et al. (2012) confirmed garlic, onion and paprika powder inhibit the development of PhIP effectively in beef patties cooked at 200^{o}C for 5min. Phenolic compounds, such as quercetin could act as nucleophiles and consequently react with active sites of phenylacetaldehyde to make the intermediate unavailable for further reaction with creatinine. As a result, the formation of PhIP was reduced (Zhu et al., 2016). All the spices demonstrated similar inhibitory efficiency in chicken meatballs, but varied efficiency in beef

meatballs on the formation of PhIP. It may indicate that involvement of iron and antioxidants in the formation of PhIP was quite complicated and need further investigation.

The six common spices could significantly reduce total HCAs in both cooked beef and chicken meatballs as indicated in Table 4-5 ($p<0.05$). Ginger showed the strongest inhibitive effect on the formation of total HCAs in cooked meatballs, with the reduction rate 78.50% in beef and 86.75% in chicken, followed by black pepper with reduction of 64.65% in beef and 71.97% in chicken, red chilli with 53.79% in beef and 64.77% in chicken. There was no significant difference of total HCAs in beef and chicken meatballs with addition of garlic, onion and paprika ($p>0.05$), which indicated that these 3 spices presented similar inhibitory efficiency on the formation of total HCAs. Inhibitory efficiency of spices on formation of HCAs was associated with their antioxidant capacity, which has been demonstrated in rosemary, cumin, turmeric (Puangsombat et al., 2011), garlic and onion (Janoszka, 2010). The relationship between total HCAs and their antioxidant capacity was confirmed by the negative correlations in Table 4-7, i.e. $r = -0.853$ ($p<0.01$) between TEAC and total HCAs; $r = -0.712$ ($p<0.05$) between ORAC and TPC. The negative correlation between antioxidant capacity and total HCAs implied that principle compounds in these spices might inhibit the formation of HCAs through both ways, i.e. quenching free radicals by hydrogen atom donation and transferring single electron to reduce active radicals (El-Badry, 2010).

The inhibitory efficiency of selected spices on radical scavenging depends on the structure and composition of phenolic compounds, i.e., the number of hydroxyl group attached on benzene ring, the position of hydroxyl group(s) and substitution pattern of hydroxyl group (El-Badry, 2010). Shahidi and Ambigaipalan (2015) explained that quercetin in black pepper and onion with high antioxidant capacity resulted from the catechol hydroxyl groups and 4-oxo group, which could donate hydrogen and delocalize the unpaired electrons to prohibit the propagation of radical reaction. The highest inhibitory efficiency of ginger could be explained by the relatively high TEAC and ORAC values, while the thermal behaviour of principle antioxidants at high cooking

temperature could also play a key role. Gingerol was the principle antioxidant in ginger, which could be degraded into shogaols and zingerone under high cooking temperature due to the presence of a β-hydroxy keto group in the structure. These two compounds had even higher free radical scavenging capacity compared with gingerol, which might lead to the increase in inhibitory efficiency (Bandyopadhyay, Chakraborty, & Raychaudhuri, 2008; Ho & Su, 2016; Puengphian & Sirichote, 2008). While piperine in black pepper acted as free radicals and reactive oxygen species quencher also showed good thermal stability at roasting condition. Chacko et al. (1996) reported piperine could remain stable at 150^{o}C for 15min. In addition, flavonoids in black pepper and onion are mainly quercetin-4'-O- monoglucoside and quercetin-3, 4'-O-diglucoside (Lee et al., 2008). At high temperature, 80% of quercetin glucosides could degrade into aglycone (quercetin) with more antioxidant activity (Khatun et al., 2006; Rohn et al., 2007). Quercetin could reduce HCAs by 60% in heated chemical model system and by 50% in meat products (Cheng, Chen, & Wang, 2007a; Zhu et al., 2016). Capsaicin was considered as the principle antioxidant in red chilli and paprika (Shahidi & Ambigaipalan, 2015; Shobana & Akhilender Naidu, 2000). Although red chilli had the highest TPC value, the inhibitory efficiency on HCAs was only moderate, which could be attributed to rapid degradation of capsaicin at high temperature (Wang et al., 2009). In addition, high tempratures could accelelerate the release of fat-soluble carotenoid and other bound phenolics into cooking oil, which could also contribute to the loss of antioxidants (Tiwari et al., 2006). Organosulfides are principle antioxidants in garlic and onion mainly including diallyl disulfide (DAD), diallyl sulfide and dipropyl disulfide. Shin et al. (2002a) reported that 70-78% of total HCAs could be reduced in garlic and onion added beef patties. In this study, garlic and onion showed the least inhibition on HCAs compared with the other spices. It could be attributed to the volatility of organosulfides during processing. In this work, garlic powder was used and large amount of sulfur compounds may be lost during garlic powder processing, such as crushing and drying (Iciek, Kwiecień, & Włodek, 2009). In addition, the bond strength of disulphide bond (S-S) is much weaker than that of phenyl-hydroxyl (-OH), the functional group of phenolics, which might make sulfide compounds

less stable compared with phenolic compounds at high temperature (Blanksby & Ellison, 2003; Franklin & Lumpkin, 1952). Therefore, garlic and onion achieved the least efficiency in prohibiting formation of total HCAs.

Meat type significantly affected the formation of HCAs ($p<0.05$). In control samples, beef meatballs had significantly higher total HCAs than chicken ones ($p<0.05$). Beef meatballs added with black pepper and paprika also contained higher level of total HCAs than these chicken ones ($p<0.05$). The difference in precursors (type and amount) between beef and chicken could explain the variation (Keşkekoğlu & Üren, 2014). Beef contains high level of nonheme iron compared with chicken, which could accelerate the oxidative process by reacting with hydroxyl and peroxyl radicals. Consequently the lipid oxidation and Maillard reaction were accelerated (Gibis, 2016). Average creatine content is 6.33mg/g in beef and 3.54-4.44 mg/g in chicken (Zöchling, Murkovic, & Pfannhauser, 2002). Being the major precursor of HCAs creatine could react with pyrazine and pyridine radicals produced from Strecker degradation to produce imidazoquinoline and imidazoquinoxaline (Vitaglione & Fogliano, 2004). Jinap et al. (2013) reported that roasted beef contained higher HCAs than in roasted chicken due to high content of creatine and low level of free amino acids. In addition, the high moisture content in chicken meatballs compared with beef might dilute the concentration of HCAs. Jinap et al. (2016) also reported low moisture content may be associated with high HCAs as well. Thus, high level of HCAs would be expected in beef meatballs.

4.4.4 Effect of common spices on the formation of PAHs in deep fried beef and chicken meatballs

In control samples, total PAHs in deep fried beef meatballs (3.87±1.44 ng/g) was similar with that in chicken meatballs (3.66±2.18 ng/g) (Table 4-6). Spice affected the formation of BaA, BaP and total PAHs, but only meat type had significant effect on the formation of BaA ($p<0.01$). Interaction between meat type and spice was not observed in both individual compounds and total PAHs ($p>0.05$) (Table 4-6). The level of BaA in beef meatballs (0.08-1.91 ng/g) was similar with commercial Swedish meatballs (2.18± 0.22 ng/g) reported by Lu, Kuhnle, and Cheng (2017a). BaP in chicken meatballs (0.1- 2.8 ng/g) was

lower than the result from El-Badry (2010), who detected 3.84 ng/g BaP in pan-fried chicken. Higher BaP in their study might be resulted from longer cooking time (10min) compared with 3min in this study. There was no difference in BaA between control and spice added beef samples, which indicated that all the spices did not have inhibitory effect on BaA in beef meatballs, however all the spices could inhibit the formation of BaA in chicken meatballs, as evidenced by a low BaA content in spice added samples compared with control ($p<0.05$). On the contrary, inhibitory effect of spices on BaP was observed in beef meatballs ($p<0.05$) but not in chicken meatballs ($p>0.05$). El-Badry (2010) reported that both spice mixture (cumin, coriander, black pepper and rosemary) and garlic paste could reduce BaP from 3.84 ng/g to 0.18 ng/g in pan-fried chicken and to 1.16 ng/g in garlic paste treated chicken. The discrepancy in the prohibitive effect of spices in chicken may be caused by the big variation in control samples. EU Commission has reduced the limit of BaP occurring in processed meat and seafood products from 5ng/g to 2 ng/g because of its high toxic potency (Ledesma, Rendueles, & Díaz, 2016). Control samples had high risk of over the limit due to the big variation, and adding spices in the meat products could greatly reduce this risk although significant reduction was not observed in spices added chicken samples.

Table 4-6: PAHs in deep fried beef and chicken meatballs added with six common spices

	Treatment	BaA (ng/g)	BaP (ng/g)	Total (ng/g)	Inhibitory efficiency (%)
Beef	Control	1.91±1.37^{ab}	1.96±0.08^{c}	3.87±1.44^{bc}	-
	Garlic	0.76±0.35^{ab}	0.59±0.01^{b}	1.35±0.34^{b}	65.12
	Onion	1.09±0.12^{a}	0.60±0.04^{b}	1.68±0.08^{b}	56.59
	Red chilli	0.47±0.73^{ab}	0.91±0.23^{ab}	1.37±0.82^{ab}	64.60
	Paprika	0.38±0.66^{ab}	0.13±0.06^{a}	0.51±0.60^{ab}	86.82
	Black pepper	1.34±0.06^{ab}	0.71±0.21^{ab}	2.05±0.17^{b}	47.03
	Ginger	0.08±0.04^{b}	Nd	0.08±0.04^{a}	97.93
Chicken	Control	2.02±0.98^{ab}	1.64±1.23^{abc}	3.66±2.18^{bc}	-
	Garlic	Nq	0.50±0.08^{b}	0.50±0.08^{a}	86.11
	Onion	Nq	0.52±0.09^{b}	0.52±0.09^{a}	85.56
	Red chilli	Nd	0.75±0.22^{b}	0.75±0.22^{ab}	79.17
	Paprika	Nd	0.92±0.32^{b}	0.92±0.32^{b}	74.44
	Black pepper	Nd	0.11±0.05^{a}	0.11±0.05^{a}	96.94
	Ginger	Nd	0.10±0.06^{a}	0.10±0.06^{a}	97.22
p (Spice)		<0.01	<0.01	<0.01	
p (Meat type)		<0.01	0.659	0.796	
p (Interaction between spice and meat type)		0.228	0.074	0.721	

Results with different letters in the same column are significantly different at the level $p<0.05$. Nd: Not detected, Nq: Not quantified. Each value is represented as mean ± SD (n = 3).

Table 4-7: Pearson correlation coefficient (p) between the level of total HCAs (ng/g) / PAHs (ng/g) and TBARS, protein carbonyl, TPC, ORAC and TEAC

	TPC	TBARS	Protein carbonyl	Total HCAs	Total PAHs
TBARS		-	0.279	0.644**	0.364*
Protein carbonyl		0.279	-	0.768*	0.598*
TEAC	0.781**	-0.532**	-0.736*	-0.853**	-0.647*
TPC	-	-0.598**	-0.657**	-0.754*	-0.507*
ORAC	0.680**	-0.343*	-0.625*	-0.712*	-0.238

* Correlation is significant at the level p<0.05 (2 tailed)

** Correlation is significant at the level p=0.01 (2 tailed)

Among all the spices, ginger powder (97.93%) was the only spice which significantly inhibited the formation of total PAHs in both beef and chicken meatballs ($p<0.05$). Black pepper, garlic and onion only showed inhibitory effect on total PAHs in chicken meatballs ($p<0.05$), but not in beef ones ($p>0.05$). Red chilli and paprika did not show significant inhibitory effect on total PAHs neither in beef or chicken meatballs. Janoszka (2011) reported that adding garlic and onion could reduce 54% and 60% of total PAHs in fried pork chop. They proposed that antioxidants, such as disulfides and polyphenols, could prevent the oxidation and polymerization of hydrocarbons produced from decomposition of fatty acids and protein, and lead to low level of PAHs. In control samples, meat type did not affect BaP, BaA and total PAHs ($p>0.05$).

The relationship between total PAHs and antioxidant capacity was also confirmed in Table 4-7. Total PAHs was negatively correlated with TEAC ($r = -0.647$, $p<0.05$) and total phenolic content of spices ($r = -0.507$, $p<0.05$), which indicated that antioxidant capacity had negative correlation with inhibitory effect on total PAHs. Viegas et al. (2014) also reported that inhibitory effects of marinade on formation of PAHs in charcoal-grilled pork were related to their ability to scavenge free radicals and destroy fatty acid hydroperoxides. However, no relationship was observed between ORAC and total PAHs ($p>0.05$), which indicated that the inhibitory effect of antioxidants in spices on the formation of PAHs was mainly through electron atom transfer instead of hydrogen atom transfer. Janoszka (2011) mentioned that the progress of forming PAHs involves a series of radical reaction, which presented in the form of active cations. *In vivo* study, organosulfides (Singh & Shukla, 1998) and phenolic compounds in ginger powder (Nirmala, Krishna, & Polasa, 2007) were also reported with capacity of detoxification of BaP during metabolism.

4.4.5 Correlation between lipid oxidation, protein oxidation and the formation of HCAs and PAHs

TBARS values of cooked beef and chicken meatballs were showed in Figure 4-1. TBARS was determined up to 0.68mg/kg in cooked beef meatballs and up to 0.5 mg/kg in cooked chicken meatballs. Such a degree of lipid oxidation

would not affect sensory quality of the meatballs, since the highest level of TBARS (up to 0.68 mg MDA /kg meat, which equals to 9.44 µmol/kg) in control beef meatballs was below the sensory limit for rancidity around 10–20 µmol/kg meat (Racanicci et al., 2004). In cooked beef meatballs, low TBARS was only observed in ginger added samples ($p<0.05$). In chicken samples, both ginger and black pepper offered lower TBARS values than other spices ($p<0.05$). The reduction of TBARS could be contributed to antioxidants, including gingerol, shogaol and zingerone in ginger. Tanabe, Yoshida, and Tomita (2002) reported that dried ginger had the highest reduction (75%) of lipid oxidation in pork sausages compared with sage, rosemary and black peppercorns, while the inhibitory efficiency on lipid oxidation was correlated with the number of antioxidants and their activity in spices. Ginger contained the highest number of antioxidants (40), compared with rosemary (26), thyme (26), oregano (26) and allspice (25), which might explain why ginger had the highest inhibitory efficiency. Phenolic compounds in ginger and alkaloid piperine in black pepper could inhibit lipid oxidation by quenching hydroxyl radicals or fatty acid radicals and preventing propagation of lipid peroxidation in cooked meat (Brewer, 2011). They could also chelate metals (iron and cooper in meat) and then turn them into non-reactive forms (Shobana & Akhilender Naidu, 2000).

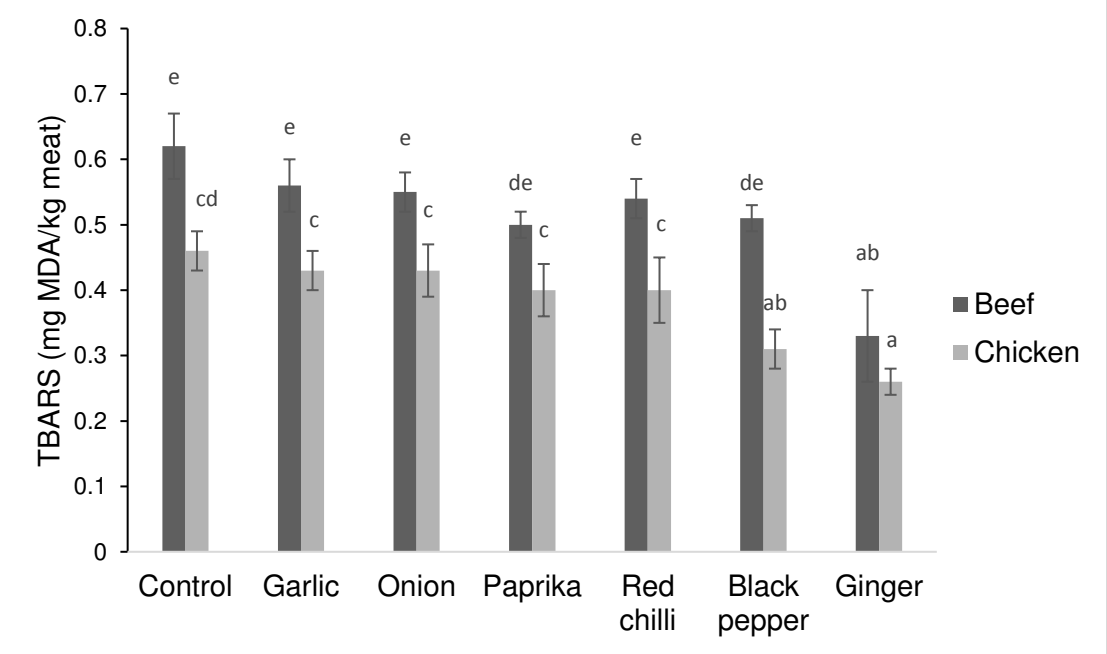

Figure 4-1: TBARS values in cooked beef and chicken meatballs[1]

[1]Results with different letters are significantly different at the level $p<0.05$.

There was no difference in TBARS value of beef and chicken meatballs added with red chilli and paprika ($p>0.05$), which indicated that both spices could not reduce the lipid oxidation of meat products. Low thermal stability of functional compounds in these spices could explain the result. Shobana and Naidu (2000) reported that capsaicin in red chilli and paprika degraded rapidly when temperature over 150°C. Addition of garlic and onion did not reduce TBARS in both chicken and beef meatballs ($p>0.05$). DAD in garlic powder showed ability of scavenging hydroxyl radicals, however, it had no effect on superoxide radicals, which is a primary and the most harmful reactive oxygen species in lipid oxidation (Chung, 2006; Shahidi & Zhong, 2010). In addition, the frying temperature (180°C) was close to the boiling point of organosulfur compounds, i.e. 190°C for DAD and 164-166°C for alliin. The volatilization of functional compounds under high temperature would make them less effective to prohibit the lipid oxidation (El-Badry, 2010). Overall, TBARS in cooked beef meatballs were higher than those in cooked chicken meatballs ($p<0.05$), which could be explained by the difference of their composition. High content of nonheme iron in beef could be responsible of severe lipid oxidation by reacting with denatured myoglobin at high temperature (Vuorela et al., 2005).

Protein carbonyls are produced from protein oxidative degradation in meat products, which were used to analyse degree of protein oxidation (Figure 4-2). Significant effect of spices on the protein oxidation was observed ($p<0.05$). Control beef and chicken meatballs had a significantly higher level of protein carbonyls (2.3 nmol/mg protein in beef and 2.01 nmol/mg in chicken) than other 6 spices-added meatballs ($p<0.05$). Similar results was found by Duthie et al. (2013) that adding dry vegetable powder (7%, w/w), especially red pepper, spinach and celery could reduce protein oxidation in cooked turkey patties ($p<0.05$). The inhibition of protein oxidation could be explained by the antioxidants in spices, which was disclosed by the negative correlation between protein carbonyl level and antioxidant capacity of spices, i.e. r =-

0.736 with TEAC and -0.625 with ORAC as indicated in Table 4-7 ($p<0.05$). The inhibitory efficiency of spices on protein oxidation is as followed: ginger> black pepper=red chilli> paprika= onion= garlic in cooked beef meatballs and ginger= onion> paprika= garlic> red chilli= black pepper in cooked chicken meatballs. Inconsistent inhibitory efficiency of spices in beef and chicken meatballs might be not only associated with the capability of antioxidants, but also the level of substrates in protein oxidation. For example, beef contained high content of iron comparing with chicken.

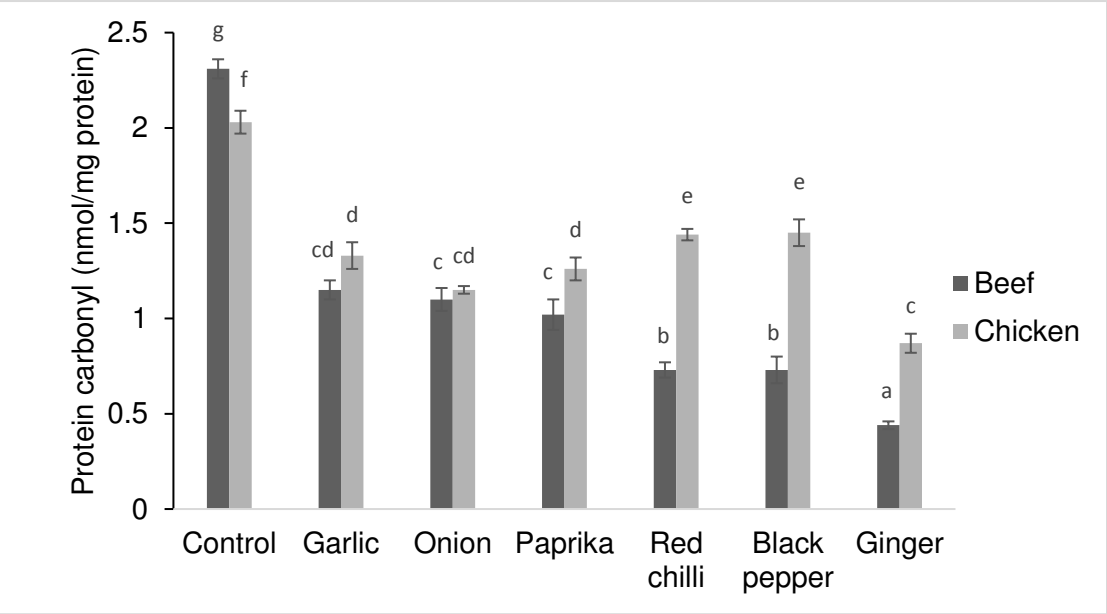

Figure 4-2: Protein carbonyl values in cooked beef and chicken meatballs[1]

[1]Results with different letters are significantly different at the level $p<0.05$.

Lipid oxidation and protein oxidation were found associated with the development of HCAs and PAHs through interactions of radicals generated from lipid oxidation, lipid pyrolysis and Maillard reaction (Chen & Chen, 2001; Gibis, 2016; Lu et al., 2017b). Thus, it is necessary to investigate the relationship between lipid/protein oxidation and the formation of HCAs/PAHs. Correlation analysis in Table 4-7 disclosed significant positive correlation existed between total HCAs and TBARS ($r =0.664$, $p<0.01$), and between HCAs and protein carbonyl ($r =0.768$, $p<0.05$), which indicated that both lipid oxidation and protein oxidation contributed to the formation of HCAs during

cooking process. Aldehydes and ketones generated from peroxyl radicals (ROO•) in the initial step of lipid oxidation might promote the formation of pyrazine via reacting with amino acids (Johansson, Skog, & Jagerstad, 1993). At the same time, peroxyl radicals could also react with hydrogen atoms on protein residues to trigger protein oxidation (Falowo, Fayemi, & Muchenje, 2014). Ganhão, Morcuende, & Estévez (2010) also suggested that lipid oxidation could enhance protein oxidation by reacting with heme iron that released from myoglobin. The incomplete combustion or pyrolysis of organic components including fat, protein and carbohydrates at temperature over 200^{o}C, especially at 500-900^{o}C was the main pathway for developing PAHs. In this study, a weak correlation between total PAHs and TBARS was observed ($r=0.364$, $p<0.05$), low frying temperature (180^{o}C) could be the main cause.

4.5 Conclusions

All 6 spices could reduce total HCAs formation in both beef and chicken meatballs, while ginger and black pepper demonstrated the highest inhibitory efficiency. Strong negative correlation between total HCAs and antioxidants capacity including TEAC and ORAC indicated that antioxidant capacity of spices was the key indicator of inhibitory efficiency in reducing formation of total HCAs. Antioxidants in the spices may be interfered with the formation of HCAs through both hydrogen atom donation and single electron transfer to reduce or quench active radicals. However, inhibition of PAHs of spices may be only involved with electron transfer as no correlation was observed between ORAC and PAHs. In beef meatballs, PAHs was only inhibited by ginger, but garlic, onion, black pepper and ginger were all effective in reducing PAHs in chicken samples. In conclusion, spices used in processed meat products could reduce HCAs and PAHs, and their antioxidant capacity was key indicator of their inhibitory efficiency.

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Chapter 5. Understand the inhibitory effect of diallyl disulfide and gallic acid on the formation of heterocyclic amines and polycyclic aromatic hydrocarbons using meat model system

This chapter will be submitted to 'Food Chemistry'.

5.1 Abstract

Effect of diallyl disulfide (DAD) and gallic acid (GA) on the formation of heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) in deep-fried beef meatballs was examined. DAD at 0%, 0.05% and 0.1% combined with GA at 0%, 0.01% and 0.02% were added into beef meatballs and cooked at 180oC for 3min. Result showed the inhibitory effect of DAD on total HCAs was dose-dependent ($p<0.05$). GA at 0.01% also showed inhibitory effect on total HCAs, but increasing GA from 0.01% to 0.02% did not show further reduction of total HCAs ($p>0.05$). Regression model between total HCAs, DAD and GA was established, i.e. predicted total HCAs (ng/g) = 52.30 - 22.34 GA - 21.62 DAD + 10.17 GA * DAD (Adjusted R^2= 0.74, $p<0.05$). Both DAD and GA could reduce PAHs, especially BaP and BaA could be completely inhibited at 0.1% DAD. Regression model between total PAHs, DAD and GA was: predicted total PAHs (ng/g) = 2.32 - 1.21 GA - 1.29 DAD + 0.696 GA * DAD (Adjusted R^2= 0.78, $p<0.05$). Regression models revealed that DAD and GA contributed similar inhibitory efficiency on the formation of HCAs and PAHs, while the interaction between DAD and GA may promote the formation of HCAs and PAHs. Addition of DAD and GA could also reduce lipid and protein oxidation in cooked meatballs.

5.2 Introduction

Intake of red meat and processed meat products has been found associated with colon and rectum cancer in World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) report (2007). The occurrence of mutagens, such as HCAs, PAHs and N-nitroso in red/processed meat

products might contribute to the increase health risk from intake of meat products, as they could induce DNA adducts and cause adenomas in colon (Aune et al., 2013; Gibis, 2016). HCAs are mainly formed with the presence of free amino acids, carbohydrates and creatine under high cooking temperature (Turesky, 2010). 5 aminoimidazoarenes (AIAs) compounds, including 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,4-methylimidazo[4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4, 8-DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) are commonly presented carcinogens in meat cooked at household cooking temperature (180-250^{o}C). In the meantime, polycyclic aromatic hydrocarbons (PAHs) can be generated in meat processing through: (1) incomplete combustion of organic materials, such as wood or charcoal; (2) pyrolysis of carbon and hydrogen, i.e. fat and carbohydrate; (3) lipids dripping on heating source and adhering on food surface (Alomirah et al., 2011). They are generally found in barbequed, smoked, fried and roasted meat products. PAH4, sum of benz[a]anthracene (BaA), benzo[a]pyrene (BaP), benzo[b]fluoranthene (BbF) and chrysene, has been selected to present total PAHs in food. BaA and BaP are the most potent carcinogenic PAHs (Group 2A) among them (IARC, 2010).

Diet is one of the major routes to expose carcinogenic HCAs and PAHs to general public. With the increase in meat consumption, it is necessary to reduce health risk associated with dietary HCAs and PAHs exposure from cooked/processed meat. Lots of researchers have worked on identifying pathways of HCAs and PAHs, and they believed that the formation of HCAs and PAHs involves a series of free radical reactions (Singh, Varshney, & Agarwal, 2016; Wang, Raj, & Chung, 2013). Antioxidants are well known radical scavengers, which have been reported to interfere with the formation of HCAs and PAH through quenching free radicals or blocking intermediates. There are 3 forms of application to get antioxidants involved in meat products/chemical model system: whole food, bioactive extracts and pure antioxidant compounds. Studies about inhibitory effect of antioxidants on HCAs and PAHs have been conducted extensively on phenolics and organosulfur compounds. The formation of polar HCAs in meat products has

been successfully inhibited by dried apple peel powder (Sabally et al. , 2016), red pepper and black pepper (Oz & Kaya, 2011a; Oz & Kaya, 2011b), spices such as ginger, turmeric and lemongrass (Jinap, Iqbal, & Selvam, 2015), grape seed and rosemary extract (Gibis & Weiss, 2012; Rounds et al., 2012), pomegranate seed extract (Keşkekoğlu & Üren, 2014), phlorizin, epigallocatechin gallate (EGCG), quercetin, kaempferol, naringenin (Cheng, Chen, & Wang, 2007a; Zhu et al., 2016) and organosulfur, diallyl disulfide (DAD) and dipropyl disulfide (DPD) (Shin et al., 2002b). Fresh garlic and onion (Janoszka, 2011), spice and herbs marinade with lemon juice (Farhadian et al., 2012), beer marinades, including Pilsner and black beer (Viegas et al., 2014) have been reported inhibitory effect on PAHs formation in various cooked meat products.

However, inconsistent results of inhibitory efficiency were obtained between adding bioactive extract and its corresponding principle antioxidant compound. Tsen, Ameri, and Smith (2006) reported that rosemary extract and its corresponding principle compound, rosmarinic acid had similar reduction efficiency on the formation of MeIQx and PhIP in roasted beef patties. However, Zeng et al. (2016) compared the inhibitory efficiency of chilli pepper and its principle compound- capsaicin on HCAs formation in roasted beef, and they found that compounds other than capsaicin in chilli pepper might enhance the formation of HCAs. The inconclusive results might be attributed to the complexity of antioxidant food vehicle, the synergistic effect or supressing effect between compounds. As single principle compounds could not present comprehensively inhibitory effect in the whole food matrix, it is useful to explore the effect of combining different types of antioxidants to have better understanding the effect of natural ingredients.

Spice and herbs, such as garlic, onion, ginger and black pepper are essential ingredients offering unique flavour and antioxidant properties in the meat processing. The antioxidant capacity of spices was mainly attributed to two types of antioxidants, organosulfur compounds and phenolic compounds (Iciek, Kwiecień, & Włodek, 2009; Shahidi & Ambigaipalan, 2015). In order to study the inhibitory pattern and interaction between antioxidants on the

formation of carcinogenic compounds, diallyl disulfide (DAD) as the representative of organosulfur compounds and gallic acid (GA) as phenolic compounds were added in meatballs to establish the relationship.

5.3 Materials and methods

5.3.1 Materials

Diallyl disulfide (>80%) and gallic acid powder were purchased from Sigma-Aldrich (Gillingham, UK). Other chemicals used see Chapter 4.3.1.

5.3.2 Procedures for preparing and cooking beef meatballs

The formulation of control beef meatballs (as shown in Table 5-1) included 640g lean beef mince, 160g tallow, 100g breadcrumbs, 10g salt, 88.8g ice and 1.2g methanol per kilogram. According to the spices chosen from Chapter 4, organosulfides and phenolic acids are two of main principle compounds presenting antioxidant potential. DAD was chosen to represent organosulfides because DAD was the major compound (60%-70% of total organosulfides) contained in garlic and onion powder/extracts (Iciek et al., 2009). Gallic acid is a typical compound that expresses total phenolic content in spices/extracts, which could represent for phenolics.

Concentration of DAD (0%, 0.05%, 0.1%) and GA (0%, 0.01%, 0.02%) was chosen with the consideration of spices/herbs application in meat products. DAD solutions with 0%, 0.05%, 0.1% and GA solutions with 0%, 0.01%, and 0.02% were made in methanol. Before manufacturing meatballs, all the solutions were kept in the fridge (4^{o}C). Beef mince, tallow, breadcrumbs, salt and ice were homogenized at 5000rpm for 5min in the Kenwood Food processor to reach a uniform emulsion, followed by adding DAD, GA and their combination solution into the mixture with a further homogenization for 2 min to obtain even distribution of the solution. Each meatball was weighed 14.9g-15.1g, and shaped to ball with 9.0±0.2 diameters by hand. Meatballs were deep fried in a rotary deep fryer (Delonghi, Type No: F283118) with the setting temperatures 180^{o}C for 3min until core temperature 71.6-73.2^{o}C reached, measured by a thermometer (KM330 Industrial Thermometer, Comark Instruments, UK). After cooking, meatballs were placed on paper towel for 10min to remove excess oil on surface then final weight was

recorded to calculate the cooking loss. Cooking loss and the storage of samples, please see Chapter 4.3.2.

5.3.3 Proximate composition, pH and Colour analysis

5.3.3.1 Proximate composition and pH

The procedure of analysis was the same with Chapter 2.3.3.

5.3.3.2 Colour

The procedure of analysis was the same with Chapter 2.3.3.

5.3.4 Determination of HCAs

Approximate 2mm samples surface were trimmed and blended well before measuring. The procedure of analysis was the same with Chapter 2.4.3.

5.3.5 Determination of PAHs

The procedure of analysis was the same with Chapter 2.3.5.

5.3.6 LOD, LOQ and recovery rate of HCAs and PAHs

The procedure of analysis was the same with Chapter 4.3.6. The average recoveries of these 5 HCAs according to triplicates were 58.35% for IQ, 61.10% for MeIQ, 53.97% for MeIQx, 57.24% for 4,8-DiMeIQx and 55.99% for PhIP. Results were comparable with published data (Gibis, 2007; Oz, 2011). Similarly, recovery rate of BaA and BaP were 54.03% and 50.87% respectively, which was comparable with published results of 50% - 115% (Farhadian et al., 2010; Janoszka, 2011).

Table 5-1: Formulation of raw beef meatballs (per kg)

		Beef Mince (g)	Fat (g)	Bread Crumbs (g)	Salt (g)	Ice (g)	Treatment
T1	Control	640	160	100	10	88.8	Methanol 1.2g
T2	GA 0.01%	640	160	100	10	89.9	GA 0.1g
T3	GA 0.02%	640	160	100	10	89.8	GA 0.2g
T4	DAD 0.05%	640	160	100	10	89.5	DAD 0.5g
T7	DAD 0.1%	640	160	100	10	89	DAD 1g
T5	DAD 0.05%+GA0.01%	640	160	100	10	89.4	DAD 0.5g + GA 0.1g
T6	DAD0.05%+GA 0.02%	640	160	100	10	89.3	DAD 0.5g + GA 0.2g
T8	DAD 0.1%+GA 0.01%	640	160	100	10	88.9	DAD 1g + GA 0.1g
T9	DAD 0.1%+GA 0.02%	640	160	100	10	88.8	DAD 1g + GA 0.2g

5.3.7 Lipid oxidation and protein oxidation

5.3.7.1 TBARS value (Lipid oxidation)

The procedure of analysis was the same with Chapter 3.3.4.1.

5.3.7.2 Protein carbonyl value (Protein oxidation)

The procedure of analysis was the same with Chapter 3.3.4.2.

5.3.8 Antioxidant capacity

5.3.8.1 TEAC

The procedure of analysis was the same with Chapter 3.3.4.3.

5.3.8.2 ORAC

The procedure of analysis was the same with Chapter 4.3.10.2.

5.3.9 Statistical analysis

Statistical significance test was carried out by using SPSS Statistics 21. The significant difference in chemical composition, colour characters, levels of HCAs and PAHs for the 9 treatments were carried out by one-way analysis of variance (ANOVA) at the significant level 0.05, and Duncan test was selected for multiple comparison if equal variances assumed, otherwise Tamhane's T2 test was used. To analyse the effect of DAD and GA and the interaction between factors (antioxidant compounds and their concentration) on the formation of HCAs and PAHs, two-way ANOVA was employed at the significant level 0.05. Regression model between HCAs/PAHs and GA/DAD was established using General Linear Model (GLM) Univariate Analysis at the significant level 0.05.

5.4 Results and discussion

5.4.1 Effect of DAD and GA on cooking loss, pH, proximate composition and colour of cooked meatballs

5.4.1.1 Cooking loss, pH and proximate composition

Table 5-2 shows the effect of DAD and GA on physical and chemical properties of meatballs. Addition of DAD significantly affected pH, moisture and protein, but not cooking loss and fat content; however GA had no effect on any parameters, but pH ($p<0.05$). There was no interaction observed between DAD and GA in all parameters except pH ($p>0.05$).

Cooking loss ranged from 23.16-24.79% in deep fried beef meatballs. It was comparable with results reported by Odiase et al. (2013), who found that deep fried beef meatballs at 170^{o}C for 3 min had 27.33% weight loss. However, much higher cooking loss was found in Keşkekoğlu and Üren (2014), and they reported 52.43±1.17% cooking loss in deep fried beef meatballs for 5min with pomegranate seed extract (0.5% w/w). Similar result was also found by Raza et al. (2015) that deep fried beef for 10min had 42.73±2.14% cooking loss. Long frying time might partially explain the difference, as revealed in a kinetic model that mass loss of beef meatball increased by 50% with frying time increased from 3 min to 10 min (Ateba & Mittal, 1994). Effect of GA, DAD and interaction between DAD and GA on cooking loss was not significant ($p>0.05$). Effect of DAD on cooking loss was consistent with the work of Nurwantoro et al. (2011), who reported marinating beef with garlic juice containing 60-70% DAD did not affect cooking loss, compared with beef without marinade. Zeng et al. (2017) found similarly no difference of cooking loss between control and patties added with capsaicin (2-6 mg/100g).

pH ranged from 5.59 to 5.75. Both DAD and GA significantly reduced pH in deep fried meatballs ($p<0.05$). Meatballs with GA addition had pH 5.60-5.62 and with DAD had pH 5.59-5.60 respectively. There was interaction observed between DAD and GA ($p<0.01$). pH is determined by the amount of free hydrogen ions (H+) in system, as GA is acidic, addition of GA would increase H ions level, and a low pH would be expected (Leygonie, Britz, & Hoffman, 2011). Decrease in pH was also observed in pork sausage with garlic powder (Phromraksa et al., 2003) and beef marinated with garlic juice (Nurwantoro et al., 2011), which might be attributed to acidic properties of raw garlic (pH 5.9) (Nurwantoro et al., 2011).

Moisture content ranged from 41.39% to 43.81% in cooked beef meatballs. Generally speaking, low moisture level in the final products was associated with high cooking loss, because the weight loss caused by cooking mainly included water and water-soluble nutrients such as myofibrillar and sarcoplasmic proteins, collagen and salt (Sánchez del Pulgar, Gázquez, & Ruiz-Carrascal, 2012). Addition of 0.05% DAD significantly reduced moisture

from 43.81% to 41.39% ($p<0.05$), while effects of GA and interaction between DAD and GA were not significant ($p>0.05$). Nurwantoro et al. (2011) reported that marinating beef with garlic juice (pH 5.9) initiated the decrease of pH close to the isoelectric point of meat proteins (pH 5.1). Since lowering meat pH could lead meat proteins including actin and myosin to approach to isoelectric point, which would trigger the reduction of space between protein filaments. As a result, water was squeezed out of the structure and low moisture content was expected. Adding DAD and GA did not affect fat content in deep-fried meatballs, which ranged from 23.82%-25.29%. Protein content of cooked meatball with 0.1% DAD (23.80%) was significantly lower than in meatballs with 0% (24.81%) and 0.05% DAD (24.52%), while GA inclusion did not cause significant variation of protein content. Nieto et al. (2013) reported that organosulfur compounds could interact with meat protein to generate protein thiols and cross-link, which might contribute to the reduction of crude protein content in final products.

Table 5-2: Cooking loss, pH, proximate composition and colour parameters of deep fried beef meatballs

		Cooking loss (%)	pH	Moisture (%)	Fat (%)	Protein (%)	L*	a*	b*
Effect of DAD	0	23.16±2.37^{A}	5.75±0.14^{B}	43.81±1.51^{B}	23.82±0.88^{A}	24.81±0.57^{A}	31.90±3.12^{A}	10.30±1.08^{A}	8.50±0.74^{A}
	0.05%	24.67±1.15^{A}	5.59±0.05^{A}	41.39±1.27^{A}	24.93±2.02^{A}	24.52±0.57^{A}	33.54±2.44^{A}	9.30±1.75^{B}	8.68±1.09^{A}
	0.1%	24.22±1.70^{A}	5.60±0.03^{A}	42.05±2.24^{A}	25.29±1.85^{A}	23.80±0.42^{B}	32.67±3.16^{A}	9.13±1.09^{B}	7.09±1.07^{B}
p-value		0.161	<0.05	<0.05	0.128	<0.01	0.232	<0.01	<0.01
Effect of GA	0	24.79±1.60^{a}	5.72±0.17^{b}	42.56±2.05^{a}	24.11±1.27^{a}	24.28±0.62^{a}	34.63±3.09^{a}	10.64±0.68^{b}	8.39±0.54^{a}
	0.01%	23.63±1.92^{a}	5.62±0.04^{a}	41.48±1.52^{a}	24.96±2.07^{a}	24.39±0.58^{a}	32.93±2.46^{a}	9.78±1.15^{b}	8.21±1.85^{a}
	0.02%	23.67±1.97^{a}	5.60±0.06^{a}	43.23±2.05^{a}	24.97±1.78^{a}	24.47±0.83^{a}	30.55±1.46^{a}	8.31±1.20^{a}	7.67±0.77^{a}
p-value		0.241	<0.05	0.107	0.401	0.672	0.726	<0.05	0.093
p-value (interaction DAD*GA)		0.117	<0.01	0.615	0.101	0.065	<0.01	0.113	<0.01

Results with different letters in the same column and in the same section are significantly different at the level p<0.05.
Each value is represented as mean ± SD (n = 9)

5.4.1.2 Colour

Effects of DAD and GA on colour characteristics of deep fried meatballs including lightness (L*), redness (a*) and yellowness (b*) were summarized in Table 5-2. a* and b* significantly affected by DAD, while GA only affected a* ($p<0.05$). Interactions between DAD and GA were observed in L* and b* ($p<0.05$), but not a* ($p>0.05$).

Both DAD and GA could significantly reduce a* in cooked meatballs ($p<0.05$). However, there was no significant effect of interaction between DAD and GA on a* ($p> 0.05$). Redness of meat is usually related to the degree of myoglobin denaturation (Oz & Kotan, 2016). pH in raw meat could affect the performance of myoglobin under heating, and a low pH could increase the denaturation of myoglobin (Claus, 2007). Myoglobin in raw and cooked meat exists in three forms including deoxymyoglobin (purple), oxymyoglobin (red) and metmyoglobin (brown) (Boles & Pegg, 2010). These 3 forms of myoglobin are constantly interconverted, and adding DAD/GA into raw beef mince might break the equilibrium, and led to significant variation in cooked products. In this work, adding DAD and GA caused a decrease in pH, which might increase the denaturation of myoglobin during cooking. Therefore, a lower a* was expected in meatballs added with DAD or GA.

L* was not affected by DAD or GA, but the interaction between DAD and GA ($p<0.01$). Boles and Pegg (2010) stated that pH could affect the charge on the proteins presented in muscle, which resulted in the alteration of space between the fibres of the meat. Horita et al. (2016) showed there was no significant difference of lightness in frankfurters sausages with the addition of garlic powder /commercial garlic oil, compared with control ones. Adding antioxidants could scavenge free radicals and suppresse the formation of brown pigments in Maillard reaction, however, antioxidants such as organosulfides could retard metmyoglobin and decrease L* (Ferna´ndez-Lo´pez et al., 2005). Thus, the lightening effect of suprsessing Maillard reaction could be neutrolized by the effect of reducing metmyoglobin.

Although L* was not significantly affected by DAD or GA, their interaction may modify the physical structure of meat and led to changes in light reflection and

absorption. b* was affected by DAD and the interaction between DAD and GA($p<0.01$), but not GA. The variation of b* may be due to pH, oxidation extent and water activity (Frank, Xu, & Xia, 2014).

5.4.2 Effect of DAD and GA on the formation of HCAs in deep fried beef meatballs

HCAs content, including IQ, MeIQ, MeIQx, 4, 8-DiMeIQx and PhIP in deep fried meatballs with DAD and/or GA at different concentration were expressed ng/g in Table 5-3. DAD and GA had significant effect on individual HCAs compounds and total HCAs ($p<0.05$), expect MeIQx ($p>0.05$). Effect of interaction between DAD and GA was significant on IQ, MeIQ, 4, 8-DiMeIQx and total HCAs ($p<0.05$), but not MeIQx and PhIP.

5.4.2.1 Effect of DAD on the formation of HCAs

Effect of DAD on the formation of IQ, MeIQ, MeIQx, 4, 8-DiMeIQx, PhIP and total HCAs was significant ($p<0.05$). It was also observed that effect of DAD on total HCAs and all 5 individual HCAs compounds except MeIQx were dose-dependent ($p<0.05$). IQ in meatballs decreased from 5.98±5.52 ng/g to 3.90±2.03 ng/g with the concentration of DAD increased to 0.05%. With the concentration of DAD increased to 0.1%, IQ was further reduced to 1.45±1.25 ng/g in meatballs. Tsai, Jenq, & Lee (1996) observed similar results that adding 0.067mM and 0.67mM DAD into boiled pork juice could suppress IQ by 62.2% and 96.7% in pork juice. Similar to IQ, MeIQ decreased from 8.51±8.71 ng/g to 1.80±0.92 ng/g in meatballs with adding DAD up to 0.1%. The effect of pure antioxidant compounds on the formation of MeIQ has been limited documented, most of studies focused on the inhibitory effect of whole food or extracts. MeIQx increased from 0.11±0.42 ng/g to 4.57±2.12 ng/g in meatballs with the addition of 0.05% DAD, but there was no difference observed in MeIQx with DAD at 0% and 0.1% ($p>0.05$). Shin, Strasburg, and Gray (2002c) reported that adding DAD (0.67mM) could reduce MeIQx by 82% in a heated chemical model system containing phenylalanine, creatine and glucose. However, lower concentration of DAD could somehow promote the formation of MeIQx in this study. Further investigation is needed to explore the relationship between MeIQx and DAD.

Does-dependent effect between reducing 4, 8-DiMeIQx and addition of DAD was observed in meatballs. With the increase of DAD from 0% to 0.1%, 4, 8-DiMeIQx decreased from 7.49±8.04 ng/g to 1.08±0.63 ng/g in meatballs with 0.1% DAD ($p<0.05$). Shin et al. (2002a) reported that concentration of 4, 8-DiMeIQx decreased with the increase of DAD concentration from 0.17mM - 1.01mM in cooked ground beef. The inhibitory effect of DAD on the formation of PhIP was also significant ($p<0.05$). Adding 0.05% DAD into meatballs could decrease PhIP from 6.79±4.07 ng/g to 4.54±2.89 ng/g, and with 0.1% DAD achieving the lowest PhIP with 1.01±0.79 ng/g ($p<0.05$). This was consistent with the results reported by Shin et al. (2002a) and Moon and Shin (2013) in heated chemical model system.

Addition of DAD reduced the total HCAs significantly ($p<0.05$), and the inhibitory efficiency of DAD increased with the concentration. Total HCAs was prohibited from 28.58±25.14 ng/g to 21.26±7.84 ng/g and 5.69±4.09 ng/g with the concentration of DAD increased to 0.05% and 0.1%, respectively. The mechanism has been proposed that the organosulfur compounds could prohibit intermediates such as pyrazine and pyridine by interfering early stage of Maillard reaction in both chemical heated model system and meat system (Dong, Lee, & Shin, 2011). Organosulfur compounds could get involved with Maillard reactions through the following two routes: (1) trapping free radicals through thiol group; (2) competing with substrate in Maillard reactions by direct interaction with glucose (Meurillon & Engel, 2016; Shin et al., 2002c). Friedman and Molnar-Perl (1990) stated that organosulfur compounds could interfere with Maillard reaction through reactions between thiol group and aldehydes on reducing sugar, to generate carbinol sulphide and thioketal, instead of Schiff base and Amadori rearrangement to block further reaction. Tsai et al. (1996) explored the inhibitory mechanism of organosulfur compounds including DAD, on HCAs by determining the amount of Maillard Reaction Products (MRPs), such as pyridines, pyrazines, thiophenes and thiazoles, the intermediates during in the formation of HCAs. They confirmed that the amount of MRPs was significantly reduced with adding DAD into the system.

Table 5-3: Effects of DAD (0, 0.05% and 0.1%), GA (0, 0.01% and 0.02%) and their interaction on the formation of HCAs in deep fried beef meatballs

		IQ (ng/g)	MeIQ (ng/g)	MeIQx (ng/g)	4, 8-DiMeIQx (ng/g)	PhIP (ng/g)	Total HCAs(ng/g)
Effect of DAD	0	5.98±5.52^{C}	8.51±8.71^{C}	0.11±0.42^{A}	7.49±8.04^{C}	6.79±4.07^{C}	28.58±25.14^{C}
	0.05%	3.90±2.03^{B}	2.76±1.38^{B}	4.57±2.12^{B}	4.56±1.60^{B}	4.54±2.89^{B}	21.26±7.84^{B}
	0.1%	1.45±1.25^{A}	1.80±0.92^{A}	1.10±0.98^{A}	1.08±0.63^{A}	1.01±0.79^{A}	5.69±4.09^{A}
p-value		<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Effect of GA	0	7.07±5.00^{a}	8.51±8.49^{a}	2.59±2.83^{a}	8.40±7.43^{b}	6.89±4.90^{b}	33.45±23.22^{b}
	0.01%	2.78±1.35^{b}	2.76±1.97^{b}	1.97±2.45^{a}	2.32±1.80^{a}	3.13±1.87^{a}	12.96±6.12^{a}
	0.02%	1.48±1.28^{b}	1.80±1.91^{b}	1.11±1.75^{a}	2.40±2.23^{a}	2.32±1.94^{a}	9.12±7.50^{a}
p-value		<0.05	<0.05	0.07	<0.05	<0.05	<0.05
p-value (interaction DAD*GA)		<0.01	<0.01	0.476	<0.01	0.31	<0.01

Results with different letters in the same column and in the same section are significantly different at the level p<0.05.
Each value is represented as mean ± SD (n = 9).

5.4.2.2 Effect of GA on the formation of HCAs

Effect of GA on the formation of all individual and total HCAs except MeIQx was significant ($p<0.05$). Adding 0.01% GA could significantly inhibit IQ, MeIQ, 4, 8-DiMeIQx, PhIP and total HCAs ($p<0.05$). However, the inhibitory efficiency of GA on the formation of HCAs did not increase with concentration from 0.01% to 0.02% (Table 5-3).

IQ declined from 7.07±5.00 ng/g to 2.78±1.35 ng/g in meatballs with the supplementation of 0.01% GA ($p<0.05$), however, increasing GA to 0.02% (1.48±1.28 ng/g) had no enhancement of inhibitory efficiency. The inhibitory efficiency of phenolic acid including gallic acid was documented by Ahn and Grün (2005), who found that IQ was reduced by more than 50% in cooked ground beef with addition of 1.0% Activin (grape seed extract). Similar trend was also found in MeIQ, MeIQ decreased greatly from 8.51±8.49 ng/g to 2.76±1.97 ng/g in meatballs when 0.01% GA was added, but no further reduction in MeIQ was observed when GA addition increased to 0.02%. Control beef meatballs fried at 225ºC had MeIQ with 2.66 ng/g, but MeIQ in meatballs was completely inhibited when 1% black pepper containing 3.8-5.1 mg/g GA equivalent phenolic compounds was added (Embuscado, 2015; Oz & Kaya, 2011a). Balogh et al. (2000) reported that adding oleoresin rosemary (1% and 10%) containing phenolic compounds into fried ground beef could achieve 47.9-87% reduction of MeIQ. The presence of GA, regardless of its concentration, had no significant inhibitory effect on MeIQx in meatballs. However, previous research showed that phenolic acids, which have similar chemical structure to gallic acid, such as ferulic acid and p-coumaric acid could inhibit 100% MeIQx in chemical heated model (Zeng et al., 2016). Although chemical model could be used to understand the formation of HCAs in principle, the complexity of real meat system including the presence of lipids, amino acids and metals may interfere with Maillard reaction and limit the effect of phenolic acids.

Compared with samples without GA (8.40±7.43 ng/g), sample with 0.01% GA reduced 4, 8-DiMeIQx formation (2.32±1.80 ng/g) significantly ($p<0.05$), but increasing level of GA to 0.02% did not reduce the formation of 4, 8-DiMeIQx

(2.40±2.23 ng/g) further in cooked beef meatballs ($p>0.05$). PhIP was reduced from 6.89±4.90 ng/g to 3.13±1.87 ng/g and 2.32±1.94 ng/g in meatballs added with 0.01% and 0.02% GA ($p<0.05$). It has been reported that polyphenol could inhibit PhIP formation through directly trapping phenylacetaldehyde, a key intermediate product of PhIP (Cheng, 2007). They also further confirmed that hydroxyl group on benzene ring in polyphenol could form adducts with phenylacetaldehyde (Cheng et al., 2008). In addition, Moon and Shin (2013) reported that the presence of epigallocatechin gallate (EGCG) in chemical heated model resulted in high energy requirement for initiating the reaction to form PhIP, as a result, it might delay the formation of PhIP. In addition, they also found that the inhibitory efficiency of EGCG on the formation of PhIP increased with concentration, where 1000ppm EGCG achieved the highest inhibition on PhIP.

Effect of GA on the formation of total HCAs was significant ($p<0.05$). Deep fried beef meatballs without GA had total HCAs 33.45±23.22ng/g, but significant reduction in total HCAs (12.96±6.12 ng/g) was achieved in meatballs when 0.01% GA was added ($p<0.05$). There was no difference in total HCAs between samples added with 0.01% GA and 0.02% GA ($p>0.05$). The inhibitory effect of phenolic compounds on reducing HCAs has been explained by free radical scavenging properties (Cheng et al., 2007a; Dong et al., 2011; Meurillon & Engel, 2016). Kato et al. (1996) proposed that unstable pyrazine cation radicals and carbon-centred radicals could be eliminated with the addition of phenolics in chemical heated model.

5.4.2.3 Effect of interaction between DAD and GA on the formation of HCAs

Effect of interaction between DAD and GA on the formation of IQ, MeIQ, 4, 8-DiMeIQx and total HCAs was significant ($p<0.01$). Since IQ, MeIQ and 4, 8-DiMeIQx were all affected by the interaction between DAD and GA, they could contribute to interactive effect on total HCAs. Figure 5-1 illustrated the interaction between DAD and GA on total HCAs. Without DAD, adding 0.01% GA could greatly reduce total HCAs ($p<0.05$), but the inhibitory rate of GA seemed remained same even concentration increased to 0.02% ($p>0.05$).

With the addition of 0.05% DAD, it did not enhance inhibitory effect of GA at both concentrations (0.01% and 0.02%). Total HCAs could be reduced by almost 50% by adding 0.05% DAD, and with the addition of 0.01% / 0.02% GA, it could further decrease total HCAs ($p<0.05$). Synergistic effect was observed between GA and DAD at high concentration in reducing formation of total HCAs. Previous studies have reported that single or mixed antioxidants inhibit the formation of HCAs through various routes (Oz and Cakmak, 2016). Since organosulfur compounds could compete with glucose in Maillard reaction to block the pathway of forming pyrazines and pyridines (Kato et al., 1998), while polyphenols could act as free radical scavengers to suppress the reaction between pyridine radicals and creatine (Cheng et al., 2007a; Vitaglione & Fogliano, 2004). As a result, these two types of compounds could work synergistically in Maillard reaction to reduce HCAs.

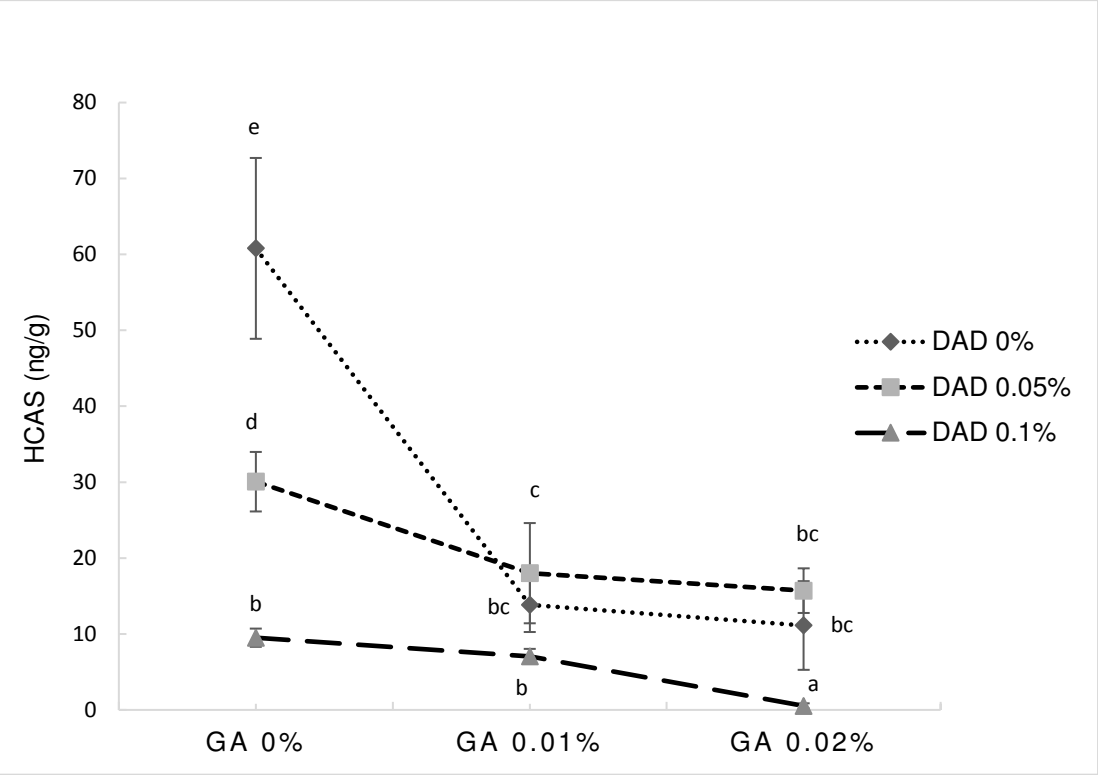

Figure 5-1: Interaction between DAD (0%, 0.05% and 0.1%) and GAE (0%, 0.01% and 0.02%) on the formation of total HCAs in cooked beef meatballs[1].

[1]Results with different letters in the figure are significantly different at the level $p<0.05$.

Predicted total HCAs = 52.30 - 22.34 GA - 21.62 DAD + 10.17 GA * DAD (Adjusted R^2= 0.74, $p<0.05$)

A regression model was fitted to reveal that the contribution of DAD, GA and interaction between DAD and GA to the formation of total HCAs ($p<0.05$). Regression coefficients of DAD (-21.62) and GA (-22.34) were similar, which indicated that both DAD and GA contribute almost equally to reduce the total amount of HCAs. The coefficient of interaction between DAD and GA was positive, but in consideration of the low concentration level for both compounds, the interaction effect was negligible in relation to the formation of total HCAs. The inhibitory effect of both DAD and GA could be associated with antioxidant capability, which was usually measured by TEAC assay with the free radical scavenging mechanism of electron transfer (radical reduction) and ORAC assay with that of hydrogen atom transfer (radical quenching) (Shahidi & Ambigaipalan, 2015).

Table 5-4 showed the antioxidant capacity of DAD solution (0, 0.05% and 0.1% w/w in methanol), GA solution (0, 0.01% and 0.02% w/w in methanol) and their combination solution. In TEAC assay, the highest TEAC values was found in solution with 0.02% GA inclusion (25.41-29.31 µmol of Trolox/ 100g), while lower TEAC values were found in solution with 0.05% DAD (1.38±0.29 µmol of Trolox/ 100g) and 0.1% DAD (9.87±0.11 µmol of Trolox/ 100g). Low TEAC value of DAD (1.7 µmol of Trolox equivalent) was also found by Kim et al. (2006). Tabart et al. (2009) reported TEAC value of GA dissolved in ethanol was approximately 78 µmol of Trolox/ 100g, which was twice higher than the result in this work. Samples containing GA with higher TEAC indicated they had stronger scavenging ability through transferring electron to reduce radicals. Leopoldini, Marino, Russo, and Toscano (2004) stated that gallic acid could stabilize free radicals due to the presence of the electron-donating OH group in the ortho position and the electron-withdrawing −COOH group in the para position.

Table 5-4: TEAC and ORAC values of DAD solution (0, 0.05% and 0.1% w/w in methanol), GA solution (0, 0.01% and 0.02% w/w in methanol) and solution of their combination

Treatments		TEAC (µmol of Trolox/ 100g)	ORAC (µmol of Trolox/ g)
T2	GA 0.01%	15.28 ± 0.57^{c}	39.45 ± 23.40^{a}
T3	GA 0.02%	26.86 ± 0.58^{d}	199.44 ± 97.23^{bcd}
T4	DAD 0.05%	1.38 ± 0.29^{a}	123.67 ± 62.99^{abc}
T5	DAD 0.1%	9.87 ± 0.11^{b}	371.01 ± 164.76^{e}
T6	DAD 0.05%+ GA0.01%	8.73 ± 1.07^{b}	108.08 ± 36.00^{ab}
T7	DAD 0.05%+ GA 0.02%	26.72 ± 0.29^{d}	186.02 ± 106.11^{bcd}
T8	DAD 0.1%+ GA 0.01%	25.41 ± 0.29^{d}	332.81 ± 125.01^{de}
T9	DAD 0.1%+ GA 0.02%	29.31 ± 0.39^{d}	287.34 ± 83.34^{cde}

Results with different letters in the same column are significantly different at the level $p<0.05$.
Each value is represented as mean ± SD (n = 3)

In ORAC assay, the highest value was found in sample with 0.1% DAD (371.01± 164.76 µmol of Trolox/g), followed by the mixture of 0.1% DAD and 0.01% GA (332.81±125.01 µmol of Trolox/g), while 0.01% GA showed the lowest ORAC with 39.45±23.40 µmol of Trolox/g. Samples containing DAD with high ORAC values could act as strong radical scavenger through hydrogen transfer. Pyrazine and pyridine radicals have been recognized as key intermediates for the formation of HCAs (Gibis, 2016; Vitaglione & Fogliano, 2004). According to Adams et al. (2001), the interchange between thiol and disulfide group could take place in meat system. The transformation of DAD into thiol could provide hydrogen atom to the unstable pyrazine cation radical and turn radical into non-radical species to prohibit the formation of HCAs (Kikugawa, 1999).

The TEAC and ORAC values of the two compounds indicated that GA might mainly scavenge free radicals through electron transfer, while DAD might mainly act as hydrogen donor to reduce the radicals during formation of HCAs. However, in order to have a comprehensive profile of antioxidant capacity of

these antioxidants, assays with various free radicals, such as OO^{-}, $OH\cdot$, H_2O_2 and $ONOO^{-}$ are required to specify antioxidant capacity of these compounds.

5.4.3 Effect of DAD and GA on the formation of PAHs in deep fried beef meatballs

Concentration of PAHs including BaA and BaP in deep fried meatballs added with GA and DAD were listed in Table 5-5. The dominating compound of PAHs was BaA in most samples. Temperature plays a major role on the formation of PAHs, the higher cooking temperature (>300 ºC), the more aromatic rings could be generated within PAHs structure (Kubátová et al., 2011). In this work, a relatively low frying temperature at 180ºC was used for cooking beef meatballs, therefore, more BaA with 4 fused rings, instead of BaP with 5 rings was detected in the samples. The EU Commission Regulation EC No 835/2011 states that the maximum allowance for BaP in cooked and smoked meat is 2 μg/kg in European countries. The highest level of BaP was 0.35±0.46 ng/g in this study, which was within the safety range. The sum of BaA and BaP in all samples was relatively low (0.11-1.19 ng/g), compared with Farhadian, Jinap, Abas, and Sakar (2010) and Farhadian et al. (2012), who reported that 4.51-4.46 ng/g BaP in grilled beef. The variation might be explained by the difference in cooking time and cooking method between the two studies. Addition of DAD, GA and interaction between GA and DAD affected the formation of BaA, BaP and total PAHs ($p<0.05$).

5.4.3.1 Effect of DAD on the formation of PAHs

DAD could significantly reduce both BaP and BaA, and the reduction increased with the concentration ($p<0.05$). Adding 0.05% DAD could reduce BaP from 0.34±0.47 ng/g in control samples to 0.13±0.11 ng/g, while no BaP was detected in samples with 0.1% DAD. Similarly, adding 0.05% DAD could reduce BaA from 0.85±0.95 ng/g in control samples to 0.24±0.06 ng/g, while adding 0.1% DAD could completely inhibit formation of BaA in meatball. Total PAHs could be inhibited from 1.19±1.33 ng/g to 0.38±0.16 ng/g in cooked meatballs with 0.05% DAD, and totally reduced by adding 0.1% DAD ($p<0.05$). Impact of pure compounds on the formation of PAHs in cooked meat has not been well documented. Previous researches have focused on the effect of

marinades on forming PAHs in cooked meat, and results were comparable to this study. El-Badry (2010) found that BaP was reduced from 3.84ng/g to 1.56 ng/g in grilled chicken when chicken was pre-treated using garlic paste, which was rich of DAD. Similarly, Janoszka (2011) reported that garlic (15%) could significantly reduce BaA by 50% and BaP by 71% in pan-fried pork collar. PAHs with five or more rings, such as BaP, can be formed via the intramolecular cyclization (Kubátová et al., 2011). During deep frying, alkenyl radicals containing double bond and unpaired electron can be formed in pyrolysis. The cyclization could be achieved by the movement of unpaired electron via hydrogen atom abstraction along the carbon chain with increasing temperature (Kubátová et al., 2011). In Table 5-4, high ORAC value of DAD inclusion mixture showed the strong hydrogen transferring ability of DAD at high concentration. The cyclization in forming PAHs might be interfered by DAD through hydrogen donation. In addition, DAD could also enhance the decomposition of fatty acids hydroperoxides, in order to terminate the propagation of free radicals (Shah, Bosco, & Mir, 2014).

Table 5-5: Effects of DAD (0, 0.05% and 0.1%), GA (0, 0.01% and 0.02%) and their interaction on the formation of PAHs in deep fried beef meatballs

		BaP (ng/g)	BaA (ng/g)	Total PAHs (ng/g)
Effect of DAD	0	0.34 ± 0.47^{B}	0.85 ± 0.95^{B}	1.19 ± 1.33^{B}
	0.05%	0.13 ± 0.11^{A}	0.24 ± 0.06^{A}	0.38 ± 0.16^{A}
	0.1%	Nd	Nd	Nd
p-value		<0.05	<0.05	<0.05
Effect of GA	0	0.35 ± 0.46^{b}	0.78 ± 1.00^{b}	1.13 ± 1.37^{c}
	0.01%	0.12 ± 0.10^{a}	0.20 ± 0.16^{a}	0.32 ± 0.25^{b}
	0.02%	Nd	0.11 ± 0.09^{a}	0.11 ± 0.09^{a}
p-value		<0.05	<0.05	<0.05
Interaction (DAD*GA)		<0.01	<0.01	<0.01

Results with different letters in the same column and in the same section are significantly different at the level $p<0.05$.
Each value is represented as mean ± SD (n = 9).

5.4.3.2 Effect of GA on the formation of PAHs

The efficiency of GA was comparable with DAD on reduction of BaP (Table 5-5). BaP was decreased from 0.35±0.46ng/g in control to 0.12±0.10 ng/g in meatballs with 0.01% GA and it was inhibited completely in samples with 0.02% GA. Total PAHs decreased significantly with the increasing concentration of GA in deep fried meatballs ($p<0.05$). Food extracts contain polyphenols, such as tea extract and spice powder, which equivalent to 44-228 mg GA/g was found effective reduction of carcinogenic PAHs in cooked meat (Lianh et al., 2015; Salah et al., 1995). Park et al. (2017) applied green tea and yerba mate tea marinade (0.25-1.0%) on grilled pork belly, and found BaP was reduced by 12.9% and 31.5%. BaA could be inhibited from 0.78±1.00 ng/g to 0.20±0.16 ng/g in meatballs with 0.01% GA. Black beer, rich in prenylflavonoids, has also been reported to reduce BaA and BaP in grilled pork (Viegas et al., 2014). They also found a weak correlation between radical scavenging ability of beers and the reduction of total PAHs. However, increasing GA from 0.01% to 0.02% did not significantly reduce BaA ($p>0.05$). Similar result was found in Min, Patra, and Shin (2017), they reported that BaA and BaP were reduced by adding 100μg/kg EGCG into heated meat model system, and no further reduction with EGCG increasing from 200 to 300μg/kg.

Lipid/fat pyrolysis could contribute to the formation of PAHs through their degradation products with heat (Chung et al., 2011; Purcaro, Moret, & Conte, 2013). With the presence of heat, oleate in frying medium and beef tallow can be degraded into short-chain alkanes, alkenes, aldehydes, ketones and several hydroperoxides. They could be broken down to form cyclic compounds, such as cyclohexene, then further oxidized to form benzene to provide the skeleton structure of PAHs, and/or in turn reacts with C4 compound for propagation of fused rings (Chen & Chen, 2001; Kubátová et al., 2011; Marikkar et al., 2002). Shah et al. (2014) demonstrated the protective effect of polyphenol compounds on oxidation in meat products through free radical scavenging, which might prevent severe fatty acids oxidation during cooking, in order to prevent cyclization of degradation products and accumulation of more fused rings of PAHs.

5.4.3.3 Effect of interaction between DAD and GA on the formation of PAHs

Effect of interaction between DAD and GA on the formation of BaA, BaP and total PAHs was significant ($p<0.01$) (Table 5-5). Addition of 0.01% GA significantly inhibited total PAHs compared with control meatballs (with no DAD) ($p<0.05$), while addition of 0.05% DAD did not enhance inhibition on total PAHs. Total PAHs could be inhibited completely by adding 0.1% DAD, and combination with the mixture of GA had no further effect on reducing PAHs. There was no synergistic effect observed between DAD and GA on the inhibition of PAHs, although enhancing effect between spice and garlic was mentioned by El-Badry (2010). They found that spices (cumin, black pepper and coriander) and garlic paste could inhibit 95% and 70% BaP respectively, and the mixture of them could completely inhibit BaP in grilled chicken.

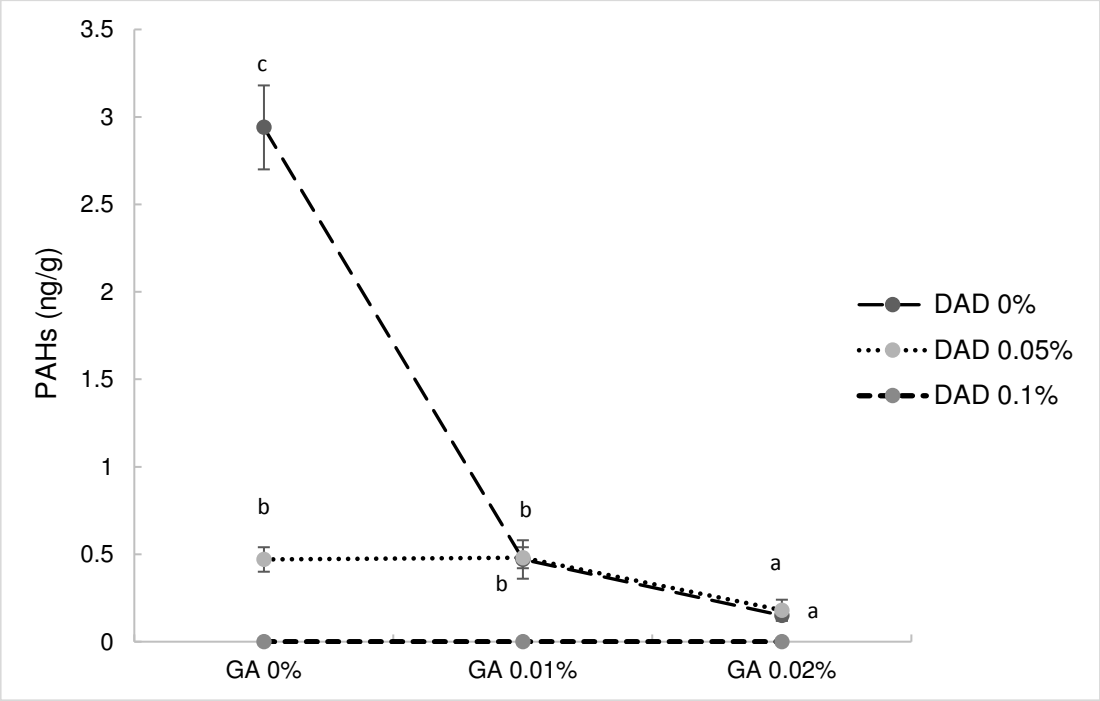

Figure 5-2: Interaction between DAD (0%, 0.05% and 0.1%) and GAE (0%, 0.01% and 0.02%) on the formation of total PAHs in cooked beef meatballs[1].

[1]Results with different letters in the figure are significantly different at the level $p<0.05$.

Predicted model conducted from statistical analysis revealed the contribution of DAD, GA and interaction between DAD and GA to total PAHs (adjusted $R^2 = 0.78$, $p<0.05$). Regression coefficients of DAD, GA and interaction were

presented below. Similar to the regression model of HCAs, both DAD and GA played equal role on reducing total PAHs, while interaction between DAD and GA was positively contributed to total PAHs.

Predicted total PAHs = 2.32 – 1.21 GA – 1.29 DAD + 0.696 GA * DAD (Adjusted R^2= 0.78, $p<0.05$)

The pathway of forming PAHs has been proposed that small organic compounds including amino acids, such as aspartic acid and lipids are partially broken down into smaller unstable fragments under heat, which could recombine to form PAHs through free radicals (Sharma, Chan, & Hajaligol, 2006). The formation of PAHs can be initiated via fat/oil and/or hydrocarbon incomplete combustion, followed by intramolecular cyclization path, where cyclopentanes were formed primarily by folding of alkenyl radicals, such as monoalkyl- and ortho-methyl-substituted six-membered monocyclic radicals with terminal double bonds (e.g., RH2·-C-C-C=C) through endo cyclization. Phenolics could reduce PAHs through prohibiting lipids degradation and eliminating fatty acids hydroperoxides (Min et al., 2017), and organosulfur might prevent cyclization by stabilizing free radicals (Janoszka, 2011). Thus, organosulfur and polyphenol might block or interfere with the pathway of forming PAHs by different approaches.

5.4.4 Effect of DAD and GA on lipid oxidation and protein oxidation in cooked meatballs

Table 5-6 showed TBARS and protein carbonyl values of control cooked meatballs and meatballs added with DAD and GA. Adding DAD, GA and interaction between DAD and GA could significantly affect lipid oxidation and protein oxidation in deep fried beef meatballs ($p<0.05$).

Table 5-6: Effects of DAD (0, 0.05% and 0.1%), GA (0, 0.01% and 0.02%) and their combination on lipid oxidation (TBARS) and protein oxidation (protein carbonyl) in deep fried beef meatballs.

		TBARS (mg MDA/kg)	Protein carbonyl (nmol/mg protein)
Effect of DAD	0	0.61 ± 0.15^{B}	5.26 ± 0.81^{B}
	0.05%	0.66 ± 0.05^{A}	1.43 ± 0.17^{A}
	0.1%	0.58 ± 0.04^{B}	0.92 ± 0.34^{A}
p-value		<0.05	<0.05
Effect of GA	0	0.71 ± 0.09^{c}	3.00 ± 2.49^{b}
	0.01%	0.62 ± 0.03^{b}	2.35 ± 1.77^{a}
	0.02%	0.53 ± 0.08^{a}	2.25 ± 1.94^{a}
p-value		<0.05	<0.05
Interaction (DAD*GA)		<0.01	<0.01

Results with different letters in the same column and in the same section are significantly different at the level $p<0.05$.
Each value is represented as mean ± SD (n = 9).

It was noticed that 0.05% DAD could significantly increase TBARS value from 0.61±0.15 mg MDA/kg in samples without DAD to 0.66±0.05 mg MDA/kg, while TBARS decreased to 0.58±0.04 mg MDA/kg when concentration of DAD increased to 0.1%. Yin and Cheng (2003) found out that adding 5-20μM DAD showed moderate inhibitory efficiency on lipid oxidation in raw ground beef. The antioxidant effect of organosulfur compounds has been proved in vivo through the activation and modification of several enzymes, such as 3-hydroxy-3-methylglutaryl- CoA reductase, glutathione-s-transferase and catalase (Borek, 2001). However, GA showed superior retardation on lipid oxidation in deep fried meatballs, compared with DAD. With the increasing GA from 0.01% to 0.02%, TBARS was significantly reduced from 0.71±0.09 mg MDA/kg (no GA) to 0.62±0.03 mg MDA/kg and 0.53±0.08 mg MDA/kg, respectively. GA has showed inhibitory effect on TBARS in vitro metal-catalysed oxidation of myofibrillar proteins, which was owing to its hydroxyl radical scavenging properties (Utrera & Estévez, 2013). TEAC values of GA and mixture contained GA in Table 5-4 could provide its strong antioxidant capacity. There was significant effect of interaction between DAD and GA on

lipid oxidation ($p<0.01$). With the presence of divalent metal ions or reducing compounds, GA could be formed into gallic phenoxyl radical through an autoxidation process of GA. The gallic phenoxyl radicals could interact and neutralise other radicals such as ·OH (Utrera & Estévez, 2013). The addition of DAD into GA might provide and enhance the reducing environment for GA, which could partially explain their interaction. Yang et al. (2011) reported that adding mixture of 0.1% garlic and 0.5% onion into raw beef could significantly reduce TBARS during storage, compared with 0.1% garlic or 0.5% onion addition in beef. From the aspect of sensory, cooked meatballs generated moderate garlic aroma, which was mainly attributed to strong intensity of DAD (gallic acid is odourless). Yang et al. (2011) reported that addition of garlic (0.1%) to ground beef produced a garlic aroma and flavour after cooking, it could also mask warmed over flavour.

The inhibitory effect of DAD and GA on protein oxidation in cooked meatballs was also showed in Table 5-6. Addition of both DAD and GA could significantly reduce protein oxidation, while increasing concentration of both antioxidants did not further reduce protein oxidation in cooked meatballs ($p>0.05$). The effect of interaction between DAD and GA on protein oxidation was significant ($p<0.01$). Protein oxidation can be initiated by transition metals, myoglobin and oxidized lipids, especially protein carbonylation (Jia et al., 2012). Iron released from myoglobin during the cooking could react with proteins and act as an oxidizing compound. Yin, Hwang, and Chan (2002) found organosulfur compounds including DAD had strong iron chelating ability, which could contribute to preventing protein oxidation. They also reported that antioxidant activity of DAD was enhanced at lower pH. On the other hand, addition of DAD could cause thiol/disulfides interchange in meat, and generate protein thiol and protein-protein cross-links (Singh & Whitesides, 1993). These protein thiols and cross links could be readily oxidized and lead to severe protein oxidation (Nieto et al., 2013). Therefore, further validation between organosulfides and protein oxidation in meat should be conducted in the future. Although limited literature has focused on pure antioxidant compounds on protein oxidation, effect of plant extracts that contain diverse types of antioxidants on protein oxidation in meat products has been well

explored, including strawberry and blackberry extract (Ganhão, Morcuende, & Estévez, 2010), black currant extract (Jia et al., 2012) and white grape extract (Jongberg, Skov, Tørngren, Skibsted, & Lund, 2011). Researchers proposed that phenolic compounds inhibited myofibrillar protein oxidation via metal chelating and radical scavenging. They could also protect proteins from attracting free radicals by covalent and non-covalent interactions with proteins (Jia et al., 2012).

5.5 Conclusions

Both DAD and GA could reduce significantly total HCAs and PAHs in deep fried beef meatballs, which could be attributed to their antioxidant capacities measured in TEAC and ORAC assay. Regression models revealed that DAD and GA had similar inhibitory efficiency on the formation of HCAs and PAHs. Synergistic effect of DAD and GA was observed on the formation of HCAs, but not on PAHs. Addition of DAD and GA could also prohibit lipid oxidation and protein oxidation in cooked meatballs, which could potentially help to extend shelf life of meatballs.

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Chapter 6. General discussion and future work

6.1 General discussion

Previous literature contributes to the understanding of HCAs and PAHs formation, characterization and quantification in meat. However, some aspects are still inconclusive due to the complexity in reaction system, such as formation of precursors and inconsistent results for causal relationship between dietary intake of meat and cancer risk (Ferguson, 2010; Gibis, 2016). In order to have a comprehensive evaluation on health risk associated with meat intake, it is necessary to determine the occurrence of HCAs and PAHs in a wide range of meat products for precise assessment about the health risk. Estimation of dietary intake of HCAs and PAHs in worldwide has been reported in US (Keating & Bogen, 2001; Layton et al., 1995), Switzerland (Zimmerli et al., 2001), Sweden (Olsson & Pickova, 2005), Spain (Busquets et al., 2004), Singapore (Salmon et al., 2006), Malaysia (Jahurul et al., 2010) and Japan (Kobayashi et al., 2002). However, few researches have focused on the occurrence of HCAs and PAHs in RTE meat products on UK's market. Therefore, the level of HCAs and PAHs in cooked meat and fish products in UK were determined (Chapter 2), and risk assessment of dietary exposure to HCAs and PAHs for UK consumers were also carried out based on diet survey.

Results from market research (Chapter 2) showed that most RTE meat products would not pose any health risk. However, increasing intake of chargrilled chicken and ham could increase risk of colorectal adenoma and breast cancer because the level of IQ and MeIQ in these meat products were much higher than the safety thresholds for cancer risk according to epidemiological studies. From a societal perspective, it is important to prevent cancer occurrence rather than receive treatments, as the low health care cost would be beneficial to individuals and government (Patel, 2015). Thus, it is necessary to explore dietary prevention strategies in meat processing to reduce the exposure of HCAs and PAHs.

Altering cooking practice has been popular strategy to reduce the formation of HCAs and PAHs for meat processors and researchers, but effects of meat composition and ingredients on the formation of the carcinogens are not consistent, especially food ingredients have evolved greatly in past decades. In Chapter 3, impact of fat replacement with vegetable oil on formation of HCAs and PAHs was evaluated. Result showed that replacing animal fat with vegetable oils in the formulation could not only improve fatty acids profile in the final meat products, but also help to inhibit the formation of HCAs and PAHs in meat products. The inhibitory effect of vegetable oil was attributed to antioxidants, such as tocopherols and polyphenols within the oils. Different type of antioxidants might have different function in prohibiting the formation of HCAs. Olive oil and sunflower oil could inhibit formation of imidazoquinolines, while grape seed oil could completely inhibit imidazoquinoxalines.

Spices are common ingredients used in meat products. Research showed spices had great potential on inhibiting the formation of carcinogen. Inhibitory effect of 6 common spices including garlic, onion, paprika, red chilli, black pepper and ginger powder on HCAs and PAHs formation in different meat system was assessed in Chapter 4. Results showed that ginger and black pepper containing high level of phenolic had the highest inhibitory efficiency on HCAs, whereas garlic and onion with organosulfur compounds had the lowest efficiency. To further understand the inhibitory efficiency of the two types of antioxidants on carcinogens formation and related mechanisms, gallic acid presented as principle compound for phenolics and diallyl disulfide for organosulfides were applied to meat model system. Results indicated that gallic acid (0-0.02%) had same inhibitory efficiency with diallyl disulfide (0-0.1%) on the formation of both HCAs and PAHs. Synergistic effect between diallyl disulfide and gallic acid was observed on reducing HCAs, but not on PAHs.

In general, low level of HCAs and PAHs are detected in meat products prepared by boiling, steaming, or microwaving. However, frying, grilling/barbecuing and oven roasting at high temperature with long cooking

time usually generate high level of HCAs and PAHs in cooked meat products, which might lead to high cancer risk (Chung et al., 2011; El-Badry, 2010; Farhadian et al., 2010). Effect of pan-frying and grilling/barbequing on generating HCAs and PAHs has been studies previously (Alomirah et al., 2011; Gibis, Kruwinnus, & Weiss, 2015; Gibis & Weiss, 2015; Viegas et al., 2014), while limited attention has been given on oven roasted and deep-fried meat products. Therefore, this project focused on meat products prepared by oven roasting and deep-frying. Products and cooking process in Chapter 3, 4 and 5 followed home cooking recipes and instructions (Rodríguez-Carpena, Morcuende, & Estévez, 2012; Zeng et al., 2014). The amount of HCAs and PAHs were determined respectively up to 141 ng/g and 3.8 ng/g in roasted pork patties (Chapter 3), up to 60 ng/g and 3.9 ng/g in deep-fried beef meatballs (Chapter 4 & 5) and up to 50 ng/g and 3.7 ng/g in deep-fried chicken meatballs (Chapter 4). Total HCAs in above samples was generally higher than previous results (Iwasaki et al., 2010; Oz & Kaya, 2011a; Zeng et al., 2017), which could be attributed to the sampling procedures (detailed explanation in Chapter 3). Among all the observed HCAs, 4, 8-DiMeIQx was frequently detected in almost all pork, beef and chicken samples in Chapter 3, 4 & 5, followed by IQ, MeIQx and PhIP. In literature, PhIP and MeIQx are the compounds mostly studied in cooked beef, fish and poultry (Kobayashi et al., 2002; Layton et al., 1995; Oz & Kaya, 2011a). However, some other aminoimidazoarenes, such as 2-Amino-1,6-dimethyl-furo[3,2-e]imidazo[4,5-b]pyridine (IFP), 2-Amino-1,5,6-trimethylimidazo[4,5-b]pyridine (1,5,6 TMIP) and 2-Amino-3,5,6-trimethylimidazo[4,5-b]pyridine (3,5,6 TMIP) have been paid limited attention (Gibis, 2016). Therefore, it is recommended to consider the occurrence of these HCAs in order to have a more inclusive HCAs profile in meat products. Total PAHs were lower than those in grilled/barbequed meat products in literature (Alomirah et al., 2011; Chung et al., 2011; Farhadian et al., 2010; Purcaro, Moret, & Conte, 2013; Wongmaneepratip & Vangnai, 2017). The difference in sampling procedures, analytical methods, and cooking conditions for maximizing the formation of HCAs and PAHs without considering consumer acceptance might contribute to the variation (Oz and Kaya, 2010; Shabbir et al., 2015). The obtained data could provide

information about occurrence of HCAs and PAHs with a wider range of meat products cooked by domestic procedures.

Impact of antioxidants on the formation of PAHs in cooked meat has been investigated by few researchers including Farhadian et al. (2012), El-Badry (2010), Janoszka (2011) and Viegas et al. (2014). They proposed that the reduction of PAHs might be associated with free radical scavenging, as the formation of PAHs involves with forming free radicals. Correlation analysis between antioxidants and PAHs was conducted in Chapter 3 and 4 to further explore the inhibitory mechanism. The correlation between TEAC of oils and PAHs was not observed in Chapter 3 ($r=0.301$, $p>0.05$). However, significant negative correlation between TEAC of spices and PAHs was disclosed in Chapter 4 ($r= -0.647$, $p<0.05$). The discrepancy might be explained by the antioxidant capacity between oil and spices. Oils had TEAC level up to 0.75 μmol Trolox/100g, while spices had much higher TEAC with range of 6.55-13.57 μmol Trolox/100g. The high TEAC level in spices was strong enough to show the inhibitory efficiency on the formation of PAHs through scavenging free radicals, whereas TEAC of oils might be too low to reveal the potential correlation.

Several studies have mentioned that lipid oxidation could interact with protein oxidation and Maillard reaction (Wongmaneepratip & Vangnai, 2017; Zamora & Hidalgo, 2007). However, limited papers have reported the association between lipid/protein oxidation and the formation of HCAs and PAHs during cooking. Only Zamora, Alcón, and Hidalgo (2012) reported that lipid oxidation was significantly associated with the formation of PhIP. In this project, both lipid oxidation and protein oxidation were found interacted with the formation of HCAs, while lipid oxidation played a predominant role. However, inconsistent correlation between lipid oxidation and formation of PAHs was observed in different meat products from Chapter 3 and Chapter 4. In Chapter 3 (fat replacement), more interfering factors including fatty acid profile, antioxidants in fat replaced meat system may affect the association between PAHs and lipid oxidation (Wongmaneepratip & Vangnai, 2017; Min, Patra, & Shin, 2017). Oils/lipids with high linoleic acid (C18:2) content could promote

the formation of PAHs and cause severe lipid oxidation, compared with those with high oleic acid (C18:1) content (Chen & Chen, 2001). In addition, heating stability of tocopherols was stronger in lipids/oils with high linoleic acid content than with oleic acid during prolonged cooking time (Rossi, Alamprese, & Ratti, 2007), which could prevent lipid oxidation at different extent. Thus, unclear correlation was observed owing to the complexity of the whole system. Therefore, the complicated effect caused by promoting factor and inhibitory factor would lead to null correlation observed between lipid oxidation and PHAs in Chapter 3.

To validate the regression model of predicting HCAs and PAHs established in Chapter 5, data of garlic and ginger obtained in Chapter 4 were used to verify the regression equations (see below).

Predicted total HCAs (ng/g) = 52.30 - 22.34 GA - 21.62 DAD + 10.17 GA * DAD

Predicted total PAHs (ng/g) = 2.32 - 1.21 GA - 1.29 DAD + 0.696 GA * DAD

For example, the average amount of DAD was 390 μg/g and phenolic content was 0.9mg/g GA equivalent in garlic (Yu, Wu, & Chen, 1989). Thus, 5g garlic powder would contain 1950μg DAD and 4.5mg GA equivalent phenolic. According to the regression model, predicted HCAs and PAHs were 40.47 ng/g and 1.68ng/g respectively (calculation details see Appendix 23), which slightly higher than the results determined in Chapter 4 (31.67 ±6.96 ng/g HCAs and 1.35 ±0.34 ng/g PAHs in garlic beef meatballs). Similarly ginger powder contained 3.4mg/g GA equivalent phenolic (results showed in Chapter 4), and 5g ginger powder would contain 17mg GA equivalent phenolic. Results of predicted HCAs and PAHs were 14.32 ng/g and 0.42 ng/g, which was comparable with the results in Chapter 4 (12.99±1.52 ng/g HCAs and 0.08±0.04 ng/g PAHs). This verification showed that the selected principle compounds DAD and GA could be able to present the major antioxidants in the whole food matrix on inhibiting HCAs and PAHs in cooked meat products, while there might be other antioxidants or synergistic effect between antioxidants also contributed to minor variation.

6.2 Limitation of studies and future work

Dietary intake of HCAs and PAHs from selected RTE meat products from market were estimated, however, RTE meat could not only be comsumed alone, but also be in prepared dishes. Therefore, estimation of carcinogens intake should be more comprehensive, since dietary pattern of the general public was much more complex according to the NDNS survey. In addition, takeaway dishes from shops or restaurants are also highly consumed from the survey and few studies have focused on these products in the UK. Thus, it is useful to assess the occurrence of HCAs and PAHs from these meals as well in order to assess the exposure of HCAs and PAHs more accurately and further clarify the relationship between meat intake and related cancer risk.

Correlation analysis in Chapter 3 showed that antioxidant capacity of oils and formation of HCAs was negatively related ($p<0.05$). However, the association (indicated in Figure 6-1) between antioxidant capacity (TEAC) of 3 oils and total HCAs was more complicated than a negative relation, further exploration is needed to consolidate whether a linear or non-linear relationship between them. Due to the difference in fatty acids profile and antioxidants level in oils, it would be interesting to explore the effect of a mixture of oils with optimum fatty acids and antioxidants profile on the formation of HCAs and PAHs in meat products.

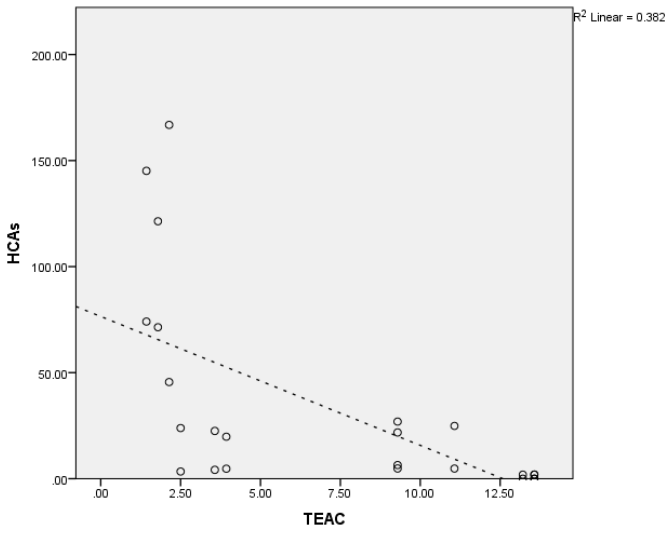

Figure 6-1: Scatter plot between total HCAs and antioxidant capacity of the 3 vegetable oils including sunflower oil, olive oil and grape seed oil.

Results in Chapter 4 reported that addition of spices could inhibit the formation of HCAs and PAHs in cooked meat. Although the level of spices added in meat was carefully selected according to commercial/home recipes, sensory evaluation should be conducted to evaluate the impact of spices on eating quality of the final products. In practice, application of antioxidants into meat products can be through (1) blending with raw meat (Puangsombat, Jirapakkul, & Smith, 2011), (2) coating/spraying on the surface of raw meat (Balogh et al., 2000; Sabally et al., 2016), (3) encapsulating with bioactive layers (Lorenzo et al., 2016). However, the approach with the most antioxidant potential and high consumer acceptance has yet been confirmed.

Results in Chapter 5 showed that the inhibitory efficiency of gallic acid on the formation of HCAs remained same when concentration increased from 0.01%-0.02%. Further verification in chemical model system with wider range of gradient of antioxidants is useful to identify their inhibitory pathway theoretically. To explore the effect of gallic acid and diallyl disulfide on the formation of PhIP in chemical model system, it would be useful to quantify phenylacetaldehyde, as it is the key intermediate in the formation of PhIP. Pyrazine cation radicals and pyridine cation radicals could react with creatine to form imidazoquinoline and imidazoquinoxaline, which could be measured by Electron spin resonance. The determination of precursor and intermediate in chemical model system would help validate the inhibitory mechanism and understand the pathway of forming HCAs and PAHs during meat processing.

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Chapter 7. Main conclusions

This project has investigated the occurrence of carcinogenic HCAs and PAHs in highly consumed meat products, including RTE meat products, patties and meatballs. Results from market research showed that chargrilled chicken contained the highest level of both HCAs and PAHs. Increase intake of chargrilled chicken and ham could increase breast cancer and colorectal adenoma risk, but other types of meat had relatively lower health risk.

Due to the carcinogenicity and related health risk, strategies to reduce exposure of HCAs and PAHs from meat products have been developed. Replacing pork back fat with vegetable oils including sunflower oil, olive oil and grape seed oil could not only improve fatty acids profile in cooked meat products, but also reduce HCAs, which could be attributed to the existence of tocopherols and polyphenol compounds in the vegetable oils. However, antioxidants in the oils could not reduce the total amount of PAHs effectively, while the complexity of oil decomposition and antioxidants performance at high temperature could partially explain the case.

All 6 spices powder including garlic, onion, red chilli, paprika, black pepper and ginger reduced the formation of total HCAs, while ginger powder achieved the highest inhibition efficiency compared with all other spices. Antioxidant capacity of spices determined their efficiency in prohibiting formation of HCAs and PAHs in great extent, while meat type only affected the formation of HCAs, but not PAHs. Regression model suggested that both diallyl disulfide and gallic acid contributed similar inhibitory efficiency on the formation of HCAs and PAHs. Synergistic effect between diallyl disulfide and gallic acid was observed on reducing HCAs, but not on PAHs.

Result in this thesis suggested that adding spices with high antioxidant potential could help to reduce dietary intake of HCAs and PAHs from meat products cooked in household kitchens. It also offered a better understanding about fat replacement because using vegetable oils to replace animal fat could not only improve nutritional value of products but also food safety from the aspect of minimizing HCAs and PAHs formation. In addition, these data

will provide important information for estimating dietary HCAs and PAHs exposure, and will contribute to verify the role of HCAs and PAHs from intake of meat products in the ecology of cancer population in the UK.

Appendix:

Appendix 1: Amount of IQ in cooked meat and fish products

	Cooking process	Cooking temperature[1]	Cooking time (min)	IQ (ng/g)	References
Chicken breast	Grilled	200	20	Nd	Liao et al., 2010
		270-350	6-8	4.09-41.84	Jinap et al., 2013; Hasnol et al., 2014
	Pan-fried	180	10	1.76	Liao et al., 2010
	Fried	180	10	Nd	
	Roasted	200	20	Nd	
Beef	Grilled	200	18	Nd	Viegas and Novo, 2012
		270-350	6-8	29.68-73.96	Jinap et al., 2013
	Pan-fried	180	6	Nd-6.45	Viegas et al., 2012
	Fried	150	8	Nd	Knize et al., 1994
		170-225	15	Nd-5.46	Oz and Kaya, 2011; Britt et al., 1998
		180	20	10.2	Murkovic et al., 1998
		175-225	12-20	0.7-5.3	Balogh et al., 2000
Beef meatball	Grilled	150-250	8	Nd	Oz and Cakmak, 2016
		150-250	9	Nd-1.34	Oz and Kaya, 2016
Pork Bacon	Salted, smoked and fried	150	5-10	3.8-10.5	Johansson and Jagerstad, 1994
Loin	Roasted	175-200	10	<0.1	Busquets et al., 2004
Salmon	Grilled	200	23	Nd	Viegas and Novo, 2012
	Fried	210	8	0.04	Khan et al., 2013
		260	-	0.6	IARC, 1993
	Microwaved		4	Nd	Oz and Kotan, 2016
	Barbequed	180	8	0.09	
	Roasted	180	10	Nd	

[1] Surface temperature (°C)

Appendix 2: Amount of MeIQ in cooked meat and fish products

	Cooking process	Cooking temperature [1]	Cooking time (min)	MeIQ (ng/g)	References
Beef steak	Grilled	195	4	nd	Busquets et al., 2004
Beef	Charcoal grilled	285-325	7	34.76-36.4	Jinap et al., 2013
	Microwave heated and char grilled	285-325	3	37.65-43.67	
Beef meatballs	Grilled	150-250	8	Nd-0.09	Oz and Cakmak, 2016
		150-250	9	Nd	Oz and Kaya, 2016
Chicken	Fried	185	12	Nd	Busquets et al., 2004
	grilled	185	13	<0.1	
	charcoal grilled	285-325	5	52.12-62.65	Jinap et al., 2013
	microwave heated and char grilled	285-325	2	48.58-58.47	
	Fried	100-200	10	0.1-0.5	Skog and Solyakov, 2002
Pork	fried	185	10	Nd	Busquets et al., 2004
Bacon	Cured and fried	150	5-10	Nd-1.7	Johansson and Jagerstad, 1994
Pork chop	Fried	170	12	9.28	Janoszka et al., 2009
Salmon	Microwaved		4	Nd	Oz and Kotan, 2016
	Barbequed	180	8	0.42	
	Roasted	180	10	Nd	

[1] Surface temperature (°C)

Appendix 3: Amount of MeIQx in cooked meat and fish products

	Cooking process	Cooking temperature[1]	Cooking time (min)	MeIQx (ng/g)	References
Beef meatball	Fried	175-225	15	Nd-2.66	Oz and Kaya, 2011
Beef steak	Grilled	195	4	2.9	Busquets et al., 2004
Beef	Char grilled	285-325	7	15.12-15.6	Jinap et al., 2013
	Microwave and char grilled	285-325	3	9.99-11.9	
	Microwave and fried	160	4	Nd	
Beef Pattie	Fried	200-250	12	Nd-3	Felton et al., 1994
	Microwave and fried	200-250	12	Nd-5.1	
	Roasted	230	20	0.34-1.32	Zeng et al., 2014
Chicken	Fried	180-185	10-12	Nd-0.77	Liao et al., 2010; Busquets et al., 2004
	Pan fried	180	10	1.83	Liao et al., 2010
	grilled	185-200	13-20	0.3-1.16	Busquets et al., 2004; Liao et al., 2010
	Char grilled	285-325	5	5.18-11.3	Jinap et al., 2013
	Microwave and fried	160	3	3.44	
	Roasted	200	20	nd	Liao et al., 2010
Pork	fried	185	10	Nd-1.9	Busquets et al., 2004
Ham	Smoked and cooked	74-80[2]	70-72	0.03	Puangsombat et al., 2011
ham	Baked	230-250	12-20	0.1-3.1	Gibis and Weiss, 2012
Bacon	cured and fried	150	5-10	Nd-1.7	Johansson and Jagerstad, 1994
Salmon	Pan-fried	102[2]	18.3	0.66-1.07	Iwasaki et al., 2010
	Charcoal grilled	84[2]	72	0.22-0.87	
	Microwaved		4	0.17	Oz and Kotan 2016
	Barbequed	180	8	2.13	
	Roasted	180	10	0.13	
Sardine	Pan-fried	101[2]	11.3-13.7	0.36-0.7	Iwasaki et al., 2010

[1] Surface temperature (°C)
[2] Internal temperature (°C)

Appendix 4: Amount of 4, 8-DiMeIQx in cooked meat and fish products

	Cooking process	Cooking temperature [1]	Cooking time (min)	4, 8-DiMeIQx (ng/g)	References
Beef	Fried	175-225	15	Nd-3.35	Oz and Kaya, 2011
Beef steak	Grilled	195	4	1.1	Busquets et al., 2004
Beef patties	Fried	200-250	12	0.1-0.3	Felton et al., 1994
	Microwave and fried	200-250	12	0.1-1.2	
	Roasted	230	20	0.24-1.04	Zeng et al., 2014
	Charcoal grilled	285-325	7	Nd-5.54	Jinap et al., 2013
	Microwave and char grilled	285-325	3	Nd-4.5	
Chicken breast	Fried	180	10	0.38	Liao et al., 2010
	Charcoal grilled	285-325	5	3.06-5.55	Jinap et al., 2013
	Microwaved and char grilled	285-325	2	Nd-8.06	
	microwaved and fried	160	3	Nd	
	Fried	185	12	0.8	Busquets et al., 2004
	Pan-fried	180	10	1.05	Liao et al., 2010
	Grilled	200	20	3.55	
		185	13	0.4	Busquets et al., 2004
	Roasted	200	20	nd	Liao et al., 2010
Pork	fried	185	10	Nd-0.9	Busquets et al., 2004
Pork loin	Chargrilled	200-230	10	Nd-4.78	Viegas, 2015
Cured Ham	Smoked and cooked	74-80	70-72[2]	0.03	Puangsombat et al., 2011
	Baked	230-250	12-20	0.5-2.1	Gibis and Weiss, 2012
Bacon	Cured and fried	150	5-10	Nd-1.7	Johansson and Jagerstad, 1994
Salmon	Pan-fried	92-102[2]	18.3	Nd-0.45	Iwasaki et al., 2010
	Charcoal grilled	84[2]	72	Nd-0.42	
Sardine	Pan-fried	101[2]	11.3-13.7	0.26-0.35	

[1] Surface temperature (°C)
[2] Internal temperature (°C)

Appendix 5: Amount of PhIP in cooked meat and fish products

	Cooking process	Cooking temperature [1]	Cooking time (min)	PhIP (ng/g)	References
Chicken breast	Pan-fried	197-211	14-36	0-70	Skog and Solyakov, 2002; Liao et al., 2010
	Boiled	100	23	nd	Skog and Solyakov, 2002
	Fried	180-204	20	2.16-6.06	Liao et al., 2010
	Roasted	200	20	0.04	
Beef	Grilled	200	18	1.45	Puangsombat et al., 2012
		204	12-24	1.58-5.63	Jinap et al., 2013
	Fried	175-225	30	nd-9.47	Oz and Kaya, 2011
		200	6	6.13-7.44	Wong et al., 2012
		204-230	24	5.27-31.8	Gibis and Weiss, 2012
	Roasted	230	20	6.06-28.6	Zeng et al., 2014
Pork	Fried	204	16	1.8	Puangsombat et al., 2012
	Pan-fried	175-200	10	2.5	Busquets et al., 2004
Bacon	Fried	172	6	6.91	Puangsombat et al., 2012
Pork chop	Fried	170	12	Nd	Janoszka et al., 2009
Pork loin	Char grilled	200-230	10	1.60-6.09	Viegas et al., 2015
Ham	Baked	230-250	12-20	0.2-0.8	Gibis and Weiss, 2012
Sausage	Fried	175-200	9	<0.2	Busquets et al., 2004
Salmon	Pan-fried	92-102 [2]	18.3	6.17-7.37	Iwasaki et al., 2010
	Charcoal grilled	84 [2]	72	22.55-28.8	
	Microwaved		4	Nd	Oz and Kotan, 2016
	Barbequed	180	8	2.67	
	Roasted	180	10	Nd	
	Fried	204	12	9.11	Puangsombat et al., 2012
		210	8	26.2	Khan et al., 2013
Sardine	Pan-fried	101 [2]	11.3-13.7	0.53-2.28	Khan et al., 2013
Cod	Fried	210	8	19.1	Khan et al., 2013

[1] Surface temperature (ºC)
[2] Internal temperature (ºC)

Appendix 6: Amount of BaP in cooked meat and fish products

	Cooking process	Cooking temperature[1]	Cooking time	Concentration of BaP (ng/g)	References
Chicken breast	Grilled	350-400	6min	0-86.4	Wongmaneepratip and Vangnai, 2017
	Sugar smoked		3-6min	Nd	Chen et al., 2013
Beef ham	Smoked	Not clear	3-6 days	0.07-0.04	Djinovic et al., 2008
Beef lion	Char grilled	290	9 mins	0.6	Viegas et al., 2012
		Not clear	30mins	0.055	Chung et al., 2011
	Roasted	200	30mins	Nd	
Steak	Sugar smoked		3-6min	Nd	Chen et al., 2013
Beef ribs	Char grilled	Not clear	30mins	0.199	Chung et al., 2011
	Roasted	200	30mins	0.032	
Pork ham	Smoked	Not clear	3-6 days	0.12-0.18	Djinovic et al., 2008
Bacon	Smoked	Not clear	3-6 days	0.22-0.66	Djinovic et al., 2008
Pork loin	Char grilled	Not clear	30mins	2.99	Chung et al., 2011
		200-230	10min	1.07-2.71	Viegas 2014
	Roasted	200	30mins	0.018	
Pork belly	Roasted	200	30mins	0.014	Chung et al., 2011
	Grilled	230	8min	5.51-8.04	Park, 2017
Pork chop	Fried	170	12mins	0.41-1.61	Janoszka, 2012
	Grilled	270	17mins	0.38-0.5	Janoszka, 2012
Sausage	Dry-cured	23	0-40days	0.26-3.53	Roseiro et al., 2011
Salmon	Char grilled	290	15 mins	1.66	Viegas et al., 2012
	Sugar smoked		3-6min	Nd	Chen et al., 2013
Iran fish	Smoked	Not clear	7days	0.30-1.86	Mohammahi et al., 2013

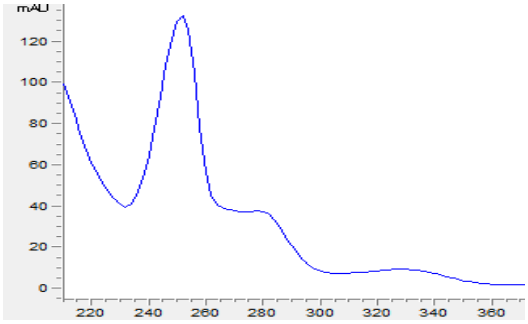
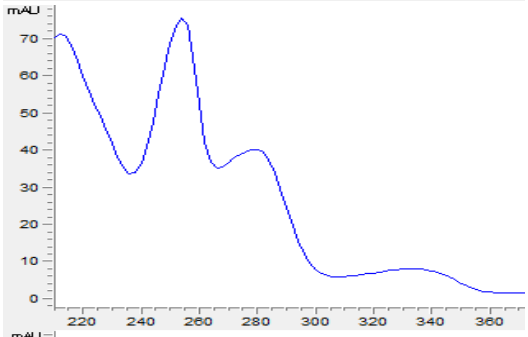
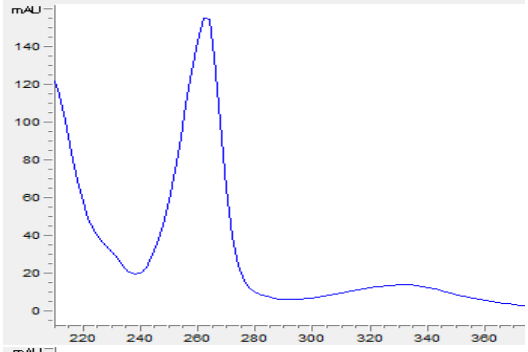
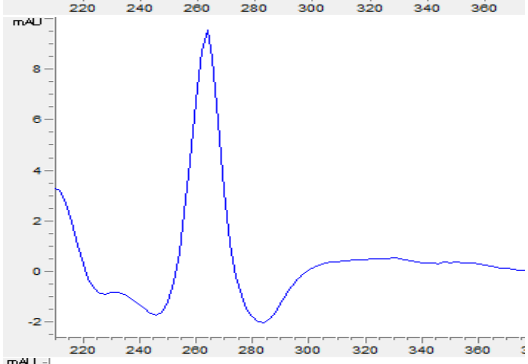
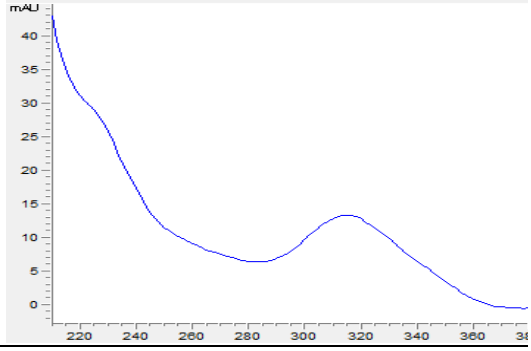
[1] Surface temperature (°C)

Appendix 7: Amount of BaA in cooked meat and fish products

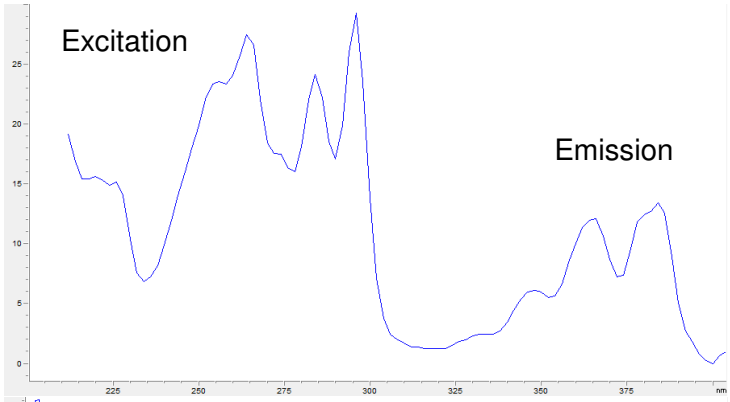
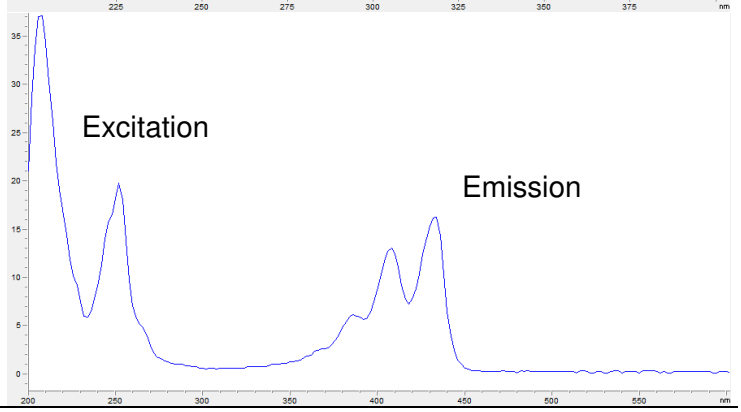
	Cooking process	Cooking temperature[1]	Cooking time	BaA (ng/g)	References
Chicken breast	Grilled	350-400	6min	0-137.6	Wongmaneepratip amd Vangnai, 2017
Beef	Smoked	Not clear	3-6 days	0.2-1.04	Djinovic et al., 2008
	Char grilled	290	9 mins	0.68	
Pork ham	Constantly exposed in burning of beech wood (smoking)	Not clear	3-6 days	0.32-0.53	Djinovic et al., 2008
Bacon	Smoked	Not clear	3-6 days	0.53-1.71	Djinovic et al., 2008
Pork chop	Fried	170	12mins	1.16-2.63	Janoszka, 2011
Minced pork chop	Grilled	270	17mins	1.1-1.53	Janoszka, 2011
Pork loin	Char grilled	200-230	10min	1.6-3.9	Viegas et al., 2014
Salmon	Char grilled	290	15 mins	3.98	Viegas et al., 2012
Sausage	Dry-cured	23	0-40days	0.79-17.98	Roseiro et al., 2011

[1] Surface temperature (ºC)

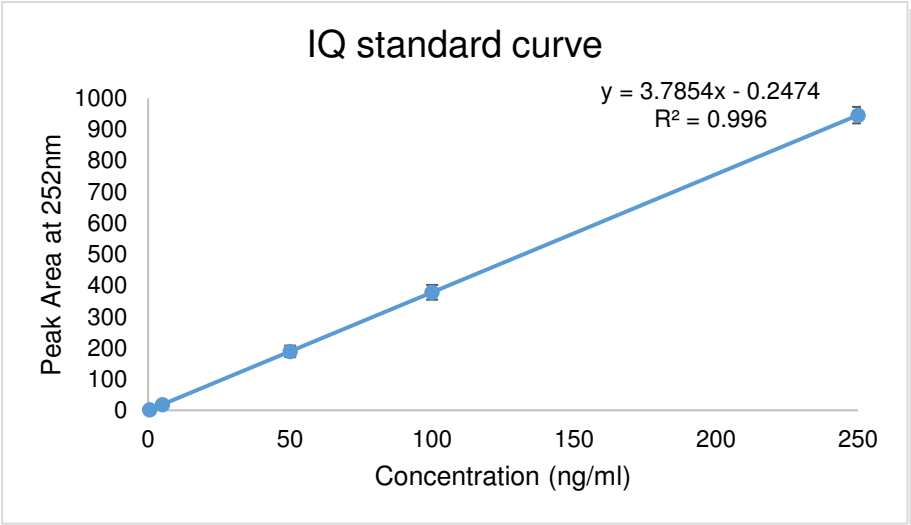
Appendix 8: Retention time and UV spectrum of 5 HCAs

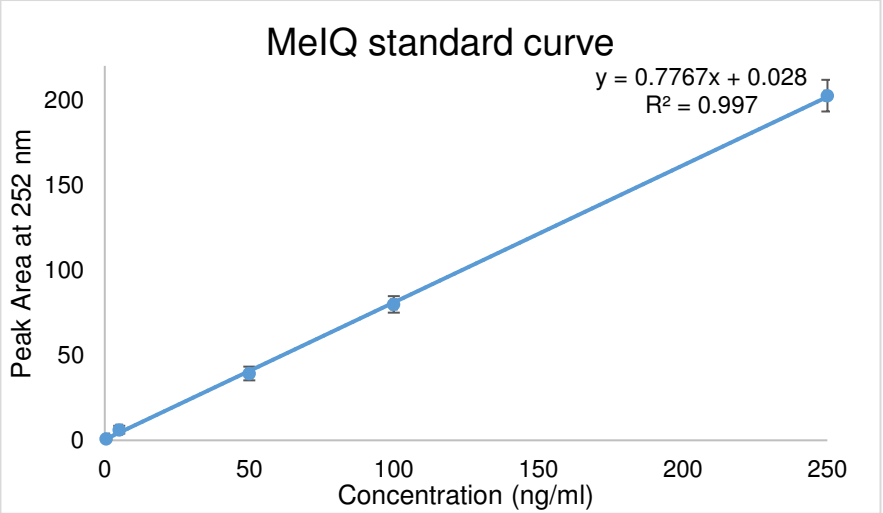
HCAs	Retention time (min)	RSD (%)	UV spectrum
IQ	10.5	0.62	
MeIQ	12.8	0.32	
MeIQx	13.5	0.99	
4,8-DiMeIQx	17.1	0.55	
PhIP	25.0	0.22	

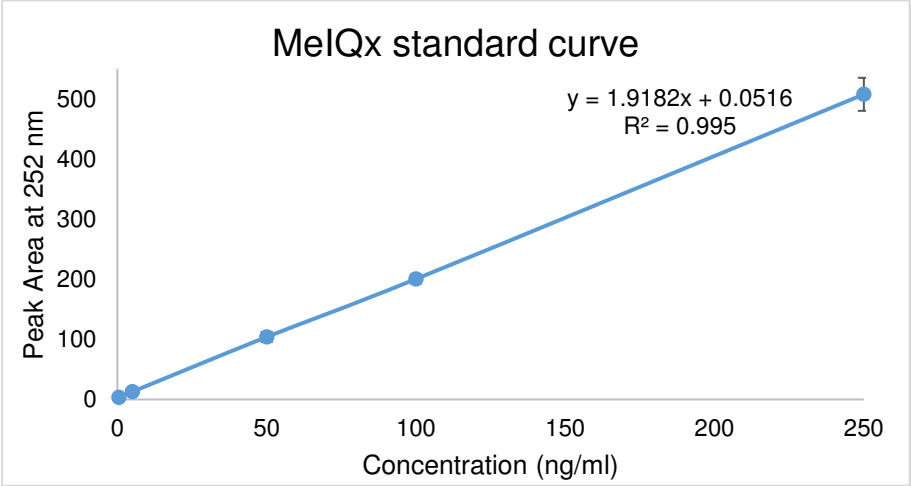
Appendix 9: Retention time and spectrum of 2 PAHs

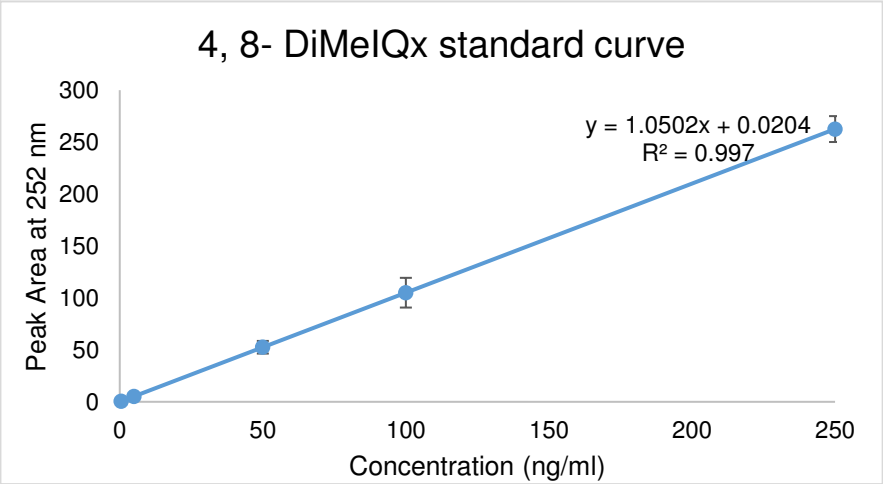
PAHs	Retention time (min)	RSD (%)	Spectrum
BaA	7.3	0.15	
BaP	9.5	0.66	

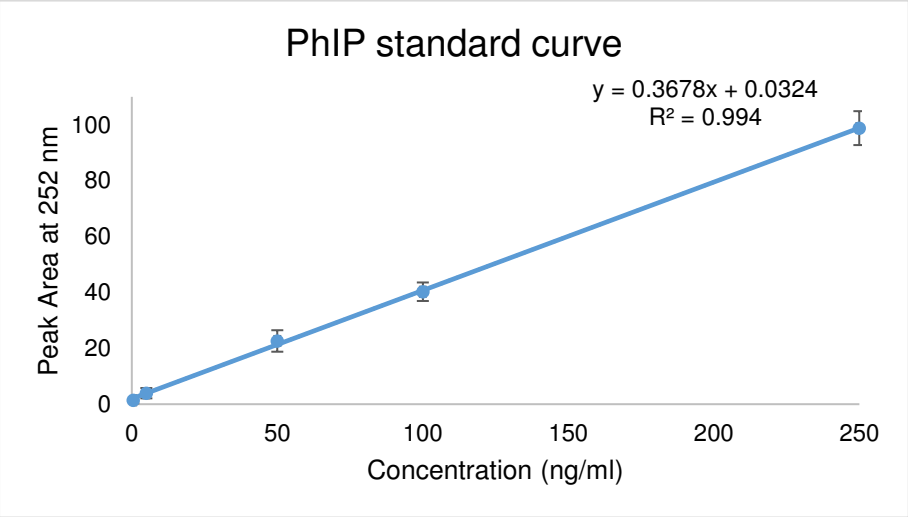
Appendix 10: Calibration curves of 5 HCAs and 2 PAHs in HPLC

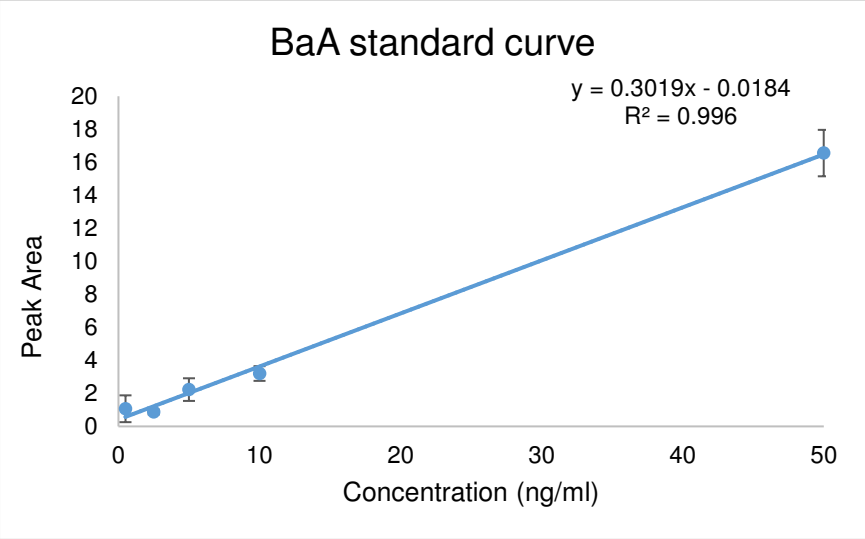


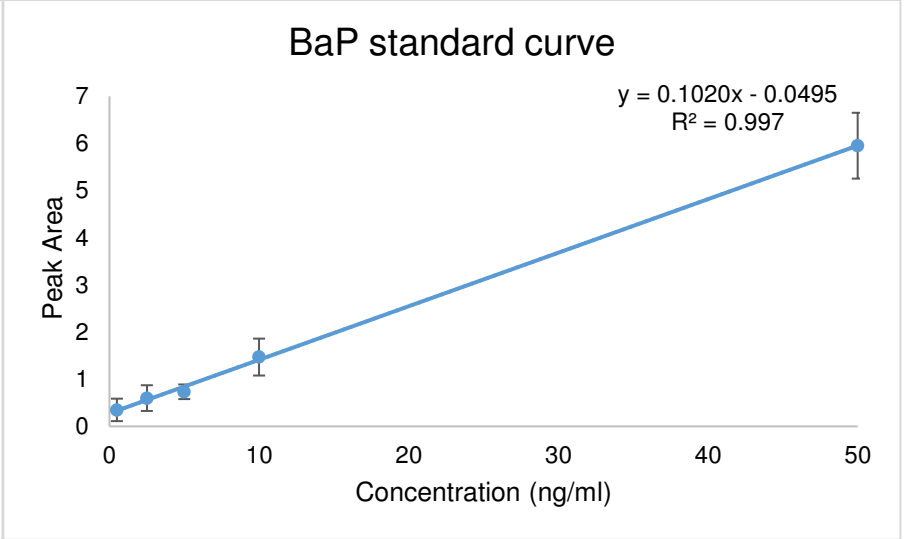






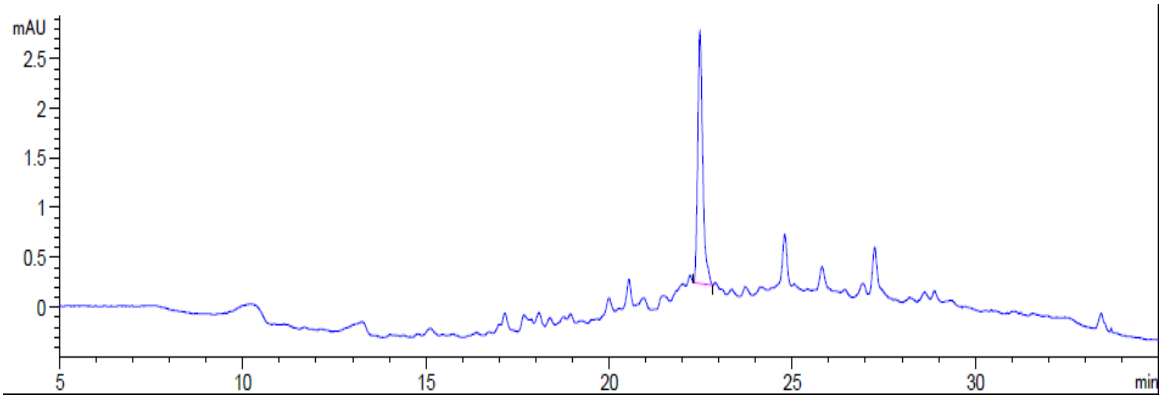

PhIP standard curve
y = 0.3678x + 0.0324
R² = 0.994
Peak Area at 252 nm
0
20
40
60
80
100
0
50
100
150
200
250
Concentration (ng/ml)


BaA standard curve
y = 0.3019x - 0.0184
R² = 0.996
Peak Area
0
2
4
6
8
10
12
14
16
18
20
0
10
20
30
40
50
Concentration (ng/ml)

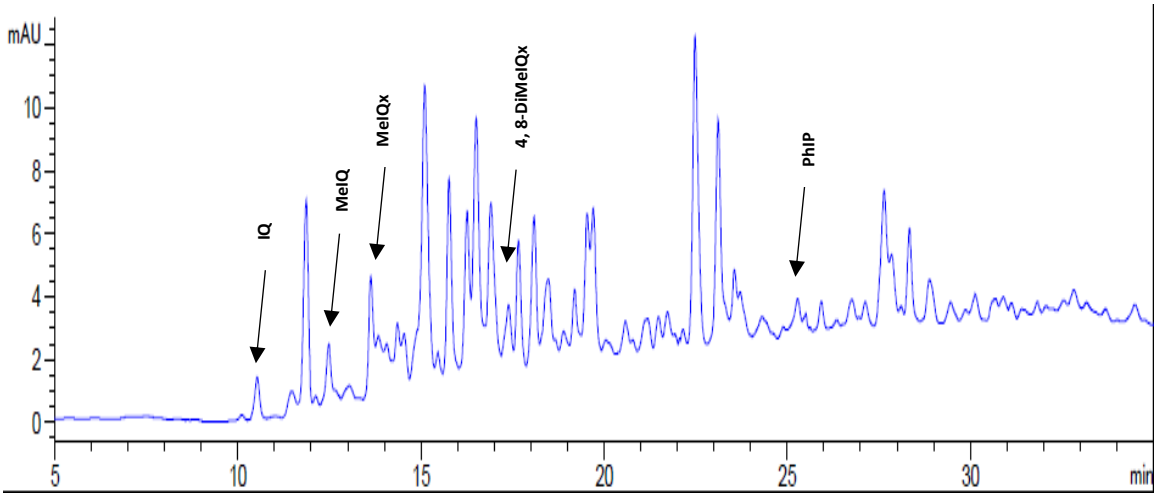

BaP standard curve
y = 0.1020x - 0.0495
R² = 0.997
Peak Area
0
1
2
3
4
5
6
7
0
10
20
30
40
50
Concentration (ng/ml)

Appendix 11: HPLC chromatograms of sample, sample with spiked 5 HCA standards (5ng/ml and 50 ng/ml each)

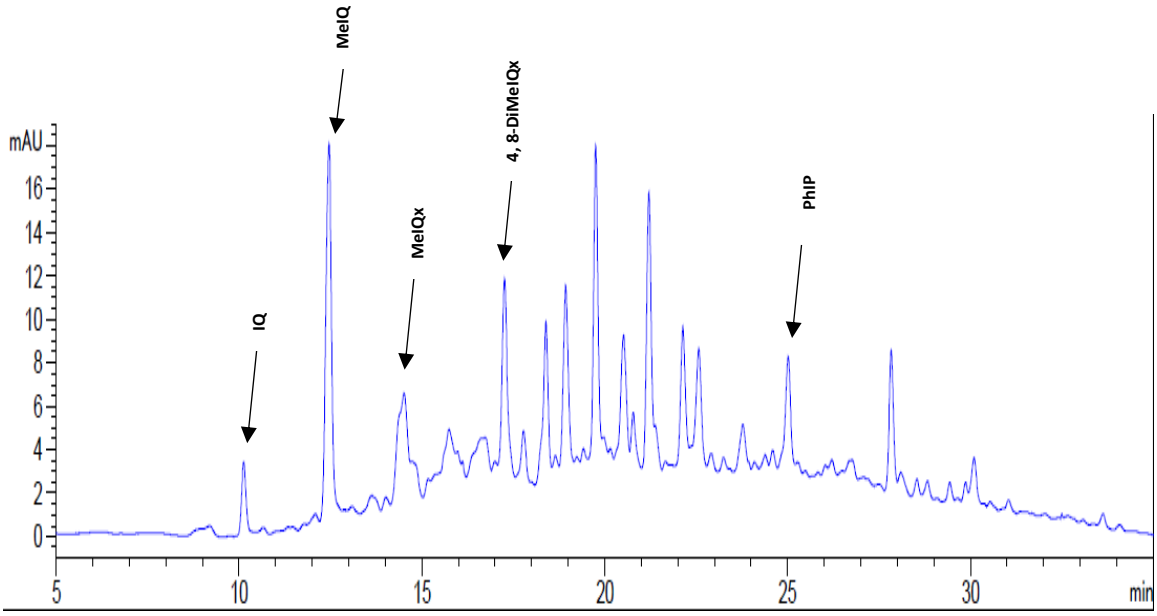
Sample



Spiked sample (5ng/ml)

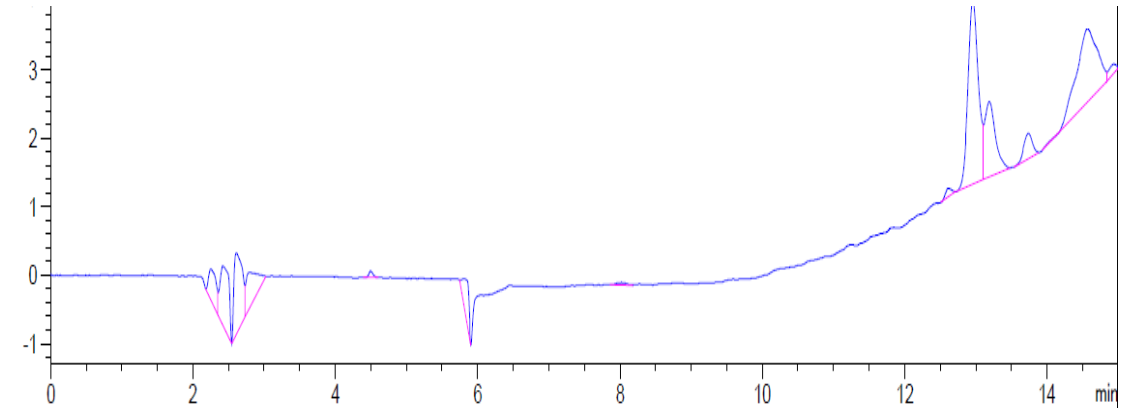


Spiked sample (50ng/ml)

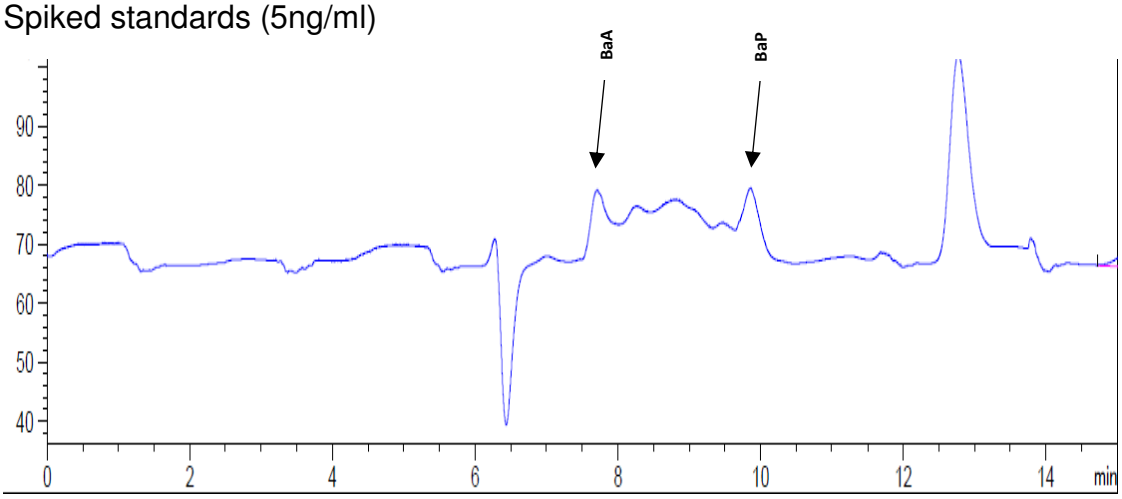


Appendix 12: HPLC chromatograms of sample, sample with spiked 2 PAHs standards (5ng/ml and 50 ng/ml each)

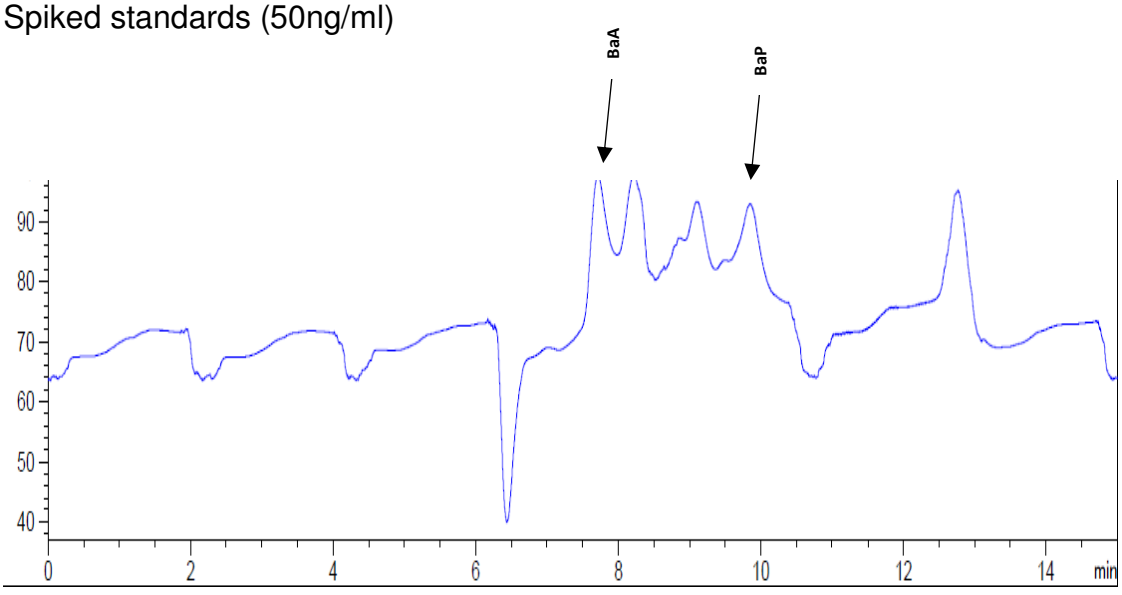
Sample:



Spiked standards (5ng/ml)

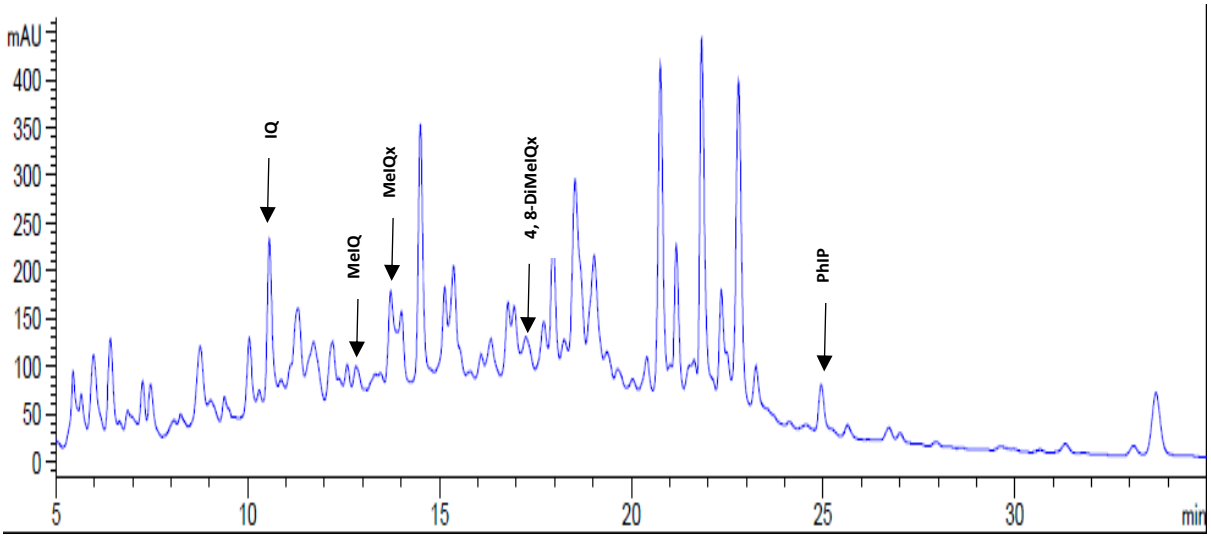


Spiked standards (50ng/ml)

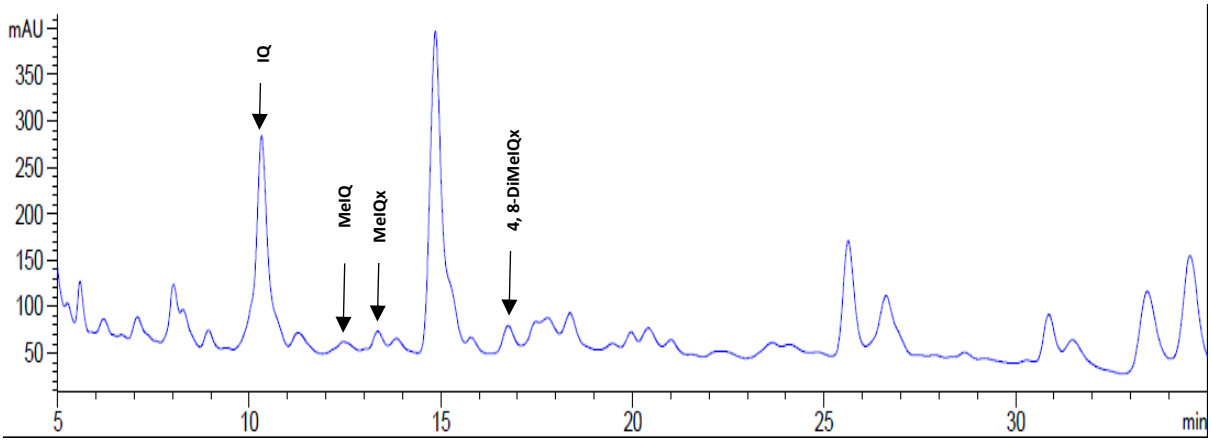


Appendix 13: HPLC chromatograms of HCAs (IQ, MeIQ, MeIQx, 4, 8-DiMeIQx and PhIP) detected with a UV detector (252nm) from selected RTE meat samples

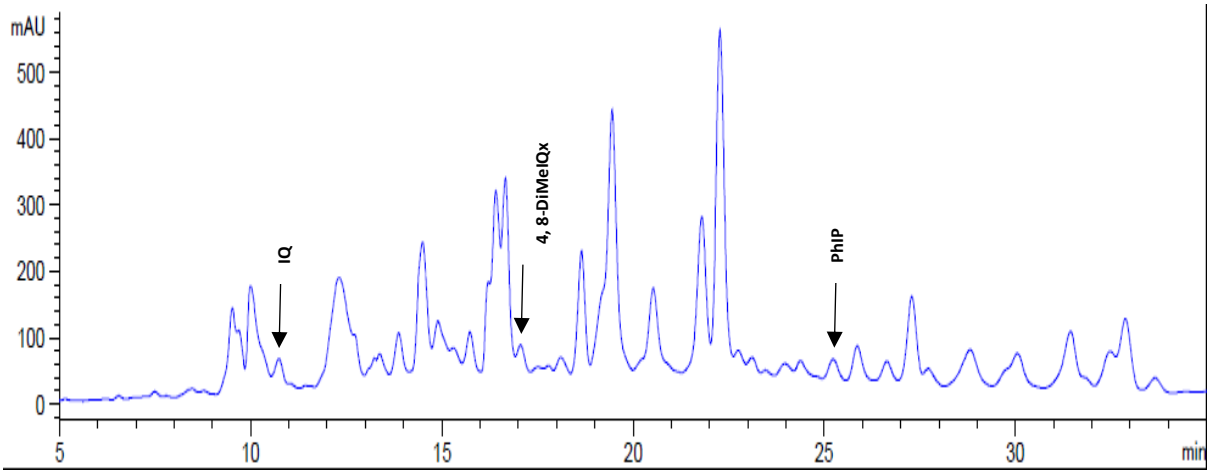
BBQ chicken



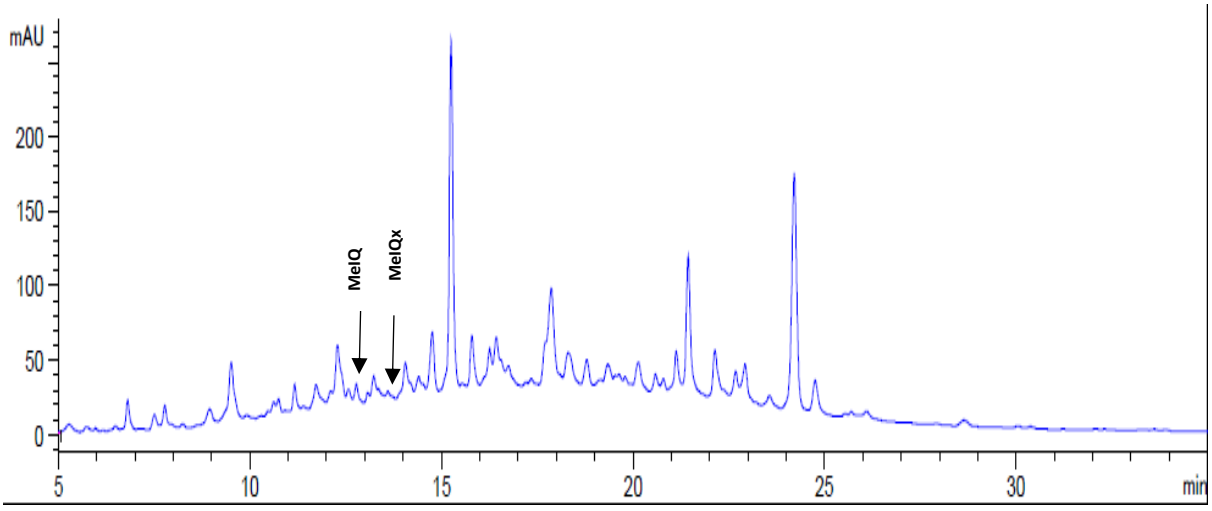
Chargrill chicken



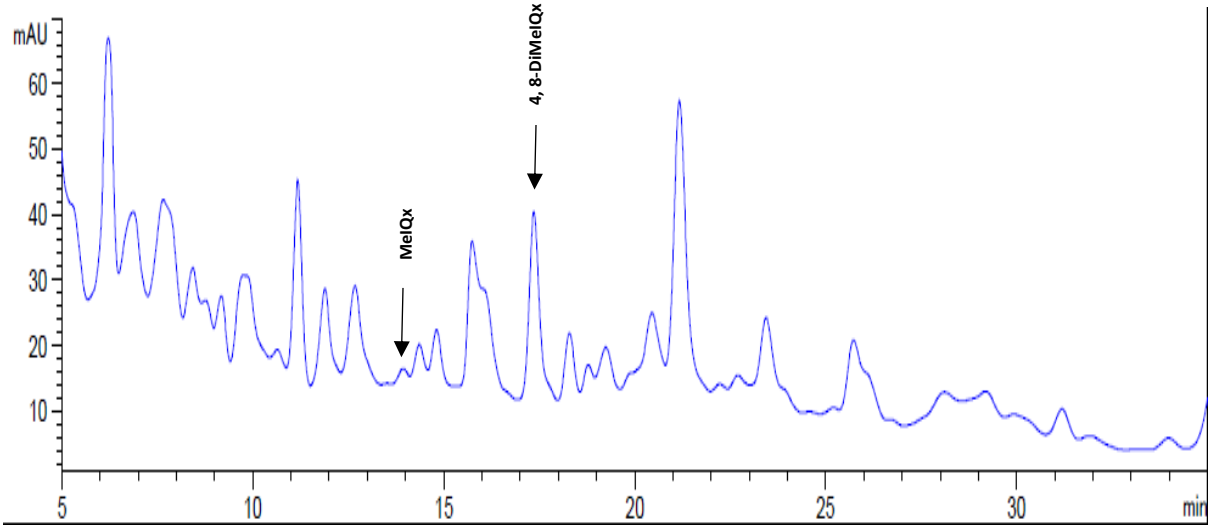
Tikka chicken



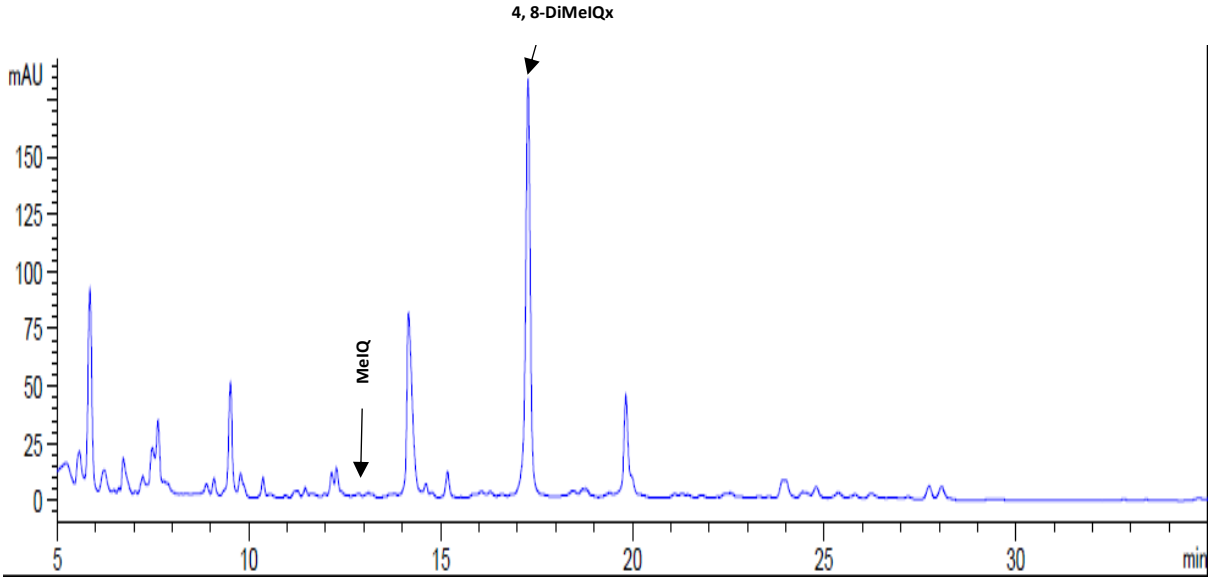
Ham



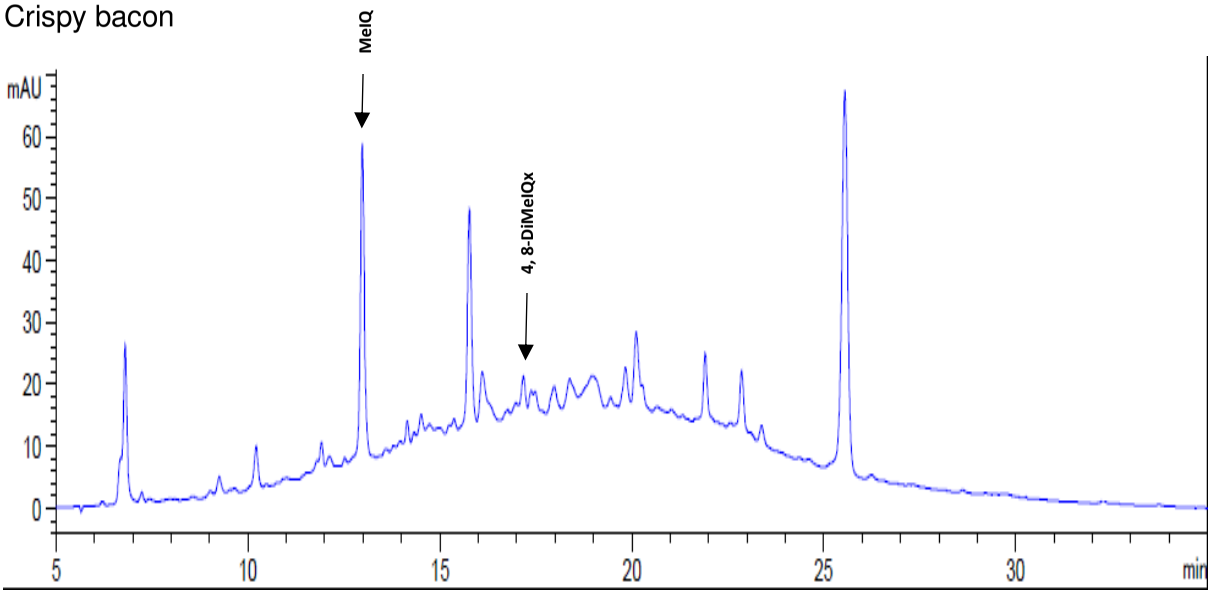
Smoked ham



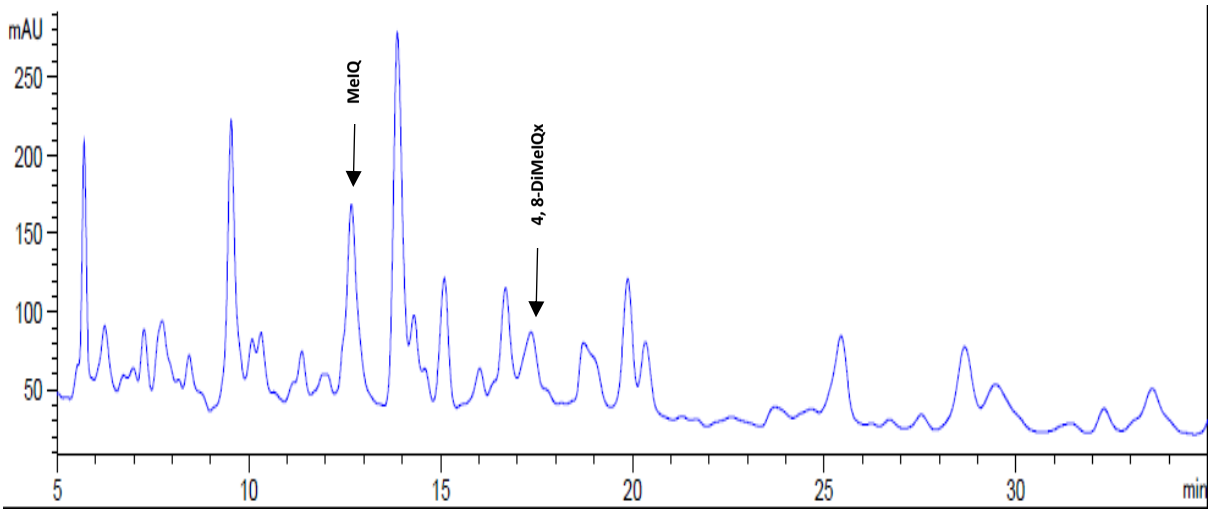
Roasted bacon



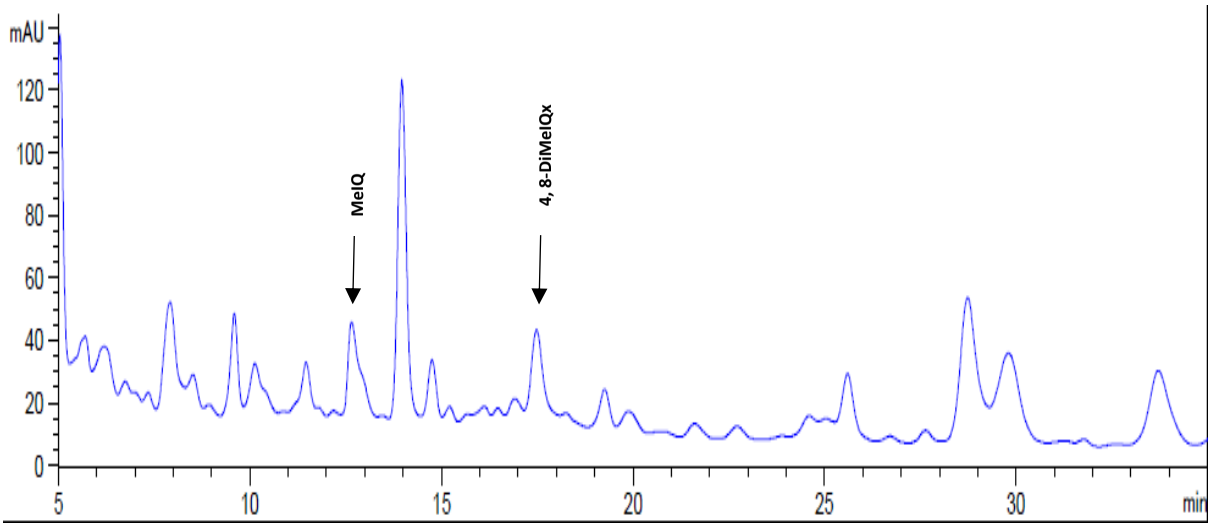
Crispy bacon



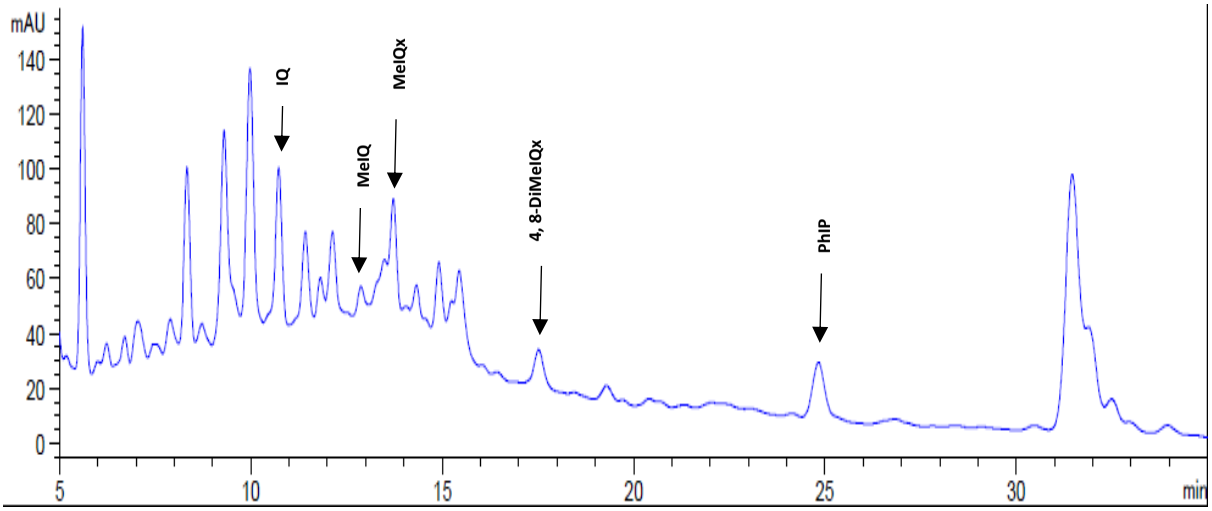
Pork sausage



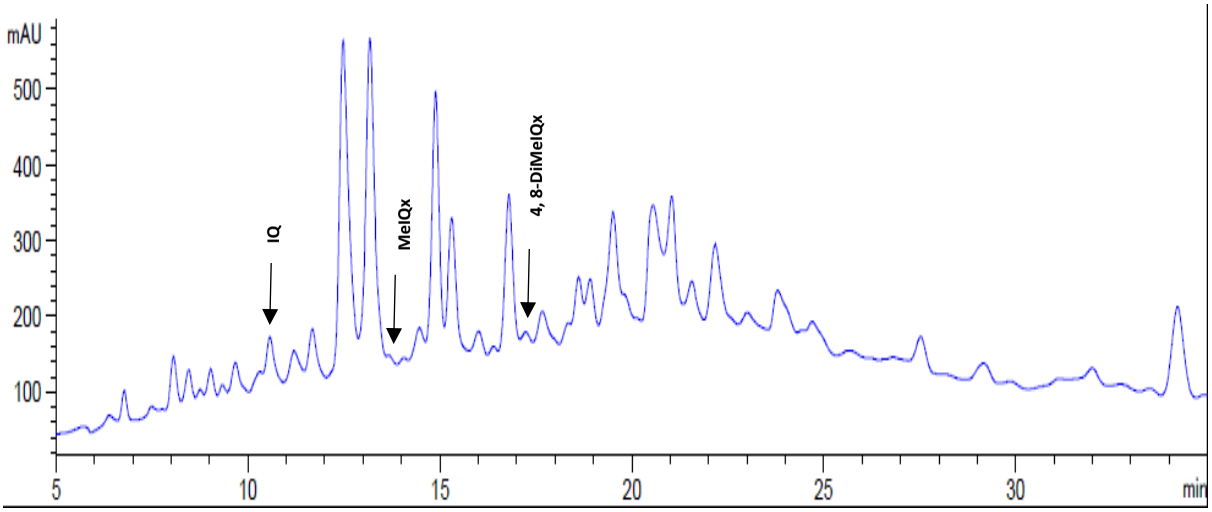
Swedish meatball



Honey roasted salmon



Sweet chilli salmon



Appendix 14: Toxicity Equivalency Factor of PAHs in 11 RTE samples[1]

RTE Samples	Measured concentration of BaP (ug/kg)	Measured concentration of BaA (ug/kg)	TEQ = Sum (C*TEF) (ug/kg)
BBQ chicken	Nq	Nq	N/a
Tikka chicken	Nq	Nq	N/a
Chargrilled chicken	Nq	3.06	0.306
Ham	Nd	Nq	N/a
Smoked ham	Nq	0.19	0.019
Roasted bacon	1.09	0.66	1.156
Crispy bacon	0.71	0.37	0.747
Pork sausage	0.21	0.67	0.277
Swedish meatballs	0.18	2.18	0.398
Honey roasted salmon	0.35	Nq	0.35
Sweet chilli salmon	Nd	Nd	N/a

[1]Toxicity Equivalency Factor (TEF, Unitless), TEF_{BaP}=1, TEF_{BaA}=0.1

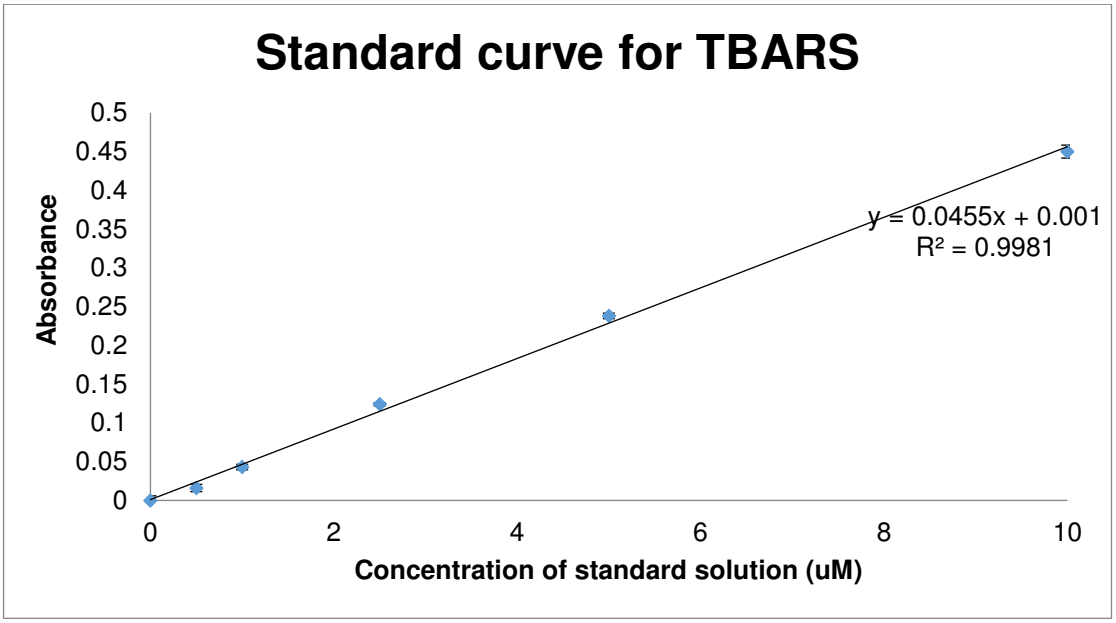
Appendix 15: Sum and Average consumption of selected RTE meat products from age group of 19-64 years old and over 65 years old in UK diet survey. Raw data obtained from the National Diet and Nutrition Survey[1] (2015).

Selected RTE meat	Male				Female			
	Sum of intake in the survey (g)	Average intake[2] (g/day)	Sum of intake in the survey (g)	Average intake (g/day)	Sum of intake in the survey (g)	Average intake (g/day)	Sum of intake in the survey (g)	Average intake (g/day)
	19-64ys		Over 65ys		19-64ys		Over 65ys	
BBQ chicken	118.7	0.03	0	0.00	767.25	0.27	0	0.00
Tikka chicken	5988	1.58	0	0.00	3165	1.11	1521.28	1.60
Chargrilled chicken	24593.5	6.51	2667.88	3.49	23710.75	8.35	4915.6	5.19
Ham	15722.16	4.16	2870.5	3.76	16798.34	5.91	7288.1	7.69
Smoked ham	1547	0.41	43.2	0.06	487.4	0.17	378	0.40
Roasted bacon	2008.3	0.53	473.46	0.62	1070	0.38	604.7	0.64
Crispy bacon	5276.25	1.40	280	0.37	4220.05	1.49	600	0.63
Pork sausage	180	0.05	77.4	0.10	126	0.04	198	0.21
Swedish meatballs	1118.6	0.30	0	0.00	1296	0.46	183.96	0.19

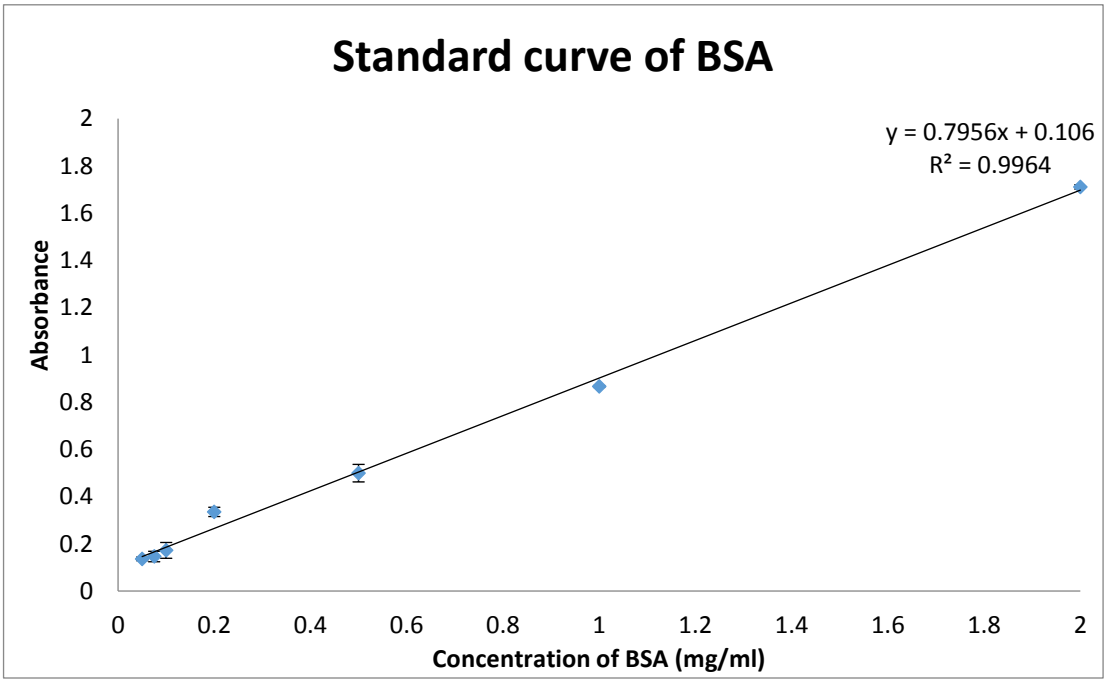
[1] 945 females and 710 males from UK took part in the survey.

[2] The food diary was involving in 4 different days with one weekend.

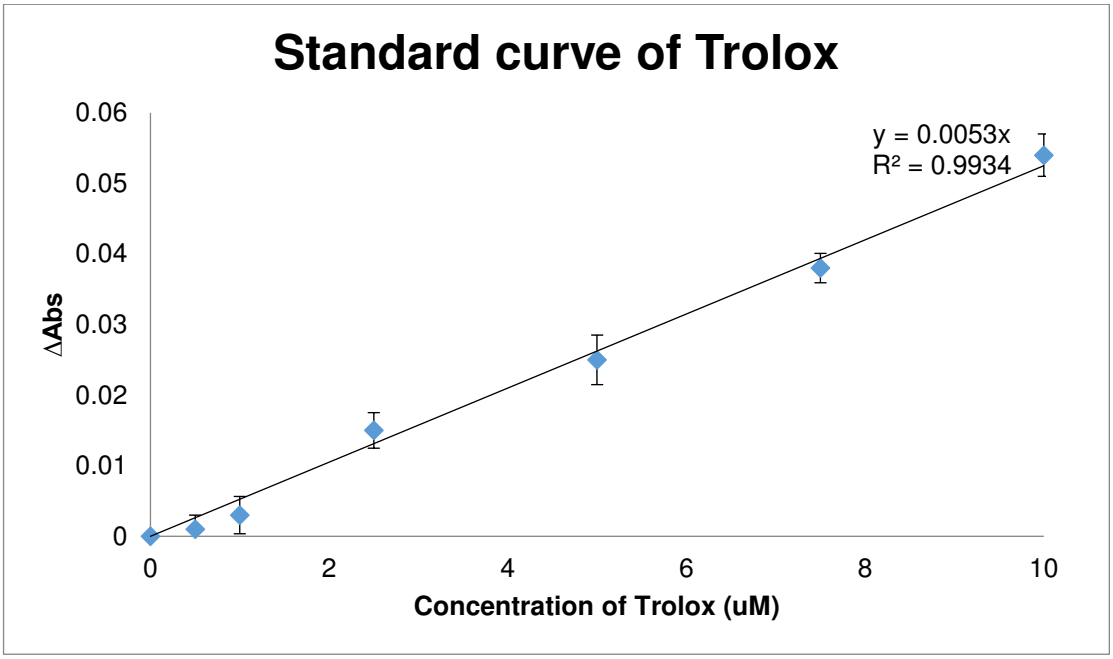
Appendix 16: Calibration curve of TEP as standard solution in TBARS assay



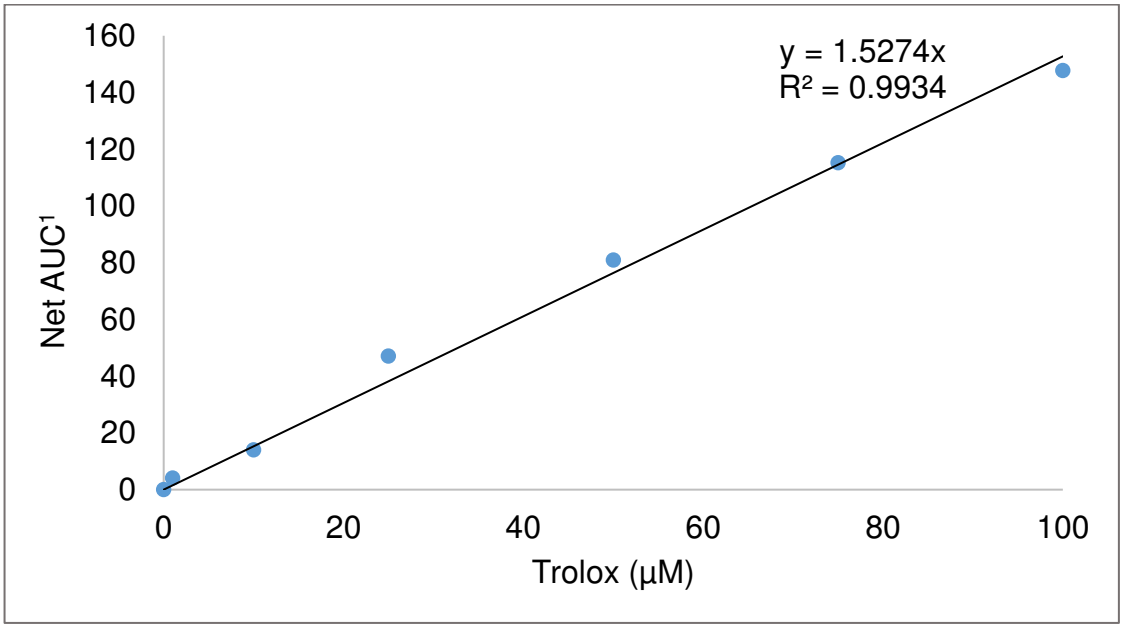
Appendix 17: Calibration curve of BSA as standard solution for calculating protein concentration in protein carbonyl assay



Appendix 18: Calibration curve of Trolox as the standard solution in TEAC assay



Appendix 19: Calibration curve of Trolox as the standard solution in ORAC assay



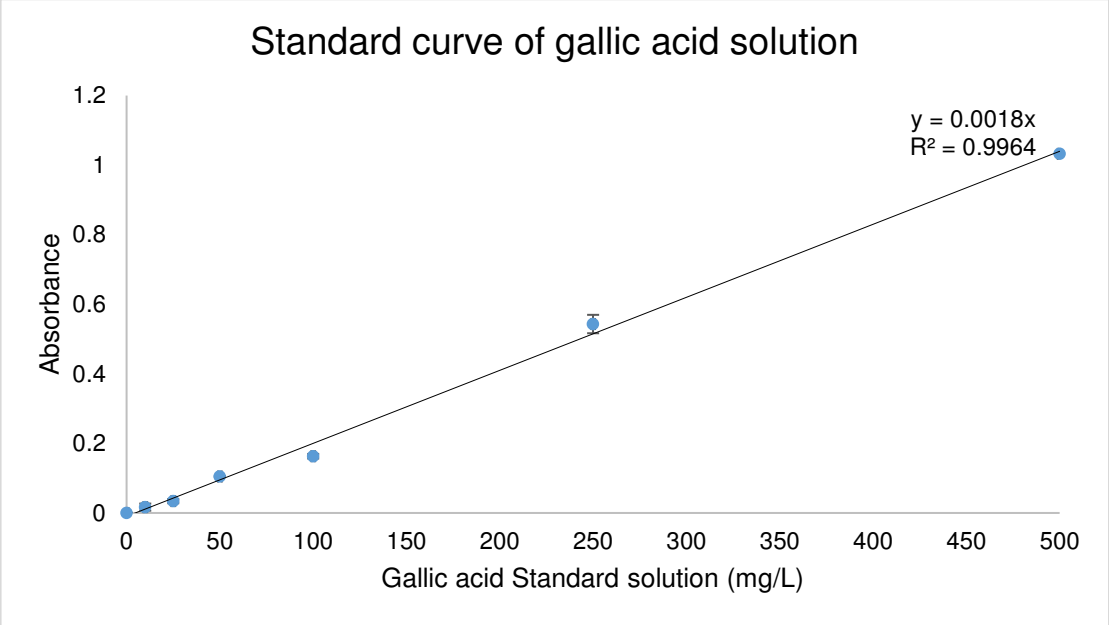
[1]Net AUC values reflecting the blank-corrected AUC values for the standard at different concentration.

Appendix 20: The concentration of spices in meatballs and their corresponding antioxidants in selected spices from previous studies

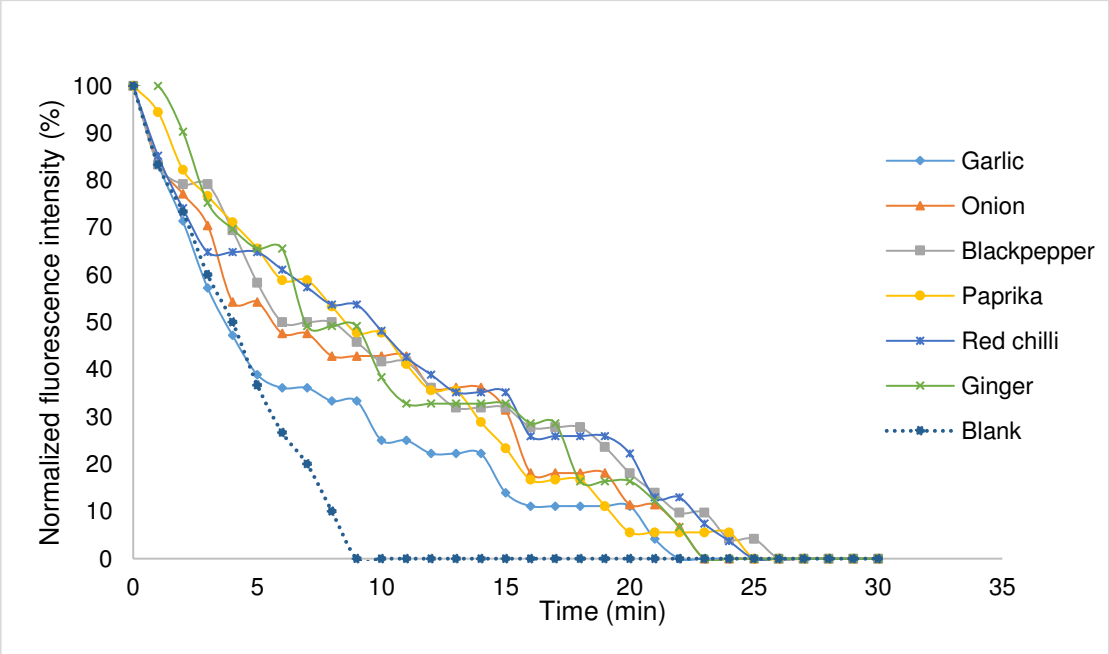
Spices	Amount in meatballs (w/w)	Antioxidants	References
Garlic	5%	Organosulfur compounds: 0.16-13.0mg/g Phenolics: 0.08-0.2 mg GAE/g DW[1]	Rounds et al. (2012) Nuutila et al. (2003); Rahman (2007)
Onion	5%	Organosulfur compounds: 0.1-0.3 mg/g Phenolics: 0.8-2.0 mg GAE/g DW	Rounds et al. (2012) Nuutila et al. (2003)
Paprika	5%	Phenolics: 13 mg GAE/g DW	Rounds et al. (2012) Vega-Gálvez et al. (2009)
Red chilli	1%	Phenolics: 7.9-26.1 mg GAE/g DW	Gurnani et al. (2016); Oz and Kaya (2011b)
Black pepper	1%	Phenolics: 3.8-27.5 mg GAE/g DW	Oz and Kaya (2011a); Shahidi and Ambigaipalan (2015); Larsen (2017)
Ginger	2.8%	Phenolics: 8.41-21.24 mg GAE/g DW	An et al. (2016); Liu et al. (2008); Lu et al. (2011); Viegas et al. (2012)

[1]Gallic acid equivalent /g dry weight.

Appendix 21: Calibration curve of gallic acid as standard solution in total phenolic content assay



Appendix 22: Average fluorescence signal curves for 6 selected spices powder (garlic, onion, paprika, red chilli, black pepper and ginger) in ORAC assay



Appendix 23: Calculations for verifying regression model estabilished in Chaper 5

Regression model in Chapter 5:

Predicted total HCAs (ng/g) = 52.30 - 22.34 GA - 21.62 DAD + 10.17 GA * DAD

Predicted total PAHs (ng/g) = 2.32 - 1.21 GA - 1.29 DAD + 0.696 GA * DAD

Assume coefficient of DAD '1'= 0.05% (50mg/100g) and of GA '1'= 0.01% (10mg/100g).

For example, garlic powder contained average 380μg /g DAD and 0.9mg GA Equivalent/g (Yu et al., 1989), so 5g garlic powder contained 1900 μg DAD and 4.5mg GA Equivalent, thus, coefficient of DAD= 0.04 and GA = 0.5.

Predicted total HCAs (ng/g) = 52.30 - 22.34 * 0.5 - 21.62 *0.04 + 10.17 * 0.5 * 0.04 = 40.47 ng/g

Predicted total PAHs (ng/g) = 2.32 - 1.21 *0.5 - 1.29 *0.04 + 0.696 *0.5 * 0.04 = 1.68 ng/g

Ginger powder contained average 3.4mg GA Equivalent/g, so 5g ginger powder contained 17mg GA Equivalent phenolic, the coefficient of DAD =0 and GA =1.7.

Predicted total HCAs (ng/g) = 52.30 - 22.34 * 1.7 = 14.32 ng/g

Predicted total PAHs (ng/g) = 2.32 - 1.21 *1.7 = 0.42 ng/g